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*To my Mum and Dad*

*Amal & Ahmad*

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## Statement of originality

The accompanying thesis submitted for the degree of Ph.D. entitled "Complement and Surfactant Protein D in the Innate Immunity to *Streptococcus pneumoniae*" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester mainly during the period between February 1999 and September 2002.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: Rania JOUNBLAT Date: 28/02/03

# Complement and Surfactant Protein D in the Innate Immunity to *Streptococcus pneumoniae*

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The aim of this project was to investigate the role of complement and lung surfactant protein D in innate immunity to *S. pneumoniae*.
Pneumolysin, a cytolytic toxin produced by *S. pneumoniae*, is able to activate the classical complement pathway. The deletion of the ability of pneumolysin to activate complement affected the early growth of pneumococcus in the lungs, the onset of bacteraemia, the histological changes and the recruitment of T lymphocytes into lung tissue during bronchopneumonia.

Lung complement C3 was substantially activated after intranasal infection with wild-type *S. pneumoniae* in comparison with the isogenic mutant strain unable to produce pneumolysin (PLN-A).

Data presented in this thesis showed that the classical complement pathway plays a critical role in the innate immunity to *S. pneumoniae* infection. Deficiency in C1q increased the susceptibility to pneumococcal infection and was associated with defects in pneumococcal clearance from lungs and blood, less severe histological changes, recruitment of T cells and a substantial decrease in the activation of complement C3 in the lung.

*In vitro* studies showed that lung surfactant protein D or its receptor gp-340 is able to bind and agglutinate several strains of *S. pneumoniae*. Sp-D did not enhance the uptake of pneumococcus by neutrophils. The capsule-type is not a determinant for *S. pneumoniae* aggregation by Sp-D or gp-340.

Sp-D-deficient mice showed increased susceptibility to pneumococcal infection. Deficiency in Sp-D was associated with decreased pneumococcal clearance in lungs and trachea, early onset and increased levels of bacteraemia. In the infected lung, accumulation of T lymphocytes and more severe inflammation were observed in the absence of Sp-D.

## Abbreviations:

| | |
|---|---|
| APAAP | Alkaline phosphatase anti-alkaline phosphatase |
| BAB | Blood agar base |
| BAL | Bronchoalveoalr lavage |
| BHI | Brain heart infusion |
| Bp | Base pair |
| BSA | Bovine serum albumin |
| CFU | Colony forming units |
| CR | Complement receptor |
| CRD | Carbohydrate recognition domain |
| CRP | C-reactive protein |
| DNA | Deoxyribonucleic acid |
| DNAse | Deoxyribonuclease |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immuno-sorbemt assay |
| Gp | Glycoprotein |
| HBSS | Hanks balanced salt solution |
| HRP | Horseradish peroxidase |
| IFNγ | Interferon gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| KDa | Kilo Dalton |
| MBL | Mannan binding lectin |
| µg | Microgram |
| mg | Milligram |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MLST | Multilocus sequence type |
| µl | Microlitre |
| ml | Millilitre |
| mM | Millimolar |
| MPO | Myeloperoxidase |
| NanA | Neuraminidase A |

| | |
|---|---|
| NanB | Neuraminidase B |
| ng | Nanogram |
| nm | Nanometre |
| OD | Optical density |
| PAF | Platelet activating factor |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PLN-A | Pneumolysin negative type 2 pneumococcal strain |
| PMN | Polymorphonuclear leukocytes |
| PsaA | Pneumococcal surface adhesion A |
| PspA | Pneumococcal surface protein A |
| PspC | Pneumococcal surface protein C |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rSp-D | Recombinant surfactant protein D |
| RT-PCR | Reverse transcriptase-PCR |
| SCD | Sickle cell disease |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SOD | Superoxide dismutase |
| Sp-A | Surfactant protein A |
| Sp-D | Surfactant protein D |
| TBS | Tris buffered saline |
| TMB | Tetramethylbenzidine |
| TNFα | Tumor necrosis factor alpha |
| V | Volts |

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# Chapter 1: Introduction

## 1.1 *Streptococcus pneumoniae* and pneumococcal disease

*Streptococcus pneumoniae*, the pneumococcus, is an encapsulated lanceolate Gram-positive diplococcus that can form long or short chains in certain culture media. It is facultatively anaerobic and belongs to the group of α-streptococci.

More than a century has elapsed since *S. pneumoniae* was first identified by Sternberg who injected a rabbit with his own saliva and recovered pneumococci from the stricken animal (Sternberg 1881). Despite the large store of knowledge that accumulated in the last century on the structure and immunological properties of the bacterium, *S. pneumoniae* persists as being a respiratory pathogen of major clinical impact, threatening the public health worldwide. The pneumococcus is a common bacterial agent in a considerable variety of infections including mucosal infections (e.g. sinusitis and otitis media) and severe invasive infections such as pneumonia, arthritis, pericarditis, peritonitis, meningitis and septicaemia (Fig.1.1) (Musher 1992).

The impact of pneumococcal diseases is very significant in terms of morbidity and mortality in both the western world and particularly the developing world where the rate of mortality is high (Austrian 1986). The levels of invasive pneumococcal disease peak at the extremes of life, before 5 years and after 75 years of age. Pneumococcal infections have been estimated to cause 1.2 million pneumonia deaths per year worldwide, and nearly 40% of all pneumonia deaths occur in children younger than 5 years of age (Mulholland 1999). Also, the pneumococcus causes 70 000 deaths per year from meningitis and a similar number from septicaemia and other infections in young children in developing countries (Mulholland 1999). Furthermore, pneumococcal pneumonia is the fifth leading cause of death worldwide (3rd International Symposium on Pneumococci and Pneumococcal Diseases 2002). In the USA, 40 000 deaths per year are caused by pneumococcal pneumonia or meningitis (Centres for Disease Control, 1997). In the UK, the rates of invasive *S. pneumoniae* disease were 48.1 per $10^5$ population less than one year of age and 36.2 per $10^5$ in adults over 65 years (Sleeman *et al.*, 2001). Generally, the incidence of invasive pneumococcal disease in the UK is 6.6-9.6 cases per$10^5$ population (Sleeman *et al.*, 2001). Pneumonia represented 19% of the invasive diseases reported in children less than five years (Shackley *et al.*, 2000).

*Streptococcus pneumoniae* is the prime cause of community-acquired pneumonia worldwide (Macfarlane 1999). In the UK, Spain and USA, the pneumococcus is responsible for between 15-48% of community-acquired pneumonia admitted to hospital (Brown and Lerner 1998, Lorente *et al.*, 2000, Lim *et al.*, 2001) and is implicated in two-thirds of bacteraemic pneumonia and fatal septicaemia in the USA (Bartlett 1997). Several studies conducted in developed countries reported that approximately 30-50% of all community-acquired pneumonia is caused by *S. pneumoniae* (Fedson *et al.*, 1994).

The pneumococcus also is a common commensal of the respiratory epithelium in healthy children and adults (Gray *et al.*, 1980). It colonizes asymptomatically and intermittently the nasopharynx of humans throughout life (Austrian 1986). The carriage of pneumococci is extremely common but the occurrence of invasive pneumococcal disease is relatively rare. Certain factors are associated with increasing the risk of pneumococcal disease, for example out-of-home child-care (Overturf 2000). Some groups are more predisposed to invasive pneumococcal diseases, such as people with humoral immune defects, patients with sickle cell disease, HIV infected children and adults and specific populations of children (Overturf 2000).
In developing countries, the prevalence of carriage approaches 95% in healthy children under the age of 3 years and 40% in adults (Austrian 1986, Lloyd *et al.* 1996). Carriage of up to four serotypes in the nasopharynx has been documented but most pneumococcal infections occur following the recent acquisition of a new serotype (Gray *et al.*, 1980; Hansman 1974). In a carriage study in children in the USA, it has been reported that the mean age of acquisition was 6 months (Gray *et al.*, 1980). In the first 24 months of life, 95 % of children were colonised at some time and 73% acquired at least two serotypes. It has been reported that local antibody protection may be important in limiting the duration of carriage since poorly immunogenic serotypes tend to be carried in the nasopharynx of young children for much longer than the more immunogenic serotypes (Gray *et al.*, 1980). In UK infants, 40% have had an episode of carriage by 3 months of age and 99% by two years (Musher *et al.*, 2000).

More than 90 capsular serotypes of *S. pneumoniae* have been characterised according to differences in the antigenic composition of their capsules (Henrichssen 1995, Kalin 1998). The pattern of serotype distribution is different between geographic locations and the carrier age groups (Henrichssen 1995, Kalin 1998). The transmission of

pneumococci can occur via airborne droplets (Fig.1), dust from dried secretions and contact with contaminated particles (Taussig 1984). It is worth noting that epidemiology and risk factors for pneumococcal diseases can be highly affected by the social and economic status of the population.

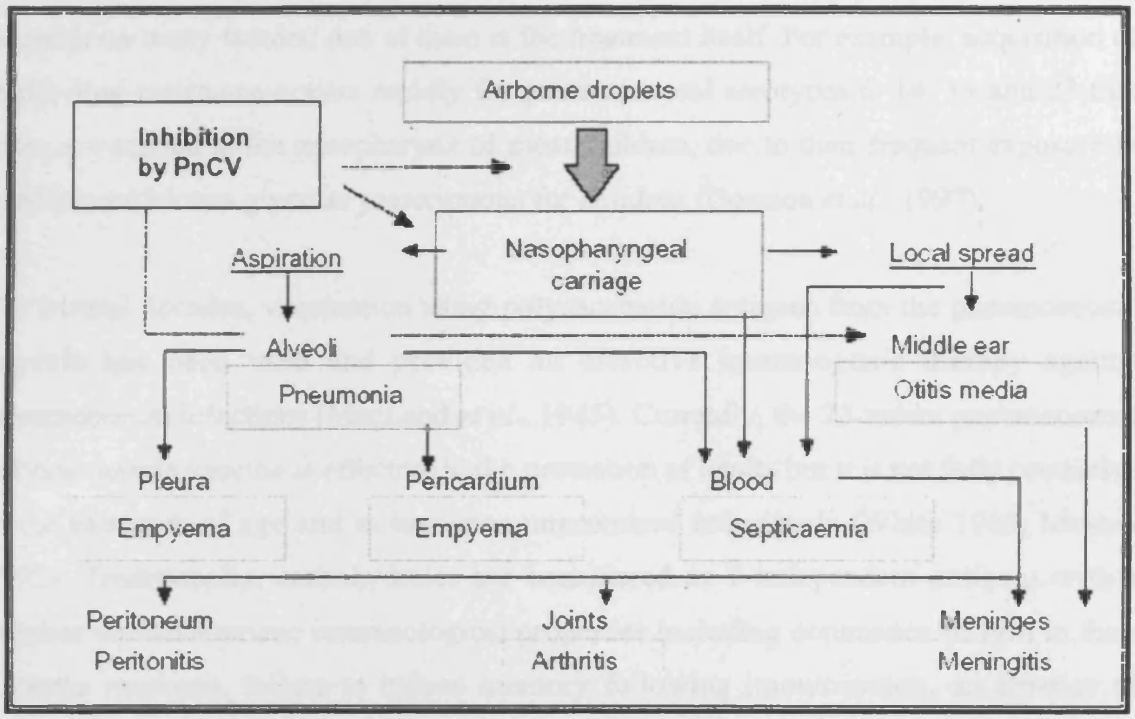



**Fig 1.1.** The pathogenesis of invasive pneumococcal disease and the sites of inhibition by the pneumococcal conjugate vaccine (Obaro and Adegbola 2002).

The translocation from the carriage status to the pneumococcal disease is not fully understood. For several decades, it has been accepted that pneumonia occurs after the aspiration of the pneumococci from the upper respiratory tract (Busse 1991, Johnston 1991) and their adherence to type II pulmonary cells (Cundell and Tournanen 1994). Also, a blood-borne route of bacterial dissemination from the upper respiratory tract has been suggested (Busse 1991).

Despite the advent of powerful antibiotics and the polysaccharide vaccine, mortality associated with invasive pneumococcal diseases has remained very high over the years. After a successful antibiotic treatment, the host can be free of pneumococci but death still occurs due to irreparable damage caused to the host (Austrian and Gold 1964).
Administration of penicillin is the most commonly used therapeutic approach in treatment of pneumococcal infection, however in the last three decades penicillin resistance in *S. pneumoniae* strains has become a clinical problem worldwide and the

level of resistance differs between geographical areas (Obaro 1996). Resistance occurs due to an alteration in penicillin binding proteins, thus reducing its ability to bind to pneumococcal cell wall components (Hoban *et al.*, 2001). Also, *S. pneumoniae* tolerance to vancomycin has been reported, one of the few antibiotics used for resistant strains (Novak *et al.* 1999). The acquisition of multi-drug resistance by pneumococcal strains depends on many factors; one of them is the treatment itself. For example, acquisition of multi-drug resistance occurs rapidly for pneumococcal serotypes 6, 14, 19 and 23 that often are carried in the nasopharynx of most children, due to their frequent exposure to multiple antibiotics given as prescriptions for children (Dowson *et al.*, 1997).

For several decades, vaccination using polysaccharide antigens from the pneumococcal capsule has been used and provided an effective immunogenic therapy against pneumococcal infections (MacLeod *et al.*, 1945). Currently, the 23-valent pneumococcal polysaccharide vaccine is effective in the protection of adults but it is not fully protective at the extremes of age and in immunocompromised individuals (White 1988, Musher 1992). Traditionally, carbohydrates are considered as T-independent antigens with a number of characteristic immunological properties including dominance of IgM in their immune response, failure to induce memory following immunisation, an absence of affinity maturation following immunisation, and poor immunogenicity in infants, the elderly and the immunocompromised (Goldblatt 1998, Eskola 2000). In addition, the protection elicited from antibodies generated after vaccination is serotype specific and there is little antibody cross-reactivity between pneumococcal serotypes (Shapiro 1991).

The conjugation of bacterial polysaccharide to carrier proteins, which has been successful for *Haemophilus influenzae* type b, was a landmark in the history of vaccine development. The conjugate vaccine converts the immune response to a T-cell dependent form, thus resulting in the development of immunological memory and improvement of the immunogenicity of polysaccharides, especially in infants (Käyhty and Eskola 1996, Adderson 2001). Conjugate-protein technology has been applied to pneumococcal polysaccharides and has shown excellent immunogenicity in children under two years of age, thus offering potential for the reduction of nasopahryngeal carriage of pneumococci and consequently transmission into disease status (Eskola 2000, Black *et al.*, 2000). Several studies in the Gambia, South Africa and Israel have shown a reduction in carriage of vaccine-type pneumococci following vaccination with

pneumococcal conjugate vaccines with increasing the risk of carriage of non-vaccine serotype (Obaro *et al.*, 1996, Mbelle *et al.*, 1999, Dagan *et al.*, 1997). A 7-valent polysaccharide-protein conjugate has recently been licensed in the UK, Europe and US after studies showed to be effective in preventing invasive diseases, pneumococcal pneumonia and otitis media (Sleeman *et al.*, 2001, McCraken 2000). However, the pneumococcal 7-valent vaccine would only provide approximately 35% coverage in Bangladesh while in South Africa, where the hugely increased burden of pneumococcal disease is related to HIV infection, it is estimated to prevent 74% of cases (3rd International Symposium on Pneumococci and Pneumococcal Diseases 2002). Research into the development of an effective pneumococcal conjugate vaccine that is effective worldwide is continuing. Ideally everyone, especially in developing countries, should have access to it. As quoted by the Global Alliance of Vaccine Immunisation (GAVI) in the 3rd International Symposium on Pneumococci and Pneumococcal Diseases 2002 'All of the world's children, without regard to socio-economic status or country of residence, have a fundamental right of life saving vaccines'. Due to the wide difference in the prevalence of disease-causing serotypes between geographical locations and the large number of pneumococcal carbohydrate serotypes that require inclusion in the pneumococcal conjugate vaccine, its formulation is far more complicated than that for *H. influenzae*. Therefore, studies on the mechanisms involved in the pathogenesis of pneumococcal disease, host-bacterial interactions and the host defences mechanisms are still underway to combat an old foe, *S. pneumoniae*.

## 1.2 Mechanisms of host defence against *S. pneumoniae*

Defence against pneumococcal infections depend upon non-immunological and immunological mechanisms involving the cooperation of humoral and cellular mechanisms (Johnston 1981, Austrian 1986, Gillespie 1989, Musher 1992). Like all respiratory pathogens, the pneumococcus will be faced first by local defences of the respiratory tract (Fig. 1.2) that are usually sufficient to eliminate the microbial invasion. Pneumococcal pneumonia occurs when the offending challenge overwhelms the resident lung defences thus leading to bacterial replication, inflammation and an immune response. The outcome of this fierce battle against the pneumococcus depends on microbial virulence, inoculum size and host susceptibility.

### 1.2.1 Local defences of the respiratory tract

Due to the anatomy of the respiratory tract, most inhaled or aspirated microorganisms will be stopped in the conducting airways before reaching the alveoli. The aerodynamic filtration and the gag reflex provide the first physical barriers against respiratory pathogens (Murray 1986). The frequent change of direction of the inhaled air before it reaches the gas exchange areas insures that most inhaled or aspirated microorganisms will be filtered and deposited on a mucosal surface and thus be subject to mechanical clearance or inactivation by mucosal secretion (Murray 1986). In addition, the integrity of the upper and lower respiratory tract epithelial surfaces composed of cells joined by apical tight junctions and backed by a basement membrane, provides a continuous barrier to microbial invasion (Murray 1986, Hogg *et al.,* 1979).

Mucosal immunity plays an important role in the first line of defence against infections with *S. pneumoniae* (Reynolds 1988). For example, the mucus, mainly composed of complex glycoproteins called mucin, covering ciliated epithelial cells prevents microbes from reaching target epithelial cells, thus leading to their removal by ciliary action (Lamblin and Roussel 1993). Other respiratory tract secretions, including lysozyme, lactoferrin and fibronectin, act by impairing the binding of microorganisms to respiratory mucosa, facilitating the interaction of infectious agents with phagocytes or by direct antimicrobial activity. For example, lysozyme was found to trigger autolysis of pneumococci by a non-enzymatic mechanism (Coonrod 1986, Cottagnoud and Tomasz 1993).

Complement components of the classical and alternative pathway have been identified in bronchoalveolar lavage fluid (Toews 1986). In the normal lung, the complement probably derives from the transudation of plasma as well as the synthesis of complement components by alveolar macrophages and epithelial cells (Toews 1986, Strunk *et al.,* 1988). Activation of complement results in the generation of a non-specific opsonin C3b, a chemotactic factor for neutrophils C5a and membrane attack complex (C5b-C9) that lyses susceptible bacteria (Densen 1990). The role of the complement system is particularly critical in the host defence against encapsulated respiratory pathogens such as *S. pneumoniae.* The interaction between complement and the pneumococcus will be reviewed in detail in section 1.3.

Immunoglobulins are present in the respiratory secretions, with IgA predominating in the upper respiratory tract and conducting airways and IgG more dominant in the alveoli (Reynolds 1991). Secretory IgA (sIgA) can be detected as early as 6 months whereas IgG is rarely detected before 18 months of age, thus sIgA is considered the most important mucosal factor in protection against *S. pneumoniae* carriage. Previous studies showed that systematic immunisation without previous exposure to the antigen through the mucosal surface does not usually lead to an IgA response (Tarkowski *et al.*, 1990). A recent study reported that pneumococcal polysaccharides induced functional secretory IgA in breast milk that can initiate killing and inhibition of adherence of *S. pneumoniae*, thus suggesting a role of sIgA in the development of mucosal vaccine (Finn *et al.*, 2002).

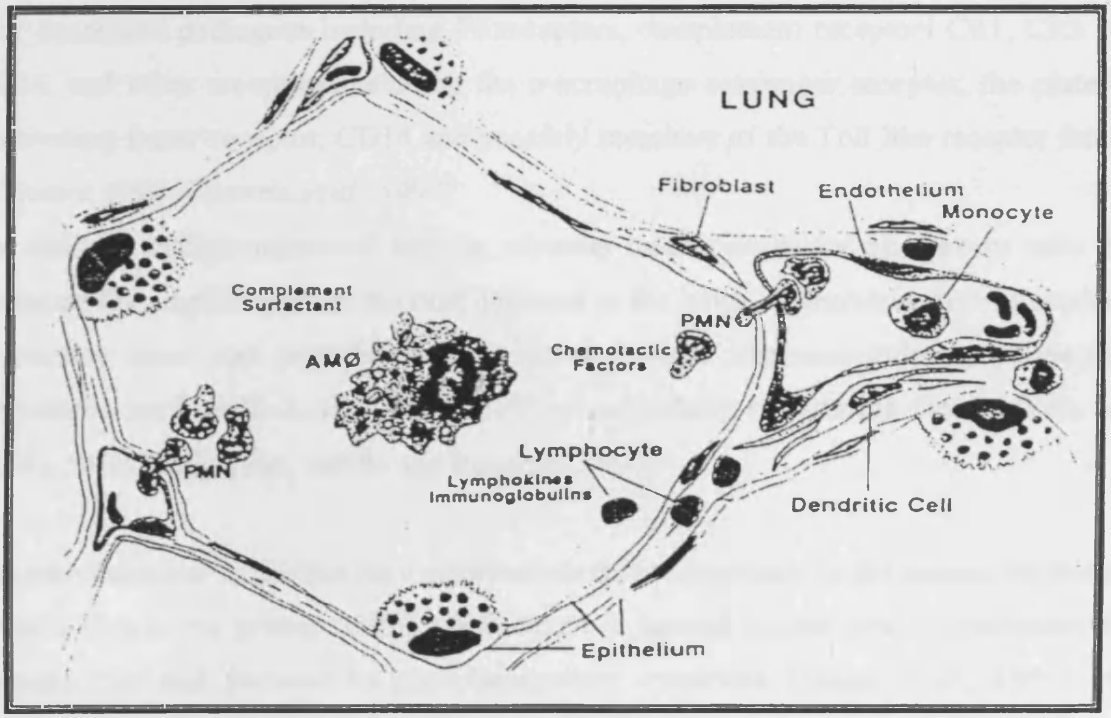



**Fig 1.2.** The resident and recruited immunological defences of the lung. AMØ = alveolar macrophage; PMN = polymorphonuclear leukocyte (Skerrett 1994).

It has been reported that effective opsonophagocytosis may take place in serum obtained from volunteers before vaccination thus giving evidence that non-type specific antibodies contribute to natural immunity against pneumococcal infections in humans (Musher *et al.*, 1986).

In the last decade, a great deal of studies highlighted the contribution of pulmonary surfactant, a lipid-protein complex lining the alveolar epithelium, as a first-line host

defence in the lungs (Wright 1997). Lung surfactants, their composition and role in innate immunity will be discussed more thoroughly in section 1.4.

The alveolar macrophages are the resident phagocytic cells of the normal lungs and are responsible, along with infiltrating neutrophils, for the removal of pathogens that invade gas-exchanging areas. Both complement and specific antibodies are required for optimal phagocytosis of encapsulated *S. pneumoniae* by alveolar macrophages and neutrophils recruited to the site of infection (Vial *et al.*, 1984, Guckian *et al.*, 1980, Jonsson *et al.*, 1985, Fels and Cohn 1986, Janoff *et al.*, 1992). In the non-immune host, bacteria will be opsonised by complement and other soluble factors for instance, surfactant protein A (Tino and Wright 1996). The surface of the macrophage displays a variety of receptors for opsonised pathogens including Fc receptors, complement receptors CR1, CR3 and CR4, and other receptors including the macrophage scavenger receptor, the platelet-activating factor receptor, CD14 and possibly members of the Toll like receptor family (Mosser 1994, Cauwels *et al.*, 1997).
In addition to their microbial activity, alveolar macrophages are pluripotent cells that regulate the amplification of the host defences in the lungs by secreting diverse products including lipid and peptides with chemotactic and immunoregulatory properties, cytokines such as IL-8, IL-1, IL-6, TNF-α and colony stimulating factors (Fels and Cohn 1986, Kelly 1990, Sibille and Reynolds 1990).

Another humoral factor that may contribute to innate immunity in the human respiratory tract is C-reactive protein (CRP). CRP, found in normal human sera, is synthesized by hepatocytes and induced by proinflammatory cytokines (Szalai *et al.*, 1997). An interesting study reported recently that CRP is present in inflamed and uninflamed secretions of the human respiratory tract in sufficient quantities for antimicrobial activity against *H. influenzae* (Gould and Weiser 2001). In addition, human respiratory epithelial cells are capable of expressing CRP thus suggesting that this protein may contribute to bacterial clearance not only in serum but as well in the human respiratory tract (Gould and Weiser 2001). Studies on the interaction between CRP, complement and the pneumococcus will be discussed in section 1.3.2.2.
Pneumococci that are not ingested and killed by neutrophils and macrophages during the inflammatory reaction in the alveoli, may escape into the interstitial tissues of the lung, where they drain to the regional lymph glands. From there, bacteria will be transported

via the lymph channels and the thoracic duct into the systemic circulation, causing bacteraemia (Austrian 1981).

### 1.2.2 Systemic defences

Systemic defences against invading respiratory pathogens involve an inflammatory response followed by a specific immune response.

The inflammatory response is initiated in the alveoli when the innate resident defences of the lung are insufficient to confront a microbial invasion thus bringing phagocytic cells (e.g. neutrophils, monocytes) and plasma proteins (e.g. specific antibodies, complement, CRP) to the site of infection. Neutrophils, which are more effective than alveolar macrophages in phagocytosis and killing of pathogens, have an essential role in pneumococcal clearance and the inflammatory reaction in the lungs (Hoidal *et al.*, 1981). After pneumococcal infection, neutrophils are rapidly recruited to the lung tissue while in normal circumstances they comprise only 2% of the cell population from human BAL (Reynolds and Newball 1974, Bergeron *et al.*, 1998, Kadioglu *et al.*, 2000). The cooperation of phagocytes is important in host defence, for instance the antimicrobial activity of neutrophils is stimulated by TNF-α, IL-1 and other factors of alveolar macrophages (Strieter *et al.*, 1993). The potent antimicrobial activity of neutrophils and macrophages include a respiratory burst that generates toxic products from the reduction of oxygen and granules containing an array of microbicidal proteins, including myeloperoxidase, lysozyme, lactoferrin, proteases and cationic proteins (Lehrer *et al.*, 1988, Sibille and Marchandise 1993, Austyn and Wood 1993).

The mechanism of pneumococcal clearance from the blood depends on the interaction of phagocytic cells (macrophages) and opsonins (e.g. type specific antibodies, complement) in the liver and spleen (Brown *et al.*, 1982, Joiner *et al.*, 1984, Frank 1987). Neutrophils are not essential in the removal of bacteria from the blood (Bruyn *et al.*, 1992). The absence of spleen or cirrhosis of the liver increases the risk of pneumococcal infections (Johnston 1981).

Similar to the non-specific immune response, humoral and cellular mechanisms of the specific immune response cooperate in the defence of the host against the encapsulated *S. pneumoniae*. In a mouse model of bronchopneumonia, accumulation of T and B cells at the site of inflammation suggested a role for lymphocytes in pneumococcal disease (Kadioglu *et al.*, 2000). Type-specific antibodies are produced directly by B lymphocytes, independently of T lymphocytes, to each capsular polysaccharide specific

for each pneumococcal strain. These antibodies are produced following an infection, colonization or vaccination with polyvalent pneumococcal vaccine. It is known that these type-specific anticapsular antibodies provide partial protection both in various animal species and in humans (Heidelberger *et al.*, 1946, Perimutter *et al.*, 1978). Antibodies to pneumococcal polysaccharides are predominantly of IgG2 subclass in adults and IgG1 subclass in children (Freijd *et al.*, 1984). In addition, antibodies directed against the cell wall protect against several pneumococcal strains in mice (Perimutter *et al.*, 1978, Briles *et al.*, 1981). Other antibodies directed against membrane-associated antigens have been identified. For example antibodies to pneumococcal surface protein A provide protection against several pneumococcal strains in mice (Szu *et al.*, 1983) while antibodies to the F polysaccharide failed to protect mice from pneumococcal infection (Au *et al.*, 1981).

Little is known regarding the role of T lymphocytes, during *in vivo* infection, in regulating the response to an intact *S. pneumoniae*. A recent study reported that the T cell help for induction of an antigen-specific *in vivo* humoral response is dependent on the type of pneumococcal antigen (Wu *et al.*, 2002). For instance, T cell help was required for an optimal anti-pneumococcal phosphorylcholine response while it was not the case for an anti-pneumococcal surface protein A response (Wu *et al.*, 2002). Furthermore, it was shown that CD4 T cells deliver help for both polysaccharide and protein specific antibody responses to intact *S. pneumoniae* in mice (Snapper *et al.*, 2001). A study on human patients infected with *S. pneumoniae* suggested that activated lymphocytes with type 1 cytokine profile are highly engaged in the *in vivo* immune response during pneumococcal infection (Kemp *et al.*, 2002).

## 1.3 Complement in pneumococcal diseases

The complement system is known to have a crucial role in the immune resistance against *S. pneumoniae*. Complement seems to be an important part of the innate humoral immunity that has been exploited by the late adaptive humoral response. The last major review on the role of complement in pneumococcal disease was by Winkelstein (1984), consequently this section will cover most of the studies that have investigated the interaction between complement and *S. pneumoniae* and the vital role of complement in host defence against the pneumococcus since that time.

### 1.3.1 The complement system

The complement system consists of a group of heat-labile self-assembling plasma proteins, which play a major role in host defence against infection and in immunopathologically-mediated inflammation. Microorganisms may activate the complement system by three pathways, in which the third component of complement C3 plays a central role, the classical, alternative and MB Lectin pathways (Fig 1.3).

#### 1.3.1.1. The classical pathway

The activation of the classical pathway, requiring the presence of calcium is usually triggered by the formation of an antigen-antibody complex. The binding of the appropriate antibody class or subclass (IgG1, IgG2, IgG3 and IgM in humans) to an antigen, such as would be found on microbial surface, activates the complement cascade. The Fc portion of the antibody molecule is then able to bind and activate the first component of the classical pathway (C1), which is a complex of three proteins, C1q, C1r and C1s. The C1q moiety is the portion of the intact molecule that binds to the antibody, leading to activation of C1r, which in turn activates C1s to generate an active serine protease. The C1s enzyme cleaves the plasma protein C4 to produce C4b, which binds covalently to the surface of the pathogen. The covalently attached C4b then binds one molecule of C2, making it susceptible to cleavage by C1s. Subsequently, C1s cleaves C2 to produce the large fragment C2a, which is itself a serine protease. The complex of C4b with the active serine protease C2a remains on the surface of the pathogen as the C3 convertase of the classical pathway. C4b2a cleaves C3 molecules to C3b, some of which bind to the pathogen surface and others are released to the fluid phase where they become biologically inactive and C3a is released to the fluid phase where it can act as anaphylatoxin initiating a local inflammatory response.

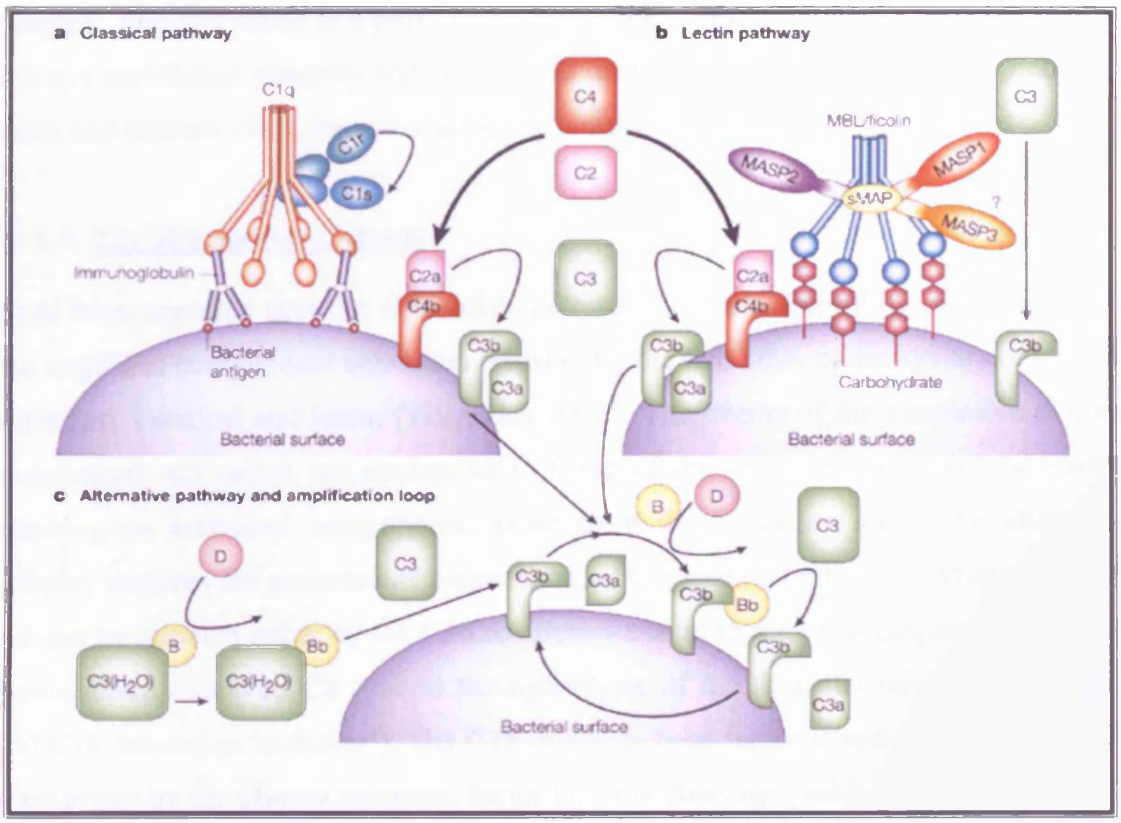



**Fig 1.3.** Activation of the classical, lectin and alternative complement pathways (Fujita 2002).

Complement component C3 is the most abundant complement protein in human plasma at 1 μg/ml and 250-500 μg/ml in murine plasma (M. Pickering, Imperial College, London, personal communication). The C3 molecule is composed of an α-chain of 115 KDa connected by a single disulfide bond to the β-chain of 75 KDa (Tack and Prahl 1976). When complement is activated, a small 10 KDa fragment of the C3 molecule, C3a is cleaved from the amino-terminus of the α-chain and the large fragment C3b having an exposed internal thioester bond binds covalently to the pneumococcal surface. The biochemical integrity of the C3 thioester bond is an absolute requirement for efficient phagocytosis that occurs when opsonically deposited C3b is recognised by complement receptors on phagocytic cells. Up to 1000 molecules of native C3 can be cleaved by just one C4b2a enzyme. Thus, the main effect of complement activation is to deposit large quantities of C3b on the surface of the initiating pathogen, where it forms a covalently bonded coat able to act either as an opsonin, or combine with C4b2a to form a new enzyme, the classical pathway C5 convertase (C4b,2a,3b). In this enzyme, the C3b acts to bind native C5, while the serine protease C2a will cleave C5 into two fragments of unequal size C5a and C5b. The small peptide C5a is a local inflammatory mediator. Finally, the generation of C5b subsequently leads to the assembly of terminal complement complex C5b, C6, C7, C8 and C9 and the formation of a membrane-attack

complex. The end result is a pore in the lipid bilayer membrane of a targeted cell that destroys membrane integrity and causes cell death (Winkelstein 1984, Complement in health and disease 1987, Immunobiology 4th edition 1999).

### 1.3.1.2. The alternative pathway

It has been reported that the alternative pathway is a self-amplifying mechanism that also amplifies complement activation initiated by the two other pathways of complement activation, classical and lectin (Volanakis 2002). The events of the alternative pathway complement activation are analogous to those of the classical pathway and involve homologous activated components, apart from the initiating step. The alternative pathway requires the presence of magnesium, is triggered directly on pathogen surfaces and can be initiated either by the C3b covalently bound to the pathogen surface or by the spontaneously cleaved C3 (due to the hydrolysis of the thioester bond in C3 to form C3(H2O) known as 'tickover'). The C3b is able to bind factor B and make it susceptible to cleavage by the plasma protease, factor D. This cleavage yields a small fragment Ba and an active protease Bb that remains bound to C3b to make the C3 convertase of the alternative pathway (C3bBb). This convertase is homologous structurally and functionally to the C3 convertase of the classical pathway. In this enzyme, C3b acts to bind native C3 and the Bb carries the active site able to cleave C3 molecule into C3a and C3b, identical to the one produced by the classical pathway. If some of the bound C3b binds to the pre-existing C3 convertase, it yields (C3bBb3b) the alternative pathway C5 convertase. C3bBb3b can cleave C5 into C5a, a potent inflammatory mediator and C5b, that initiates the generation of the membrane attack complex (Winkelstein 1984, Complement in health and disease 1987, Immunobiology 4th edition 1999).

### 1.3.1.3. The MB Lectin pathway

The mannan-binding lectin pathway (MBL pathway) is initiated by binding of a serum lectin, the mannan-binding lectin to mannose-containing carbohydrates on bacteria or viruses in a calcium dependent manner. MBL, along with C1q belong to the family of collectins, and is found in normal serum at low levels but it is also produced in increased amounts during the acute-phase response. The lectin pathway is activated by the binding of either MBL or Ficolin- associated with MBL- associated serine protease 1 MASP1, MASP2, MASP3 and small MBL associated protein (sMAP) to an array of carbohydrate groups on the surface of a bacterial cell. Similar to C1s, MASP2 is responsible for the

cleavage of C4 and C2 that leads to the generation of the same C3 convertase (C4b2a) as in the classical pathway. By contrast, MASP1 is able to cleave C3 directly thus resulting in activation of the alternative pathway. The following events are exactly analogous to the classical and alternative pathway. To date, the functions of MASP3 and sMAP are unknown (Thiel *et al.*, 1997, Dahl *et al.*, 2001, Fujita 2002). The detailed molecular mechanisms of the lectin pathway activation remain to be elucidated. The result of complement activation through its three pathways is the rapid saturation of the surface of the pathogen with C3b.

### 1.3.1.4. Control of the complement system

The activity of complement is modulated by a system of regulatory proteins that prevent tissue damage as a result of inadvertent binding of activated complement components to host cells or spontaneous activation of complement components in plasma (Winkelstein 1984, Complement in health and disease 1987, Immunobiology 4$^{th}$ edition 1999).
First, the enzymatic action of C1s can be inhibited by a control protein C1 esterase inhibitor (C1INH), which binds to activated C1r: C1s and causes it to dissociate from C1q, which remains bound to antibody on the pathogen. Secondly, the C4b fragment is rapidly inactivated in plasma at physiological temperature and the C3 convertase does not form. In addition, other control mechanisms inactivate any C3 or C5 convertase that accidently form on host cells. C2b can be displaced from the complex by either of two proteins- a serum protein called C4 binding protein (C4BP) or a cell-surface protein called decay-accelerating factor (DAF) which compete with C2b for binding to C4b. When C4BP binds to C4b, the latter becomes highly susceptible to cleavage by a plasma protein called factor (I) into the subfragments C4c and C4d. Furthermore, complement receptor (CR1) or a plasma protein factor H bind to C3b, displacing C2b or Bb and making C3 susceptible to cleavage by factor I. A second membrane associated protein called membrane co-factor protein (MCP) can bind to membrane associated C3b and catalyse its destruction by factor I. Finally, the activity of the terminal complement component is also regulated by cell surface proteins widely expressed which is the protectin or CD59 that prevents formation of the membrane attack complex on homologous cells. In addition, properdin is a control protein for the alternative pathway in normal serum that displays a stabilizing rather than a disruptive or degradative role by binding to C3b in (C3bBb) complex thus increasing the life of the C3/C5convertases. To

date, C1-inhibitor and alpha-2-macroglobulin are reported as regulators of the MBL-lectin pathway of complement (Gulati *et al.*, 2002, Petersen *et al.*, 2000).

### 1.3.2 Complement and *Streptococcus pneumoniae*

The complement system by its alternative, classical and lectin pathways, is an integral participant in the innate and acquired mechanisms of host defence in resistance to the respiratory pathogen *S. pneumoniae*. A great deal of knowledge has been generated in the last century on the interaction between the complement system and the pneumococcus. A study by Wright and Douglas (1903) was the first to show that serum elements were required for phagocytosis of *S. pneumoniae*. It was followed by another pioneer study that showed that phagocytosis of type 3 pneumococci *in vitro* was an extremely slow process in the presence of anticapsular antibodies and was not complete until nearly 8h, while by adding normal serum as a complement source, the process was completed within 2h (Ward and Enders 1933). Furthermore, depletion of complement C3 in rat serum with zymosan or cobra venom factor (CoVF) suppressed phagocytosis of encapsulated *S. pneumoniae,* thus leading to the confirmation that 'serum factors' required for this process were certainly complement proteins (Smith and Wood 1969).
Activation of complement will lead to the production of biologically active products important in generation of inflammatory response and defence of the host against pneumococcal infection. Since *S. pneumoniae* having a rigid cell wall means it is not susceptible to the lethal effect of the complement membrane-attack complex, the deposition of opsonic C3b on the pneumococcal surface is the principal mediator of pneumococcal clearance in the non-immune host (Winkelstein 1984).
A great number of studies concluded that C3 is the primary opsonin for *S. pneumoniae*.
A number of *in vitro* studies demonstrated that pneumococci are able to activate the complement system. When sensitised with the appropriate anticapsular or anti-pneumococcal cell wall antibodies, the classical pathway was activated (Johnston *et al.*, 1969, Brown *et al.*, 1982). The teichoic acid of the pneumococcal cell wall was shown to be responsible for alternative pathway activation (Winkelstein and Tomasz 1978). It is worth mentioning that the extent of complement activation differed between pneumococcal strains and serotypes (Edwards *et al.*, 1977, Brown *et al.*, 1982).
*In vivo* studies using experimental animals have been critical in establishing the biological significance of the activation of the complement system in clearance of the pneumococci from the bloodstream. When the complement system is compromised,

either by use of C4-deficient guinea pigs or by the use of CoVF-treated animals, the number of pneumococci in blood showed an initial decrease followed by an uncontrolled bacterial multiplication (Hosea *et al.*, 1980, Brown *et al.*, 1981). Furthermore, it appears that pneumococci opsonised by complement are cleared mainly in the liver and when complement-dependent opsonisation is impaired, the spleen plays an important role (Brown *et al.*, 1981).

It has been reported that complement-dependent opsonophagocytosis is a crucial defence against infection with *S. pneumoniae,* even in the presence of specific antibodies (Bruyn *et al.*, 1992). Janoff and colleagues reported that specific Ig-A mediated killing of *S. pneumoniae* by resting phagocytes was almost totally complement dependent (Janoff *et al.*, 1999). Studies showed that the opsonic potential of C3b, that is an important prerequisite for pneumococci destruction by phagocytic cells, is influenced by the mechanism by which complement is activated. Thus, classical pathway activation resulted in fixation of C3b to the capsular polysaccharide (Brown *et al.*, 1983), while in contrast activation of the alternative pathway resulted in fixation of C3b to the cell wall beneath the capsule (Winkelstein *et al.*, 1980, Hummell *et al.*, 1981), a location where the capsule could act as a mechanical barrier to the recognition of C3b by phagocytic cells.

After covalent deposition on the pneumococcal surface, C3b is cleaved by serum proteases to smaller fragments such as iC3b and C3d, which determine the subsequent interaction with complement receptors on leukocytes. Interestingly, the cleavage pattern of C3b differs among pneumococcal serotypes. The serotype-specific pattern of C3 fragmentation determines the subsequent disposition of opsonised pneumococci through their interaction with complement receptors on phagocytes or B lymphocytes (Hostetter 1986). It has been found at a cellular level that some pneumococcal surfaces halt C3b cleavage at C3d and will not generate iC3b. For instance *S. pneumoniae* serotypes 3 and 4, found mostly in adult infections, are highly resistant to phagocytosis and however potent immunogens. After opsonisation in non-immune serum, the predominant surface-bound C3 fragments was C3d that is recognised by receptors (CR2) on B lymphocytes, thus facilitating the interaction with B cells but retarding phagocytosis. Conversely, *S. pneumoniae* serotypes 6A and 14, most commonly found in childhood otitis, bacteraemia and meningitis are readily phagocytized but are poorly immunogenic. This is due to the presence of only the iC3b cleavage fragment on their surface. Therefore, iC3b readily acts as a ligand for complement receptor 3 on the surface of neutrophils,

thus enhancing phagocytosis and clearance of serotypes 6A and 14 and abolishing their interaction with B lymphocytes (Hostetter 1986). Two studies confirmed that pneumococcal polysaccharides bearing C3d could interact directly with B cells (Griffioen *et al.*, 1991, 1992). Studies also showed that surface-bound C3d is a potent adjuvant for both proteins and polysaccharides thus suggesting C3d as a candidate in the formulation of pneumococcal vaccines (Dempsey *et al.*, 1996). In addition, experiments using blockage of CR1 (C3b receptor) or CR3 (iC3b receptor) with monoclonal or polyclonal antibodies have confirmed that iC3b-bearing pneumococci are readily phagocytized via CR3 and are able to elicit the release of potent microbicidal factors (Gordon *et al.*, 1986, Hostetter and Johnson 1989).

A recent study using factor D-deficient mice demonstrated that the efficiency of phagocytosis of *S. pneumoniae* by C3 fragments depends critically on an intact alternative pathway amplification loop (Xu *et al.*, 2001). It is worth mentioning that natural antibodies, being mainly IgM isotypes, are spontaneously occurring immunoglobulins in naïve animals and in normal individuals in the absence of apparent antigen (Casali and Notkins 1989, Herzenberg and Kantor 1993). It has been shown that antiphosphorylcholine natural antibodies are protective against intravenous infection of mice with *S. pneumoniae* (Briles *et al.*, 1981).

Another component of the innate host defence is mannose-binding lectin (MBL) that is able to activate the lectin pathway of complement, in C1q- and antibody-independent manner, using MBL-associated serine protease 1(MASP-1) and MASP-2 (Sato *et al.*, 1994, Thiel *et al.*, 1997, Matsushita *et al.*, 1998). An *in vitro* study showed high levels of binding of radiolabelled MBL to *S. pneumoniae* (van Emmerik *et al.*, 1994). However, another group using flow cytometry and purified MBL isolated from immunocompromised children detected low levels of binding to *S. pneumoniae* (Neth *et al.*, 2000). Furthermore, bound MBL was able to promote C4 deposition in a concentration dependent manner (Neth *et al.*, 2000). A recent case-control study in the UK reported an association between mutant genotypes of the MBL gene and increased susceptibility to invasive pneumococcal disease in adults (Roy *et al.*, 2002). In the studied population, the frequency of homozygotes for particular MBL codon variants represented 5% (12% of patients with invasive pneumococcal disease compared to 5% of control groups were homozygotes for MBL codon variants) that could be a

substantially increased risk of invasive pneumococcal disease (Roy *et al.*, 2002). Further investigations are required to understand the mechanism by which MBL deficiency is involved in pneumococcal diseases.

Activation of complement is constantly controlled by a system of regulatory proteins. *In vitro* experiments showed that soluble complement receptor type 1 (CR1) exerts a concentration-dependent inhibitory effect on the phagocytosis of *S. pneumoniae* by neutrophils and at higher concentration, soluble CR1 also inhibits ingestion of bacteria which had been previously opsonised (Swift *et al.*, 1994). Furthermore, a significant inhibition of complement-mediated host defence against pneumococcal infection was detected in rats previously injected with soluble CR1 (Swift *et al.*, 1994).

### 1.3.2.1. Complement in the lung

At the time of primary contact with any microorganism, the role of the innate immune system including complement, natural antibodies, collectins including surfactant protein A and D, MBL and CRP is principal. Pneumonia caused by *S. pneumoniae* is a consequence of failure by the host to clear bacteria inhaled into the lung. A few studies have provided evidence that the local presence of complement components at the alveolar epithelium supports antibody-mediated killing of S. *pneumoniae.* In culture, isolated human alveolar macrophages and resting or activated type II epithelial cells synthesize and secrete specific components of the classical (C2, C3, C4, C5) and alternative (factor B) pathways (Ackerman *et al.*, 1978, Cole *et al.*, 1983, Strunk *et al.*, 1988, Zach *et al.*, 1993). In addition, low levels of some complement proteins have been detected in secretions and alveolar lining fluids obtained by lung lavage of humans (Reynolds and Newball 1974, Robertson *et al.*, 1976) and other species, such as rats (Coonrod and Yoneda 1981). Furthermore, functional complement components such as C1q, C3, C4 and factor B have been found in human saliva (Andoh *et al.*, 1997). In addition, it has been reported that complement components are secreted by recruited neutrophils (Botto *et al.*, 1992).

Watford and colleagues investigated the functional activity of the alternative and classical complement components in broncho-alveolar lavage fluid (BALF) of healthy volunteers. Using a standard haemolytic assay, a functional classical pathway was detected while in contrast there was no alternative pathway activity probably correlating with the low levels of factor B in the studied samples (Watford *et al.*, 2000).

Furthermore, since lung surfactant Sp-A and Sp-D belong to the same collectin family as C1q, their ability to substitute for C1q was investigated. However, neither Sp-A nor Sp-D restored classical pathway activity to C1q-depleted serum (Watford *et al.*, 2000). Surprisingly, western blots have showed that although MBL is easily detectable in rat serum, there is no detectable MBL in the rat lavage fluid (Watford *et al.*, 2000).

Multiple sources have been suggested for complement components detected in BAL. In addition to lung-specific production, serum complement components may exude through the alveolar epithelium during an infection (Watford *et al.*, 2000). Even though local complement components contribute relatively little to the total complement pool (the concentration of C3 at epithelial surfaces is 100 fold less than in plasma), it may play an important role in immediate protection within both alveolar spaces and parenchyma of the lung, in the early stages after infection, by providing a ready source of opsonin and initiating local inflammatory responses that recruit immune cells and serum complement to the site of infection (Watford *et al.*, 2000).

*In vivo* studies showed that complement plays a major protective role in the lung. Decomplemented mice that were infected intratracheally with virulent *S. pneumoniae*, were unable to clear the bacteria efficiently from their lungs and developed a severe pneumonia (Gross *et al.*, 1978, Coonrod *et al.*, 1983, Coonrod and Yoneda 1982). Other studies on the interaction between human alveolar macrophages and *S. pneumoniae* highlighted the importance of opsonisation by complement for an effective pulmonary clearance. A study reported that poor internalisation and killing by human alveolar macrophages occurred when the pneumococcus was not opsonised by complement (Gordon *et al.*, 2000).

In the immune host, complement and significant amounts of IgG found in the alveolar fluid (Reynolds 1991) act as pneumococcal opsonins, but in the non-immune host, phagocytosis is mainly dependent on opsonisation by complement, natural antibodies and other soluble factors such as CRP and Sp-A (Tino and Wright 1998).

#### 1.3.2.2. CRP, complement and *S. pneumoniae*

The protective effect of CRP is mediated by its ability to act as an opsonin by binding to phosphorylcholine on the pneumococcal surface and subsequently to activate the classical complement pathway through interaction with C1q (Kaplan and Volakanis 1974, Joiner *et al.*, 1984, Briles *et al.*, 1989). The importance of complement in CRP mediated host defence against pneumococcal infections has been studied (Szalai *et al.*,

1996, Nakayama *et al.*, 1983, Horowitz *et al.*, 1987) and the results indicated that CRP protection in pneumococcal infection involves both complement-dependent and complement-independent mechanisms. Using transgenic mice and immunization with CRP provided evidence on the role of this acute-phase protein during pneumococcal infection (Mold *et al.*, 1981, Yother *et al.*, 1982, Szalai *et al.*, 1995).

The innate opsonins that bind to phosphocholine on the pneumococcal surface, the human acute-phase CRP and naturally occurring anti-phosphocholine antibodies, require complement for effective protection of mice from lethal pneumococcal bacteraemia (Mold *et al.*, 2002). In addition, neither CRP nor anti-phosphocholine antibodies require FcγR (leukocyte receptors for CRP) to protect mice from infection (Mold *et al.*, 2002). This latter study supported the previous results showing that CRP activates complement through the classical pathway and increased the amount of C3b and iC3b bound to *S. pneumoniae* (Holzer *et al.*, 1984, Mold *et al.* 1982). However, the major acute phase reactant in the mouse, serum amyloid P component fails to protect mice from pneumococcal infection (Yother *et al.*, 1982).

Recently, CRP has been reported to be present in inflamed and uninflamed secretions of the human respiratory tract in sufficient quantities for antimicrobial activity (Gould and Weiser 2001). *In vitro* experiments demonstrated that purified CRP as well as CRP in sputum and nasal airway surface fluid, have antimicrobial effects on *H. influenzae* in the presence of complement (Gould and Weiser 2001).

#### 1.3.2.3 Novel mechanisms of interaction between complement and *S. pneumoniae*

The well-known mechanisms of complement resistance by *S. pneumoniae* consist of the presence of the capsular polysaccharide as well as the site, number and structure of deposited C3 fragments and their avidity for phagocytic receptors (Hostetter 1986, Gordon *et al.*, 1986, Akesson *et al.*, 1996). In addition, it has been reported that clinical and laboratory isolates of encapsulated and non-encapsulated pneumococci are able to degrade complement C3. This degradation activity is most prominent during exponential growth and results in the attack of the C3 β-chain, thus destabilising the C3 molecule (Angel *et al.*, 1994).

To date, several proteins of *S. pneumoniae* (pneumolysin, PspA and PspC) have been investigated for their interference with complement-mediated host defence, their role in pneumococcal pathogenicity and as candidate antigens for protein-based vaccine.

Pneumolysin, a cytolysin produced by all clinical isolates of *S. pneumoniae*, is a proven pneumococcal virulence factor (Paton *et al.*, 1993, Paton 1996). An *in vitro* study reported that highly purified pneumolysin (10 μg/ml) is capable of directly activating the classical complement pathway in the absence of anti-pneumolysin antibody, with a concomitant reduction in serum opsonic activity for *S. pneumoniae* (Paton *et al.*, 1984). A subsequent study showed that 1 μg/ml of pneumolysin is sufficient to activate complement, as detected by radioimmunoassay, and that this activation was mediated by the ability of pneumolysin to bind directly the Fc region of human IgG (Mitchell *et al.*, 1991). Subsequently, several *in vivo* studies investigated the effect of the ability of pneumolysin's complement activating activity and these are reviewed in section 1.5.1.

Pneumococccal surface protein A (PspA), a molecule that has been extensively studied in the last decade, was shown to be essential for virulence and to elicit protection against pneumococcal infections (McDaniel *et al.*, 1987, Briles *et al.*, 1998, Ogunniyi *et al.*, 2000). The specific mechanism by which PspA acts as a factor for virulence of pneumococci has not been identified. However, an *in vitro* study showed that heat-killed pneumococci lacking PspA fixed more complement than did heat-killed pneumococci bearing PspA (Briles *et al.*, 1998). In a subsequent study, $PspA^+$ and $PspA^-$ strains of type 3 pneumococci were used to investigate the effect of PspA on bacterial virulence and complement activation *in vivo*. Results showed that PspA reduces the amount of C3b deposited on the pneumococcal surface and/or inhibits the formation of a fully functional alternative pathway C3 convertase (Tu *et al.*, 1999). Based on this study, the functional property of PspA was concluded to be avoidance of interaction of *S. pneumoniae* with the host complement system, and furthermore PspA was proposed to function like the membrane regulators of complement activation, such as DAF, CR1, factors H and I (Tu *et al.*, 1999).

Another surface protein of *S. pneumoniae*, PspC, also known as SpsA, CbpA, PbcA and Hic, has been identified. Pneumococcal surface protein C (PspC) has been reported as a novel choline binding protein, CbpA, functioning as an adhesin (Rosenow *et al.*, 1997) and promoting invasion of epithelial cells (Zhang *et al.*, 2000), as a *S. pneumoniae* secretory IgA binding protein, SpsA (Hammerschmidt *et al.*, 1997), and as a C3 binding protein A, PbcA (Cheng *et al.*, 2000), and factor H-binding inhibitor of complement, Hic

(Janulczyk *et al.*, 2000). In addition, PspC elicits release of IL-8 from monolayers of human pulmonary epithelial cells (Madsen *et al.*, 2000).

PspC, by its different allelic forms corresponding to different biological activities, is considered to have a potential role in pneumococcal pathogenesis since it is conserved among several encapsulated clinical isolates, produced throughout pneumococcal growth and is present in both cell-bound and secreted forms (Cheng *et al.*, 2000). Furthermore, when PspC was used as an immunogen, it proved to be good candidate for a pneumococcal vaccine (Brooks-Walter *et al.*, 1999). Investigations of the functional importance of complement C3 as a substrate for PspC, reported a novel adhesive interaction between the pneumococcus and the epithelial barrier, suggesting the possibility of increase in bacterial infective capacity in the lungs and secretion of inflammatory mediators such as IL-8 (Smith and Hostetter 2000).

Finally, the interaction between complement C3 and the virulent pneumococcus decreases opsonophagocytosis and mediates attachment to type II epithelial cells, thus providing evasion from an integral part of the immune system.

Neeleman and co-workers reported that type 3 *S. pneumoniae* resistance to phagocytosis is mediated by an unknown pneumococcal surface protein binding to factor H and that PspA does not contribute to this interaction (Neeleman *et al.*, 1999). Subsequent studies identified the novel surface protein Hic (factor-H binding inhibitor of complement) in the PspC locus of type 3 pneumococci that was found to be responsible of factor H binding since mutant pneumococci lacking Hic showed no absorption of factor H (Janulczyk *et al.*, 2000, Dave *et al.*, 2001). Furthermore, utilization of type 2 *S. pneumonaie* strain D39 and its isogenic mutant strain lacking PspC (Hic) fortified the previous finding that factor H binding was PspC dependent. Moreover, PspC was shown to bind hydrophobically to the middle part of factor H, mainly the short consensus repeats 13 and 15. The consequence of factor H binding to Hic-expressing pneumococci resulted in restricted phagocytosis of the bacteria due to the enhancement of cleavage of C3b by factor I (Duthy *et al.*, 2002, Jarva *et al.*, 2002).

In 1999, Hostetter reported another two pneumococcal surface proteins of 29 and 20 KDa that have proteolytic activity against human C3. To date, one of the two proteins, the 20KDa proteinase (PhpA) was evaluated for its protective efficacy against pneumococcal infections. Intranasal immunisation with the 20KDa PhpA protein

resulted in no protection in mice while in contrast the recombinant protein of 79KDa (rPhpA-79) protected mice from death, bacteraemia and resulted in elicitation of antibodies and remarkable reduction in nasopharyngeal colonisation by type 3 and 14 *S. pneumoniae*. Thus, the workers speculated that rPhpA-79 may contribute to initial protection against not only systemic disease but also otitis media (Zhang *et al.*, 2001).

### 1.3.2.4 Complement deficiencies

Patients with complement deficiencies have been associated with an increased susceptibility to pneumococcal infection. Homozygous deficiency of the second component of complement C2 is the most common inherited deficiency of complement (Agnello 1978). Although C2 deficiency has been detected in asymptomatic individuals, these patients usually present with either autoimmune disease or recurrent pyogenic infection (especially in young children) due to *S. pneumoniae*. In the majority of these cases, the pneumococcus has been an etiologic agent causing pneumonia, osteomyelitis, sepsis and meningitis (Glovsky *et al.*, 1976, Newman *et al.*, 1979, Thong *et al.*, 1980, Hyatt *et al.*, 1981, Sampson *et al.*, 1982). An IgG subclass deficiency has been reported in these patients and furthermore one study associated C2 deficiency with low levels of factor B (Newman *et al.*, 1979). It was recently reported that a C2 deficient female child with recurrent pneumococcal infection showed impairment in specific IgG responses to vaccination against *S. pneumoniae* although IgG subclasses were normal (Attwood 2001).

Patients with genetically determined C3 deficiency were highly susceptible to recurrent pneumococcal infection and have diseases ranging from pneumonia to sepsis (Ballow *et al.*, 1975, Alper *et al.*, 1972, Davis *et al.*, 1977, Roord *et al.*, 1983, Ameratunga *et al.*, 1998).

Children with sickle cell disease (SCD) are vulnerable to invasive bacterial infection, most notably pneumococcal sepsis and meningitis (Robinson and Watson 1966, Barret-Connor 1971). Dysfunctions in IgG and IgM antibody responses, splenic clearance, alternative pathway fixation of complement and opsonophagocytosis are defects that play an important role in the predisposition of these patients to pneumococcal infections (Winkelstein and Drachman 1968, Johnston *et al.*, 1973, Bjornson *et al.*, 1977). To date, vaccination with the pneumococcal polysaccharide vaccine was not efficient in children

with SCD but preliminary data with the conjugate pneumococcal vaccines look promising in controlling pneumococcal infections in children with SCD, as well as normal children (Overturf 1999).

## 1.4 Surfactant protein D in pulmonary host defence

Early observations on the effects of natural surfactant on immune cell function were reported almost 30 years ago (LaForce *et al.*, 1973). In the 1980s, studies focused on the effect of surfactant lipid preparations. In the last decade, there has been less focus on the surfactant lipids that was associated with the finding that two of the surfactant proteins, surfactant protein A (Sp-A) and surfactant protein D (Sp-D) belongs to the collectin family that appears to be involved in the innate host defence system. Sp-A and Sp-D have received increasing attention as primary immunomodulators in the alveolar space. Numerous pieces of evidence from both *in vitro* and *in vivo* studies suggested that lung surfactant protein A and D are important elements in the first line of host innate immune defences and have an impact on later adaptive immunity. The emphasis of this section is on lung surfactant protein D and its involvement in the innate immunity.

### 1.4.1 Pulmonary surfactant

Pulmonary surfactant, a lipoprotein complex lining the airspaces, has a well-defined ability to lower surface tension at the alveolar air-liquid interface which results in enhanced lung compliance during expiration, thus reducing the work of breathing and preventing alveolar collapse at the end of the expiration (Haagsman and van Golde 1991). The presence of adequate functional amounts of surfactant covering lung epithelium is essential for this major function. Disruptions or deficiencies in alveolar surfactant pool size and composition may result in life-threatening diseases such as infant respiratory distress syndrome and acute respiratory distress syndrome (ARDS) in adults (Jobe and Ikegami 1998, Mason *et al.*, 1998).

By weight, normal surfactant isolated from humans and animals, is a complex mixture of 90% lipids and 10% proteins. The use of different methods for surfactant purification from lavage fluid, such as the density gradient centrifugation and differential centrifugation, affected the apparent composition of surfactant and thus the results obtained from different studies (King and Clements 1972, Wright *et al.*, 1984).

The lipids in surfactant are primarily phospholipids; phosphatidylcholine (PC) and dipalmitoylphosphatidylcholine (DPCC) are highly abundant. Phosphatidylglycerol

(PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are other phospholipids that contribute to lipid surfactant composition including also cholesterol, sphingomyelin and fatty acids (Wright *et al.*, 1997).

To date, four specific proteins have been found in lavage fluid and have been designated surfactant proteins: Sp-A, Sp-B, Sp-C and Sp-D. Alveolar type II cells, which cover ~ 7% of the alveolar epithelial surface, are a major source of surfactant proteins and lipids. However, all the surfactant proteins, except for Sp-C, are synthesized by not only alveolar type II cells but also by non-ciliated Clara cells of the airway (Auten *et al.*, 1990, Wong *et al.*, 1996). A major clearance pathway of surfactant appears to be uptake by type II cells that recycle, degrade and then reutilise surfactant components. Furthermore, alveolar macrophages are able to degrade a significant fraction of surfactant (Wright and Dobbs 1991).

Sp-B and Sp-C are the highly hydrophobic surfactant proteins and both are classified as proteolipids. Identification of patients who are genetically deficient in Sp-B and had also low levels of Sp-C showed that both proteins are involved in the reduction of surface tension by surfactant in the lung but to date no identification of a role in host defence has been reported for these two proteins (Ballard *et al.*, 1995, Vorbroker *et al.*, 1995).

Although having high tendency to self-associate, Sp-A and Sp-D are relatively hydrophilic (Haagsman *et al.*, 1990, Wright 1997). The most characterized component, surfactant protein-A, is involved in tubular myelin formation (Williams *et al.*, 1991), enhances the rate of adsorption of surfactant lipids to a surface and appears from *in vitro* tests to be involved in the regulation of surfactant metabolism (Hawgood *et al.*, 1987). Surprisingly, no abnormalities in histology, lung wet weight or lung volume could be detected in Sp-A deficient mice completely lacking tubular myelin, thus suggesting that Sp-A does not have an essential role in the reduction of surface tension (Korfhagen *et al.*, 1998). In contrast to Sp-A, lung surfactant protein D does not bind to the major surfactant lipids but does bind to phosphatidylinositol (Persson *et al.*, 1992). Deleting the Sp-D gene in mice resulted in an accumulation of surfactant lipids in alveolar spaces, thus indicating that Sp-D is involved in pulmonary surfactant homeostasis (Botas *et al.*, 1998).

It is worth mentioning that preparations used for surfactant replacement therapy are composed of synthetic lipids and only a few of these preparations contain a protein fraction of 1 % of Sp-B and Sp-C (Soll 1997).

**Host defence functions of 'whole surfactant'**

Before the identification of surfactant proteins, many studies have investigated the effects of 'whole surfactant' and its lipid components on immune cell function *in vitro*. Unfortunately, some of these studies yielded conflicting results probably, due to different methods used to isolate surfactant thus affecting their composition. Reported studies showed that the phospholipid component of surfactant has both immune enhancing and inhibitory effects. Surfactant, mainly lipids, isolated by centrifugation of lavage fluid enhanced killing but not phagocytosis of *S. aureus* by alveolar macrophages (LaForce *et al.*, 1973, Juers *et al.*, 1976). However, human surfactant purified by gradient centrifugation stimulated phagocytosis, but not killing of *S. aureus* by human alveolar macrophages and had no effect on *S. pneumoniae* phagocytosis and killing while rat lung lavage enhanced both phagocytosis and killing (O'Neill *et al.*, 1984, Jonsson *et al.*, 1986). Coonrod and co-workers reported that many of the immunoregulatory roles of surfactant could be attributed to phospholipids and free fatty acids found in surfactant (Coonrod *et al.*, 1986, Coonrod and Yoneda 1983). More recent studies showed that a surfactant pellet enhanced phagocytosis of *S. aureus* by alveolar macrophages (van Iwaarden *et al.*, 1990), probably due to Sp-A rather than surfactant lipids (Tino and Wright 1996).

Furthermore, surfactant lipids, mainly DPCC, have been reported to suppress the production of reactive oxygen species by stimulated alveolar macrophages and monocytes (Hayakawa *et al.*, 1989, Geertsma *et al.*, 1993). Pre-treatment of alveolar macrophages with DPCC decreased the migration of cells stimulated by activated serum (Zeligs *et al.*, 1984). In addition, DPCC and PG were reported to inhibit or to regulate lymphocyte proliferation *in vitro* and in disease state (Catanzaro *et al.*, 1988, Kremlev *et al.*, 1994, Lesur *et al.*, 1994).

A recent study reported that surfactant lipids may not always have a protective effect but may in some situations inactivate the innate defences. For instance, the ability of CRP to block the attachment to host cells of *S. pneumoniae* and *H. influenzae* expressing cell-surface phosphorylcholine can be diminished in the terminal airway by human surfactant, probably due to CRP binding to DPPC (Gould and Weiser 2002).

It is worth mentioning that surfactant lipids and proteins may have counter-regulatory effects. For instance, surfactant lipids inhibit lymphocyte proliferation while in contrast Sp-A stimulates their proliferation (Kremlev *et al.*, 1994).

### 1.4.2 Collectin family

Collectins are present in mammalian serum, lung secretions and on various mucosal surfaces where they play roles in innate immunity against bacterial, viral and fungal pathogens. The pulmonary surfactant proteins Sp-A and Sp-D are members of the collectin family (Sastry and Ezekowitz 1993).

This C-type calcium-dependent lectin family consists of Sp-A and Sp-D produced mainly in the lung, the serum mannose-binding lectin (MBL) (Drickamer *et al.*, 1986), serum bovine conglutinin (Lee *et al.*, 1991), serum bovine collectin-43 (Lim *et al.*, 1994), and the recently described human liver collectin CL-L1 (Ohtani *et al.*, 1999).

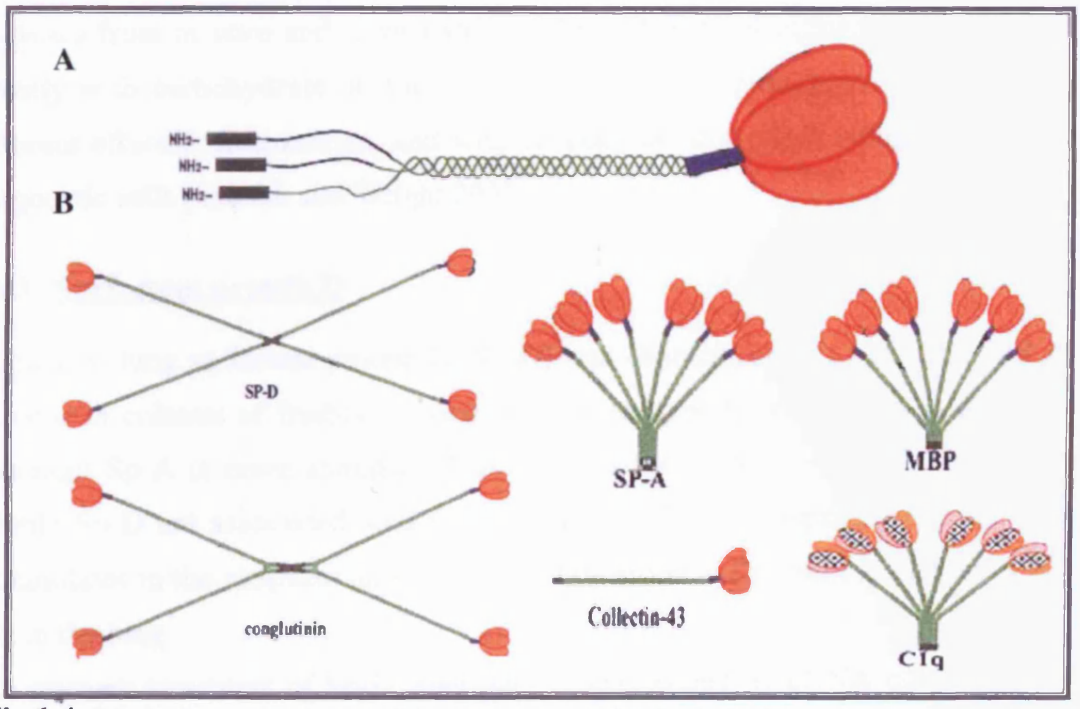



**Fig 1.4**. Schematic representations of domain structure of collectins and complement component C1q. Top panel A: collectin molecule composed of 3 polypeptide chains. Bottom panel B: molecules of the collectin family (Sp-D, conglutinin, Sp-A, MBP and collectin-43) and C1q. Drawings are to scale based on electron microscopic analysis (Hoppe and Reid 1994).

The collectins are assembled as oligomers of trimeric subunits. Each subunit consists of a short amino-terminal domain (N) involved in disulfide bond formation, followed by a triple helical long collagen domain (C), a short neck region corresponding to a trimeric coiled-coil linking domain (L) and a C-type lectin carbohydrate recognition domains

(CRD) involved in calcium-dependent binding to carbohydrates (Hoppe and Reid 1994, Holmskov *et al.*, 1994).

Multimerization seems to be an important determinant in the interaction of collectins with immune cells and pathogens (Brown-Augsburger *et al.*, 1996).

Predominantly, Sp-A and MBP form a bouquet-like octadecamers consisting of six trimeric subunits. Each monomer consists of 26-35 KDa, but also smaller oligomeric forms have been identified (Hickling *et al.*, 1998, Lipscombe *et al.*, 1995).

With their extended collagen domain, Sp-D and conglutinin are predominantly assembled as dodecamers consisting of four trimeric subunits, each composed of 43 KDa monomers. Larger and smaller forms of Sp-D have been identified (Lu *et al.*, 1993, Crouch *et al.*, 1994, Hoppe and Reid 1994). It is worth mentioning that the maximum molecular dimension for octadecamer Sp-A is approximately 20 nm compared with 100 nm for dodecamer Sp-D having a cruciform structure with four arms of equal length (Hoppe and Reid 1994).

Evidence from *in vitro* and *in vivo* studies showed that collectins specifically interact directly with carbohydrate on the surface of microbial pathogens, thereby initiating different effector mechanisms, and with leukocytes, thus modulating the function of phagocytic cells (Crouch and Wright 2001).

### 1.4.3 Surfactant protein D

Originally, lung surfactant protein D (Sp-D) was characterised as a glycoprotein (CP4) secreted in cultures of freshly isolated rat alveolar type II cells (Persson *et al.*, 1988). Although Sp-A is more abundant than Sp-D in the alveoli, a significant fraction of soluble Sp-D not associated with lipid surfactant (50-90% depending on the species) accumulates in the airspaces and candidates this surfactant protein to play an important role in the lung.

The primary structures of Sp-D have been characterized by cDNA cloning in several mammal species including human (Rust *et al.*, 1991, Lu *et al.*, 1992), rat (Shimizu *et al.*, 1992) and mouse (Motwani *et al.*, 1995). The human Sp-D gene is 11 Kb and is comprised of eight exons, encoding for different regions of the molecule (Crouch *et al.*, 1993).

As reported for protein pulmonary surfactant, the main sites for Sp-D synthesis and secretion is alveolar type II cells and non-ciliated airway epithelial cells (Clara cells) (Voorhout *et al.*, 1992). However, it has been suggested that the processing of Sp-D is

different between the two types of cells (Mason *et al.*, 1998). Interestingly, several studies reported the expression of Sp-D at extra-pulmonary sites for instance in the epithelium of the conducting airways, in the tracheal and bronchial glands of the lower airways (Wong *et al.*, 1996), extrapulmonary sites in murine tissues (Motwani *et al.*, 1995) and in rat gastric mucosa (Fisher and Mason 1995). More recent studies using immunohistochemistry and RT-PCR demonstrated that Sp-D, which unlike Sp-A restricted to the respiratory tract, is generally present on mucosal surfaces, sites in which 95% of infections are initiated. Sp-D mRNA expression was detected in trachea, brain, heart, kidney, pancreas, gastric mucosa and other human tissues (Madsen *et al.*, 2000, Murray *et al.*, 2002) and in uterus, ovary and lacrymal gland of the mouse (Akiyama *et al.*, 2002). In addition, it has been reported that Sp-D is detected in the epithelial lining of the Eustachian tube (ET) of the middle ear (Paananen *et al.*, 1999) and is expressed and secreted by specialized cells in the ET (Paananen *et al.*, 2001).

Kuan and co-workers (1992) were the first to suggest a role for Sp-D in pulmonary innate host defence. However, the localization of Sp-D at extrapulmonary surfaces suggests this collectin to participate in mucosal immunity throughout the body.
According to the Sp-D protein structure described in 1.4.2, studies showed that the CRD of Sp-D is responsible for its lectin activity and trimeric clusters of Sp-D CRDs are required for high-affinity binding to multivalent ligands (Kishore *et al.*, 1996, Hakansson *et al.*, 1999).

Several studies reported an estimation of the normal concentration of Sp-D in the airways and showed that it may increase during infection. In humans, the concentration of Sp-D in lavage isolated from healthy volunteers was 0.88 μg/ml (Honda *et al.*, 1995). Kuroki and co-workers (1991) estimated the concentration of Sp-D in the rat hypophase ranging from 36 to 216 μg/ml. However, other reported that the physiological concentration of Sp-D in rodent lung lavage fluid is about 0.552 μg/ml from a normal mouse aged 6-8 weeks (Reading *et al.*, 1997).
The lavage fluid of patients with alveolar proteinosis or silica-treated rats are often used as source for Sp-D. Mostly, Sp-D is isolated by maltose affinity chromatography by size exclusion chromatography (Persson *et al.*, 1990). Remarkably, the degree of multimerization of the purified Sp-D affects its function (Hartshorn *et al.*, 1996). A recombinant fragment of human Sp-D (rSp-D) of 60 KDa composed of the trimeric α-

helical coiled coil neck region and three CRDs, lacking the collagen-like region present in the intact Sp-D molecule, is readily produced in large amounts by *E.coli*. rSp-D was found to be similar to native Sp-D in its ability to bind simple sugars, phospholipids and malyosyl-bovine serum albumin (Kishore *et al.*, 1996).

### 1.4.3.1 *In vitro* interactions of Sp-D

A multitude of *in vitro* studies have shown that pulmonary collectin Sp-D and Sp-A bind and aggregate a wide variety of microorganisms (bacteria, fungi, virus, mycobacteria), directly activate macrophages and affect the *in vitro* phagocytosis and killing of a variety of pulmonary pathogens (Wright 1997).

**Sp-D interaction with diverse carbohydrate, lipid and conserved cell wall conjugates:**

All the *in vitro* studies showed that the carboxy-terminal domains of Sp-D and Sp-A are responsible for their lectin activity, and trimeric clusters of the Sp-D CRDs are required for high affinity binding to multivalent ligands. Furthermore, due to its structure, Sp-D has a great capacity to mediate bridging interactions between binding sites on different particulate ligands (Kishore *et al.*, 1996, Hakansson *et al.*, 1999). Lung Sp-D, along with Sp-A, can bind to mannose, glucose and show little interaction with galactose. However, each protein shows selectivity in saccharide (Haagsman *et al.*, 1987, Persson *et al.*, 1990). Human Sp-D preferentially interacts with inositol, maltose and glucose that are commonly found on bacterial surfaces (Persson *et al.*, 1990, Lim *et al.*, 1994). Furthermore Sp-D broadly recognises the core region of LPS of Gram-negative bacteria (Kuan *et al.*, 1992), cell wall conjugates of *Mycobacterium tuberculosis* (Ferguson *et al.*, 1999), LPS extracted from three *Helicobacter pylori* strains with a marked variation in the avidity of binding among the strains (Murray *et al.*, 2002) and lipoteichoic acid and peptidoglycan, two major cell wall components of Gram-positive bacteria (van de Wetering *et al.*, 2001). In these studies, binding of Sp-D took place in a calcium-dependent manner and was carbohyrate inhibitable thus indicating that the CRD is responsible for Sp-D binding to ligands. Additionally, Sp-D has been reported to interact *in vitro* with lipid ligands, thus suggesting a role for Sp-D in surfactant lipid reorganization. Sp-D interacts in a complex manner with inositol and lipid moieties of phosphatidylcholine PI (Persson *et al.*, 1992, Ogasawara *et al.*, 1994, Saitoh *et al.*, 2000, Sano *et al.*, 1998) and with glucosyl-ceramide (Kuroki *et al.*, 1992).

**Sp-D interaction with bacteria:**

Several studies demonstrated that lung surfactant protein D is able to bind to a variety of bacteria including Gram-negative, *Mycobacterium tuberculosis* and Gram-positive bacteria in a calcium- and saccharide- dependent manner. The effect of binding can be inhibited by competing sugars such as maltose or mannose. As a result of Sp-D binding, microorganisms can be aggregated and/or opsonised, in many cases leading to enhanced killing and clearance by phagocytic cells. Studies reported the ability of Sp-D to bind and aggregate several Gram-negative bacteria such as *E. coli* (Kuan *et al.,* 1992, Pikaar *et al.,* 1995), *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Lim *et al.,* 1994). More recently, Sp-D has been reported to interact in a specific lectin manner with LPS of encapsulated phase variants of *K. pneumoniae*, a common cause of nosocomial pneumonia and *Helicobacter pylori* infecting persistently the gastric mucosa (Ofek *et al.,* 2001, Murray *et al.,* 2002).

The use of flow cytometric and fluorescent-microscopic assays, it has been demonstrated that Sp-D binds and aggregates Gram–positive bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (Hartshorn *et al.,* 1998). Furthermore, dodecameric Sp-D is essential for agglutination of the virulent Erdman strain of *Mycobacterium tuberculosis* in the presence of calcium (Ferguson *et al.,* 1999, 2002). It is worth mentioning that most of the studies indicated that the degree of multimerization of the Sp-D preparation is a critical determinant of both aggregating activity and potency in enhancing bacterial uptake by phagocytes.

**Sp-D interaction with fungi and yeast:**

The lung surfactant protein D shows, in the presence of calcium, specific binding to cell wall conjugates expressed by fungi and yeast via its CRD. Sp-D binds to the surface of the cyst, precyst and trophozoite of *Pneumocystis carini* (O'Riordan *et al.,* 1995). β-glucans have been implicated in the interactions of Sp-D with pneumocystis (Vuk-Pavlovic *et al.,* 2001). Interestingly, Sp-D was found to be associated with cysts and trophozoites in the airspaces of rats with pneumocystis pneumonia and the recovered aggregated organisms showed disruption in the absence of calcium and their agglutination was reversible when Sp-D was added (Kwon *et al.,* 1998). Furthermore, a recent study showed that the CRD of Sp-D interacts with *P. carini* gpA through its attached oligosaccharide and that the extent of binding is higher with dodecamers and higher order forms of Sp-D (Vuk-Pavlovic *et al.,* 2001). Rat and human Sp-D show

CRD-dependent binding to the N-linked sugars of specific cell wall glycoproteins including major glycoprotein allergens of *Aspergillus fumigatus* (Madan *et al.*, 1997, Allen *et al.*, 1999).

Recent studies suggest that Sp-D may have a direct effect on metabolism and hyphal growth of *Candida albicans*, an important respiratory fungus (van Rozendaal *et al.*, 2000). Other studies reported interaction of Sp-D in a lectin-dependent fashion with the pathogenic acapsular form of the yeast *Cryptococcus neoformans* (Schelenz *et al.*, 1995) and the wild-type *Saccharomyces cerevisiae* (Allen *et al.*, 2001). Furthermore, rSp-D and Sp-D were found to bind to the carbohydrate residues on the surface of the purified allergen Der $_{P}$ I and the whole mite extracts *Dermatophagoides pteronyssinus* (Wang *et al.*, 1996).

**Sp-D interaction with respiratory viruses:**

The specific interaction of lung surfactant protein D with various respiratory viruses is mediated, in the presence of calcium by CRD-dependent interaction with viral envelope glycoproteins. Purified Sp-D binds to and induces massive aggregation of the Influenza virus type A and inhibits its hemagglutination activity. Viral aggregation is calcium dependent, inhibited by competing sugars and correlates with the multimerization state of Sp-D (Hartshorn *et al.*, 1994, 1996, 1997). Furthermore, Sp-D and rSp-D bind to the respiratory syncytial virus (RSV) G protein and inhibit viral infectivity by CRD-dependent mechanism (Hickling *et al.*, 1999).

It is worth mentioning that the collectin surfactant protein A binds and interacts with most of the organism recognised by surfactant protein D. However, several workers based on different observations in the functional consequence of collectin binding, suggested that the mechanism of interaction of the two collectins with the microbial surfaces are often distinct. For instance, Sp-A binds to di-mannose repeating units and to lipid A domain in Gram-negative LPS whereas Sp-D preferentially recognises core oligosaccharides of LPS (Kabha *et al.*, 1997, van Iwaarden *et al.*, 1994).

Interesting recent studies investigated the contribution of the N- and C- terminal domains of the Sp-D molecule to binding, aggregation and phagocytic uptake of bacteria and virus. In these studies, chimeric collectins consisting of the Sp-D molecule in which the neck and CRDs were replaced with those of MBL and conglutinin were used.

The Sp-D/$MBL_{neck+CRD}$ and Sp-D/$Cong_{neck+CRD}$ chimeras showed viral neutralisation, aggregation and uptake by neutrophils greater than wild-type Sp-D, MBL or conglutinin (Hartshorn *et al.*, 2000, White *et al.*, 2000). It has been suggested that MBL or conglutinin CRD increased viral binding activity compared to wild-type Sp-D while the N-terminal and collagen domains of Sp-D increased viral aggregation and neutrophil uptake of the virus compared to that of wild-type MBL or conglutinin. However, testing these chimeras with several strains of Gram-positive and negative bacteria showed that they have significantly greater ability than MBL or conglutinin to aggregate and/or bind virus but in contrast, had unchanged or reduced ability to interact with bacteria when compared to the wild-type Sp-D (Hartshorn *et al.*, 2002). These studies suggest that requirement for optimal collectin binding differs between bacteria and Influenza virus.

**Sp-D interaction with immune cells:**

A variety of *in vitro* data showed that Sp-D affects immune cell functions. To date, Sp-D is not reported as a significant, effective opsonin to enhance uptake of particles by macrophages and monocytes. However, Sp-D enhances modestly the phagocytosis of *P. aeruginosa* by alveolar macrophages (Restrepo *et al.*, 1999) and inhibits phagocytosis of *P. carin_* (Vuk-Pavlovic *et al.*, 2001), *C. albicans* (van Rozendaal *et al.*, 2000) and *M. tuberculosis* by alveolar macrophages (Ferguson *et al.*, 1999). Further investigations showed that the reduction in *M. tuberculosis* phagocytosis is independent of bacterial agglutination (Fergusson *et al.*, 2002).

In addition, Sp-D stimulates chemotaxis of alveolar macrophages (Tino and Wright 1996) and has been reported to both have no effect (Tino and Wright 1999) or enhance (Crouch *et al.*, 1995) chemotaxis of monocytes.

An initial study showed that rat Sp-D, in the absence of activators, enhances the production of oxygen radicals by rat alveolar macrophages (van Iwaarden *et al.*, 1992). Furthermore, precoating of unencapsulated phase variants of *K. pneumoniae* with Sp-D enhances phagocytosis of these organisms and production of nitric oxide by rat alveolar macrophages (Ofek *et al.*, 2001) and stimulates interleukin secretion and cytokine mRNA expression by both macrophages and peripheral blood monocytes (Keisari *et al.*, 2001). It was recently reported that a truncated 60 KDa fragment of human Sp-D (rSp-D) binds preferentially to apoptotic and necrotic alveolar macrophages *in vitro* (Clark *et al.*, 2002).

In addition, Sp-D affected neutrophil responses *in vitro*. Several studies showed that Sp-D enhances neutrophil binding and/or uptake of Influenza A virus (Hartshorn *et al.*, 1994, 1996, 1997), phagocytosis of *E. coli*, *S. pneumoniae* and *S. aureus* (Hartshorn *et al.*, 1998) and oxidative burst of neutrophils in response to *Aspergillus fumigatus* conidia (Madan *et al.*, 1997). It is worth mentioning that enhanced phagocytosis was strongly dependent on the extent of multimerization of Sp-D (Hartshorn *et al.*, 1997, Brown-Augsburger 1996).

Studies reported a role for Sp-D and rSp-D in attenuating lymphocyte responses by inhibiting human T lymphocyte proliferation and IL-2 cytokine production, a potent mitogen for lymphocytes (Borron *et al.*, 1998). An investigation on this direct suppression of Th cell function demonstrated that Sp-D inhibits CD3 (+)/ CD4(+) lymphocyte proliferation via two mechanisms, an IL-2 dependent mechanism observed with accessory cell-dependent T cell mitogens and specific antigens, as well as an IL-2-independent mechanism of suppression that potentially involves attenuation of free calcium (Borron *et al.*, 2002). Interestingly, recent studies showed interaction of Sp-D with immature dendritic cells, the most potent antigen presenting cells in a dose-, calcium- and carbohydrate manner whereas binding to mature dendritic cells was reduced (Brinker *et al.*, 2001). Additionally, Sp-D binds to bacteria (*E. coli*) and enhances their association with dendritic cells. Furthermore, Sp-D uniquely enhances the antigen presentation by mouse bone marrow-derived dendritic cells of an ovalbumin fusion protein expressed in *E. coli* to ovalbumin-specific MHC class II T cells hybridomas (Brinker *et al.*, 2001, Shepherd 2002). However, another study showed that Sp-D-*M. tuberculosis* complexes are taken up by immature dendritic cells in the lung interstitium (Gonzalez-Juarrero and Orme 2001).

#### 1.4.3.2 Sp-D in the adaptive immunity

Although Sp-D, along with Sp-A, are known for almost a decade to be mediators in innate immunity, the effects of these proteins on adaptive immunity has not been highly investigated. However, the *in vitro* interactions reported for lung surfactant protein D with T lymphocytes and dendritic cells defined this collectin as a novel link between innate and adaptive immunity. Furthermore, lung Sp-D and Sp-A can interact with the glycosylated antigens and allergens of *Aspergillus fumigatus* thus inhibiting specific IgE binding to these allergens and consequently blocking histamine release from sensitised basophils (Madan *et al.*, 1997, 2001). Additionally, recent studies using a murine model

of allergic pulmonary inflammation investigated possible links between Sp-D and Sp-A and responses to allergens that require T and/or B lymphocyte responses. Results from these studies showed after aerosolised challenge, induction of Sp-D and Sp-A in wild-type mice was observed whereas in recombinase-activating gene deficient mice that lack functional lymphocytes, no induction of Sp-D or Sp-A was detected (Haley *et al.*, 2002). These findings support a new concept in which Sp-D and Sp-A are important to both innate and adaptive immune responses to allergens.

#### 1.4.3.3 Sp-D-deficient mice

The recent development of transgenic models of Sp-D and Sp-A deficiency allows assessment of the physiological importance of all the activities of lung surfactant protein D and A detected *in vitro* and its potential role in host defences. Sp-D knockout mice (Sp-D-/-) have been produced by gene targeting in two different strains of mice, NIH Swiss black strain Sp-D-/- mice showing a decrease in Sp-A levels (Korfhagen *et al.*, 1998) and CD-1 strain Sp-D -/- mice that showed no down regulation of Sp-A (Botas *et al.*, 1998). These contradictory results are probably due to the different genetic background of the generated Sp-D deficient mice.

One mouse strain deficient for Sp-A had normal lung structure, respiratory function, surfactant lipid metabolism and function (Korfhagen *et al.*, 1998, Ikegami *et al.*, 1997) but deficiencies to microbial challenge were demonstrated (LeVine *et al.*, 1998, 1999, 2000, Korfhagen *et al.*, 2000).

Based on the *in vitro* studies that showed Sp-D to have no direct effect on surfactant homeostasis, the Sp-D deficient mice exhibit a complex and surprising lung phenotype. Despite their healthy appearance, Sp-D deficient mice develop a progressive alveolar proteinosis and emphysema (at eight weeks old, the alveolar phospholipid pool was 8-fold higher than in wild-type mice), distal airspace dilation (at the age of seven months showed increased collagen deposition in the lung and elastic fibres appear shorter, thicker and more highly coiled compared to the fibres of wild-type mice), hypertrophy of alveolar type II cells (enlargement of their lamellar bodies) as well as accumulation of alveolar macrophages (10-fold higher than wild-type mice), many of them multinucleated, foamy in appearance and associated with increased levels in the murine

metalloproteinase MMP2 and MMP9 and in oxidant production (Korfhagen *et al.*, 1998, Botas *et al.*, 1998, Wert *et al.*, 2000, Yoshida *et al.*, 2001).
Knowing that deficiency in either Sp-D or granulocyte-macrophage colony stimulating factor (GM-CSF) influence surfactant homeostasis, the use of single and double transgenic mice with both Sp-D and GM-CSF showed that Sp-D and GM-CSF regulate surfactant homeostasis via distinct mechanisms (Reed *et al.*, 2000, Ikegami *et al.*, 2001). Furthermore, the transcription factor NF-κB is spontaneously activated via oxidant sensitive pathways in alveolar macrophages from Sp-D-/- mice (Yoshida *et al.*, 2001).

Interestingly, the described phenotypes could be corrected by introducing the rat Sp-D transgene into respiratory epithelial cells of Sp-D-/- mice. Furthermore, overexpression of rat Sp-D in wild-type mice did not affect lung structure or surfactant pool sizes (Fisher *et al.*, 2000). The oligomeric structure of Sp-D was found to play a role in surfactant lipid homeostasis and the development of emphysema and foamy macrophages (Zhang *et al.*, 2001).

In addition to the previous observations reported on lungs of Sp-D-/- mice, the use of morphometric analysis and flow cytometric analysis on Sp-D-/- mice lungs showed a peribronchial and perivascular accumulation of lymphocytes (CD4, CD69 and CD8) with a marked activation (Fisher *et al.*, 2002). The proinflamatory cytokines IL-12 and IL-6 were increased in the lung of Sp-D-/- mice (Fisher *et al.*, 2002). Workers suggested a local immunoregulatory role for Sp-D *in vivo*.

Furthermore, interesting recent studies reported that Sp-D, Sp-A and C1q enhanced apoptotic cell ingestion by resident murine and human alveolar macrophages *in vitro* (Vandivier *et al.*, 2002, Schagat *et al.*, 2001). The use of mice deficient in Sp-D, Sp-A and C1q showed that alteration in apoptotic cell clearance from the naive murine lung was only altered in Sp-D deficient mice, thus suggesting an important role for Sp-D in clearance of apoptotic cells *in vivo* (Vandivier *et al.*, 2002). Another recent study demonstrated that Sp-D contributes to immune homeostasis in the lung by recognizing and promoting removal of necrotic and apoptotic cells (Clark *et al.*, 2002). Sp-D deficient mice have a 5- to 10-fold increase in the number of apoptotic and necrotic alveolar macrophages compared to the wild-type mice (Clark *et al.*, 2002).

Interestingly, a mouse doubly deficient in Sp-D and Sp-A was developed. The changes observed in the lungs of these mice were qualitatively similar to the lung pathology seen in Sp-D-deficient mice (Hawgood *et al.*, 2002).
In conclusion, absence of Sp-D resulted in multiple abnormalities in surfactant form and metabolism in the lungs that can be associated with an interplay of several interacting mechanisms.

To date, there are only few reports on the host defence functions of Sp-D knockout mice. By intratracheal instillation, mice lacking Sp-D have been infected with Gram-negative bacteria (*Haemophilus Influenza*) and Gram-positive bacteria (group B Streptococcus). Although clearance of bacteria was unaltered in Sp-D-/- mice, an increased inflammation, inflammatory cell recruitment and deficiency in *in vivo* bacterial uptake by alveolar macrophages were observed in the lung of Sp-D-deficient mice (LeVine *et al.*, 2000). Furthermore, isolated alveolar macrophages from Sp-D-/- mice generated significantly greater superoxide and hydrogen peroxide compared with wild-type alveolar macrophages, thus they are likely to contribute to effective bacterial killing in the lungs of Sp-D-/- mice (LeVine *et al.*, 2000). It is worth noting that in the same study, Sp-A deficient mice were used and showed in contrast to Sp-D-/- mice, a decrease in bacterial killing and less generation of superoxide and hydrogen peroxide compared to wild-type (LeVine *et al.*, 2000). Therefore, the researchers suggested that the collectins Sp-D and Sp-A play different roles during bacterial infection in the lung.
Mice lacking Sp-D have been infected with Influenza A virus (IAV) by intranasal instillation. A decrease in viral clearance and uptake by alveolar macrophages, an increase in the production of inflammatory cytokines, superoxide and infiltration of neutrophils were detected in the lungs of Sp-D-/- mice (LeVine *et al.*, 2001). Additionally, clearance of RSV virus from the lung was impaired in Sp-D deficient mice (LeVine and Whitsett 2001).

The *in vivo* studies with Sp-D and Sp-A gene targeted mice support roles for both pulmonary collectins as opsonins that enhance bacterial and viral clearance and killing and as immunomodulators that regulate the cellular recruitment and the inflammatory response. The following table taken from (McCormack and Whitsett 2002) compares some of the roles for Sp-D and Sp-A identified to date.

| | Sp-D | Sp-A |
|---|---|---|
| **Host defence** | | |
| Agglutination | + + | + |
| Opsonisation | + | + + |
| Reduced viral infectivity | + + | + |
| Modulation of inflammation | + | + |
| **Surfactant function** | | |
| Tubular myelin formation | - | + |
| Resistance to serum inhibitors | - | + |
| Surfactant homeostasis | + | - |
| **Lung structure** | | |
| Regulation of lung remodelling | + | - |

#### 1.4.3.4 Sp-D receptors

Initial studies reported that Sp-D binds with high affinity to alveolar macrophages. Isolated human Sp-D from amniotic fluid interacts with human and bovine alveolar macrophages in a calcium-independent manner, is time, concentration and temperature-dependent and is saturable (Miyamura *et al.,* 1994) whereas another study suggested that Sp-D binds to rat alveolar macrophages in a saturable, concentration and calcium-dependent manner and is inhibited by mannose (Kuan *et al.,* 1994). According to these two preliminary studies, there is probably more than one specific site for Sp-D on alveolar macrophages.

Recently, a large molecule discovered by virtue of its affinity for surfactant protein D and designated gp-340 is a 340 KDa glycoprotein, isolated from lung washings of alveolar proteinosis patients. The protein binds specifically to lung Sp-D in a calcium-dependent manner (Holmskov *et al.,* 1997). It has also been established that binding of Sp-D to gp-340 is a protein-protein interaction rather than a lectin-carbohydrate interaction (Holmskov *et al.,* 1997). Furthermore, protein sequencing showed that gp-340 is a member of group B of the scavenger receptor cysteine-rich (SRCR) superfamily (Holmskov *et al.,* 1997). The domain structure of gp-340 is composed of 13 SRCR domains, followed by two C1r/C1s Uegf Bmp1 (CUB) domains next to the 14$^{th}$ SRCR and then a zona pellucida (ZP) domain. All these three types of domain have been

implicated in mediating protein-protein interactions (Bodian *et al.,* 1997, Bauskin *et al.,* 1998, Gal and Zavodszky 1998).

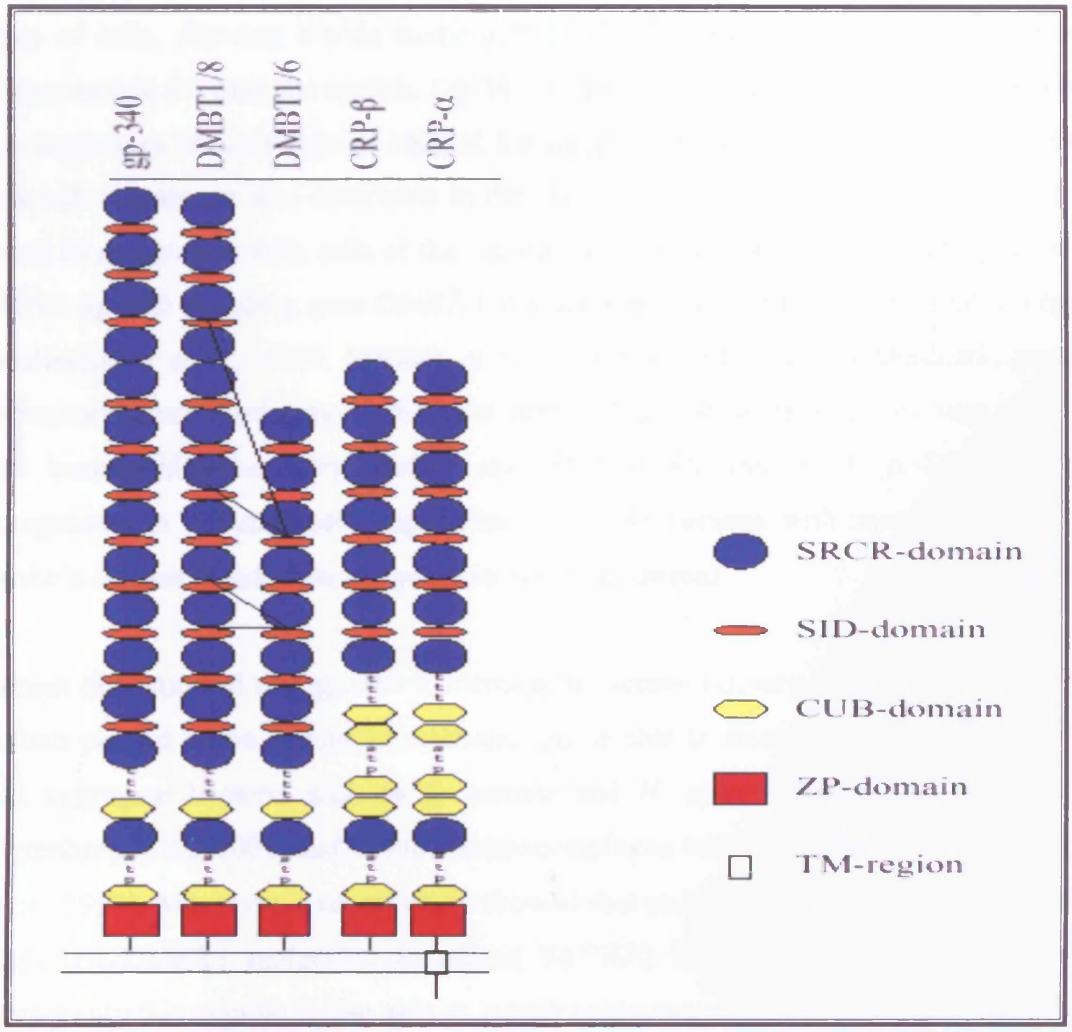



**Fig 1.5.** Domain structure of gp-340, DMBT and CRP-ductin. Abbreviations: CUB: Complement subcomponents C1r/s, Uegf and Bone morphogenic protein 1; SID: SRCR intercepted domain, SRCR: scavenger receptor cysteine rich, TM: transmembrane, ZP: zona pellucida (J. Madsen, University of Southern Denmark, Denmark, personal communication).

Moreover, gp-340 has been cloned at the cDNA level and identified to be an alternatively spliced form of the gene *DMBT*-1 (deleted malignant brain-tumor-1) (Holmskov *et al.,* 1999) that has been proposed as a candidate tumour suppressor gene for brain, gastrointestinal and lung cancer because of its frequent homozygous deletion or lack of expression in these tumours (Mollenhauer *et al.,* 1997, Wu *et al.,* 1999) and its increased susceptibility to genomic instability (Mollenhauer *et al.,* 1999).

In addition, the finding that gp-340 molecules expressed in tissue macrophages, both intracellulary and in a plasma associated membrane form (Holmskov *et al.,* 1999) bind specifically to Sp-D suggested that gp-340 may act as an opsonin receptor for Sp-D.

Tino and Wright (1999) demonstrated the chemokinetic effect of gp-340 on alveolar macrophages and showed that gp-340 may also bind to lung surfactant protein A.
RT-PCR and immunohistochemistry studies showed that gp-340 is present in various types of cells, showing a wide tissue distribution thus suggesting multiple function of this protein in the immune system. Gp-340 is synthesized mainly by epithelial cells from the respiratory (mainly type II cells of the lung), alimentary and reproductive systems, although signals are also detectable in the brain (Holmskov *et al.*, 1997, 1999) and was found in association with cells of the immune system such as alveolar macrophages and mRNA gp-340 encoding gene *DMBT*-1 is widely present in the human lymphoid organs (Mollenhauer *et al.*, 2000, Madsen *et al.*, University of Southern Denmark, personal communication). Furthermore, different forms of gp-340 were found in lung and colon and immunohistochemistry studies showed that expression of gp-340 is clearly upregulated in inflamed colon epithelial cells from patients with ulcerative colitis and Crohn's disease (Madsen *et al.*, personal communication).

Recent data showed that gp-340 is identical to human salivary agglutinin isolated from human parotid saliva, a soluble molecule that is able to interact with Sp-D and to bind and aggregate bacteria such as *S. mutans* and *H. pylori* (Prokobphol *et al.*, 2000, Ligtenberg *et al.*, 2001) and to form heterocomplexes with IgA in the mouth cavity (Oho *et al.*, 1998). Moreover, a recent study showed that only one of the fourteen SRCR of the agglutinin/DMBT1 molecule, designated SRCRP2 is able to bind to *S. mutans* and remarkably this peptide is also able to induce agglutination of *S. mutans* and a number of other bacteria (Bikker *et al.*, 2002). The use of monoclonal antibodies showed that the salivary agglutinin, identical to lung gp-340 and brain DMBT1 is distinctly localised in salivary glands (Bikker *et al.*, 2002). Furthermore, a protein identical to gp-340 was identified in a purified respiratory mucin preparation (Thornton *et al.*, 2001).

These results suggest a co-operative action between Sp-D and soluble form of gp-340 in the defence against microorganisms, as shown recently in a viral model of aggregation and neutralization (K. Hartshorn, University of Boston, USA, Personal communication). In addition, the distribution of gp-340 on epithelial surfaces suggested a role for this molecule in the differentiation of epithelial cells. Recent studies demonstrated that the expression of gp-340 is upregulated differentially in two innate-immune related epithelial cell lines, following treatment with phorbol ester (Kang *et al.*, 2002). For

instance, in A549 cells derived from lung epithelium, upregulation of gp-340 is seen in parallel with inflammatory cytokines IL-6 and IL-8, while in AGS cells derived from stomach epithelium, gp-340 is upregulated with two mature gastric epithelial specific proteins TFF1 and TFF2 (Kang *et al.*, 2002). Thus the workers concluded that gp-340 is involved not only in mucosal defence but also in growth of epithelial cells and its role differs at different body locations and during different stages of epithelial differentiation (Kang *et al.*, 2002).

Initial studies indicated that there is probably more than one specific binding site for Sp-D in alveolar macrophages. Both Sp-D and Sp-A bind to the CD14 LPS receptor (Sano *et al.*, 2000) where Sp-D binds to the associated sugars whereas Sp-A binds to the protein backbone of CD14.

**CRP-ductin**

The homologue of gp-340 in the mouse, named CRP-ductin, is synthesised by epithelial cells at different body locations (Cheng *et al.*, 1996). CRP-ductin was cloned from a jejunal cDNA library made from Balb/C mouse (Cheng *et al.*, 1996). Two different cDNA clones, α-CRP-ductin and β-CRP-ductin, were obtained with the only difference between the two transcripts being a region of 82 bp that results from an alternative stop codon (Fig 1.5).

Antibodies directed against CRP-ductin reacted with 230-260 KDa protein from extracts of mouse small intestine, and showed staining in the small intestine, colon, epithelial cells lining the pancreatic and the large hepatic duct systems (Cheng *et al.*, 1996). This protein has been reported to play roles in various physiologic processes such as epithelial differentiation in the small intestine (Cheng *et al.*, 1996) or as part of the secretions of various glands (Matsushita *et al.*, 2000). An alternatively spliced form of CRP-ductin, vomeroglandin that is expressed in the glands of the vomeronasal system of the mouse has been reported (Matsushita *et al.*, 2000).

Furthermore, CRP-ductin purified from the pancreas binds human Sp-D in a calcium-dependent and saccharide-independent manner (Madsen *et al.*, personal communication). CRP-ductin also binds, in the presence of calcium, to Gram-positive and Gram-negative bacteria and is able to aggregate many microorganisms such as *S. aureus* and *S. mutans* (Madsen *et al.*, personal communication). Using RT-PCR, mouse tissues were screened for CRP-ductin and Sp-D. Results showed that the pancreas is the main site of synthesis of CRP-ductin but the protein is detected also in other tissues such

as salivary glands, stomach, large intestine, whereas the lung is the main site of synthesis for Sp-D but also has been detected in uterus, salivary glands, pancreas and other organs (Madsen *et al.*, personal communication).
The recent reported abilities of CRP-ductin, the mouse homologue of human gp340/ DMBT1, to bind Sp-D as well as bacteria directly guided investigators to suggest a role for this protein in mucosal immunity.

### 1.4.3.5. Sp-D levels in diseases

Several studies implicated Sp-D and Sp-A as disease markers and useful indicators of lung injury. Sp-D levels are altered in many diseases but particularly in infectious diseases. The levels of Sp-D in both lavage and serum have been measured in patients with a variety of pulmonary diseases (Honda *et al.*, 1995). Sp-D levels were elevated ~ 20-fold in lavage of patients with pulmonary alveolar proteinosis (PAP), but were not elevated in lavage of patients with idiopathic pulmonary fibrosis (IPF) or interstitial pneumonia with collagen disease (IPCD). In contrast levels of serum Sp-D were elevated in patients with IPF, IPCD, PAP and pulmonary tuberculosis (Honda *et al.*, 1995). In addition, smokers have decreased Sp-D levels in their BAL fluid (Honda *et al.*, 1996). Thus, the determination of Sp-D levels may be useful in predicting disease, even if the values do not correlate with physiological lung function tests. However a study that investigated the alteration in surfactant levels in children with malignancies, immunosuppression, fever or pulmonary infiltrates showed increase in Sp-A and Sp-C levels but no difference in the levels of Sp-D or Sp-B. Furthermore, there was no difference in the ability of Sp-D and Sp-A binding to pathogens (Griese *et al.*, 2002).

Many studies showed increase of Sp-D concentrations in serum, BAL fluid and lung homogenate in case of lung injury in rats. Silica treatment (Crouch *et al.*, 1991) and LPS both increase levels of Sp-A and Sp-D in animal models (McIntosh *et al.*, 1996, Viviano *et al.*, 1995). In addition, a glucocorticoid (dexamethanose) augments Sp-D production both *in vitro* and *in vivo* (Deterding *et al.*, 1994). Levels of expression of Sp-D were increased in murine models of allergic pulmonary inflammation, Influenza A virus infection and *H. pylori*-associated gastritis (Haley *et al.*, 2002, LeVine *et al.*, 2001, Murray *et al.*, 2002). In contrast, in RSV infection, the levels of Sp-D in the BAL fluid were reduced (Kerr and Paton 1999).

It has been reported that during acute lung inflammation, the extracellular pool size of Sp-D in rats fluctuates significantly (McIntosh *et al.,* 1996), due to an increase in the clearance of Sp-D into lung tissue during inflammation and tissue-associated neutrophils significantly contribute to this process (Herbein and Wright 2001).

Allelic variants of Sp-D have also been found (DiAngelo *et al.,* 1999) but they were not linked directly to disease states. However, in a case study of children at the age of 6-9 years, data indicated substantial genetic influence on both MBL and Sp-D levels (Husby *et al.,* 2002). The genes encoding Sp-D and Sp-A, acting in the clearance of common lung pathogens, are implicated as candidates in a few infectious diseases including respiratory syncytial virus (RSV) infections and tuberculosis (Haataja and Hallman 2002).

#### 1.4.3.6 Implications for medical treatments

Studies with transgenic mice deficient in Sp-D or Sp-A and mouse models of inflammatory lung diseases gave evidence of the requirement of Sp-D for the maintenance of lung structure and most importantly highlighted the immunomodulatory functions of pulmonary collectins (Sp-D and Sp-A) as being anti-inflammatory and antimicrobial. The finding that collectin replacement in Sp-D and Sp-A mice corrected the defects in cellular function and microbial clearance (Wert *et al.,* 2002) suggests a possible role for these collectins in human treatments.

The treatment by intranasal administration of Sp-D and a 60-KDa recombinant fragment of Sp-D (rSp-D) in a murine model of allergic bronchopulmonary aspergillosis (ABPA) caused by an opportunistic fungal pathogen, *Aspergillus fumigatus*, has a protective effect and increased the survival rate of mice to 60 and 80 % respectively (Madan *et al.,* 2001). In addition, treatment with Sp-D and rSp-D lowered blood eosinophila, pulmonary infiltration and specific antibody levels considerably, decreased levels of IL-2, IL-4 and IL-5 while the level of IFN-γ was raised in the splenic supernatants of the treated mice indicating a shift from Th2 to Th1 response (Madan *et al.,* 2001). From these studies showing that Sp-D and rSp-D can rescue mice from a fatal challenge with *A. fumigatus* conidia, investigators suggested that rSp-D may be very suitable for use as an antifungal agent, perhaps by addition to surfactant mixture already in use.

Furthermore, intrapulmonary administration of rSp-D reduces the number of apoptotic and necrotic alveolar macrophages and partially corrects lipid accumulation in Sp-D

deficient mice (Clark *et al.,* 2002). In addition, the coadministration of virus and rSp-D normalised the viral clearance and the cytokine response in Sp-D-deficient mice (LeVine *et al.,* 2001). These studies highlighted the potential of the truncated form of Sp-D as novel therapy for infection and inflammatory diseases.

## 1.5 Pneumococcal virulence factors

To date, several virulence factors are known to be synthesised by *S. pneumoniae* and to be essential in the establishment of pneumococcal disease. Further research almost certainly will continue to identify other virulence factors involved in pathogenesis of the pneumococcus.

The polysaccharide capsule of the pneumococcus has long been known to be vital but not exclusive for the organism's virulence (Avery and Dubos 1931). There are over 90 capsular serotypes (Henrichssen 1995, Kalin 1998). However, the capsule itself is not inflammatory (Tuomanen *et al.,* 1987). The virulence of the capsule lies mainly in its antiphagocytic properties (Lee *et al.,* 1991) and the level of virulence differs between serotypes due to the difference in composition more than size of the capsule (Knecht *et al.,* 1970).

In contrast to the capsule, the pneumococcal cell wall is a potent inducer of inflammation (Tuomanen *et al.,* 1987) and thus is likely to contribute to tissue injury (Johnston 1991). The cell wall components, teichoic acid and lipoteichoic acids, can activate the alternative complement pathway (Winkelstein and Tomasz 1978), acute phase protein CRP and procoagulant activity on epithelium cell surfaces, induce cytokines and PAF upon binding to epithelial, endothelial cells and also macrophages and influence recruitment of leukocytes (Tuomanen and Sande 1989, Geelen *et al.,* 1992, 1993, Cabellos *et al.,* 1992, Heumann *et al.,* 1994).

A number of adhesins and surface proteins such as PspA, PsaA and PspC have been implicated in the pathogenesis of pneumococcal disease but the specific mechanisms by which they confer virulence is not known. The pneumococcal surface protein A (PspA) is produced by all pneumococci and is highly antigenically variable (Crain *et al.,* 1990). It has been reported that PspA negative mutant are less virulent than wild-type pneumococci (Briles *et al.,* 1988), immunisation with PspA protects mice from *S.*

*pneumoniae* infection (McDaniel *et al.*, 1984, Tart *et al.*, 1996) and more recently that this surface-exposed protein inhibits complement activation both *in vitro* and *in vivo* (Briles *et al.*, 1998, Tu *et al.*, 1999). Immunisation with the pneumococcal surface adhesion A (PsaA), a 37KDa protein, confers protection to challenge with pneumococci in mice (Talkington *et al.*, 1992). *In vitro* studies showed that this protein is not involved in adherence to a respiratory epithelial cell line (Talbot *et al.*, 1996) while in contrast, a role of PsaA in pneumococcal adhesion to type II pneumocytes has been reported (Berry and Paton 1996). The pneumococcal surface protein C (PspC), structurally related to PspA, is a member of the choline-binding family of pneumococcal proteins (Brooks-Walter *et al.*, 1999). As reviewed in section 1.3.2.4, PspC by its different functions, is involved in pneumococcal pathogenesis.

Some of the protein components named adhesins expressed on the cell surface of the pneumococcus, are able to mediate attachment to host cells (Andersson *et al.*, 1988, Geelen *et al.*, 1993). Pneumococcal adhesins recognize glycoconjugate-containing receptors on the surface of host cells. For instance, attachment to cells of the nasopharynx is mediated by a GlcNACβ1-3Gal specificity while preferential adherence to type II pneumocytes is mediated by receptors that show specificity for GalNACβ1-4Gal, GalNACβ1-3GAL and GlcNAC (Krivan *et al.*, 1988, Cundell and Tourmanen 1994, Cundell *et al.*, 1995).

The reported pneumococcal IgA1 protease (Male 1979) has been proposed to allow invading pneumococci to evade the local mucosal defence system for instance by cleaving mucosal IgA (Plaut *et al.*, 1974). It is worth mentioning that this enzyme (IgA1 protease) is not produced in non-pathogenic bacteria (Mitchell *et al.*, 1997).

Autolysin, a cell-wall degrading enzyme is found in the pneumococcal cell envelope (Diaz *et al.*, 1989). Under normal conditions the enzyme is inactive but during cell division, stationery phase when nutrients are low or after binding to penicillin, autolysin is activated (Tomasz 1979, Ronda *et al.*, 1987). The role in virulence of autolysin is the release of cell wall degradation products, peptidoglycan and teichoic acid, in addition to cytoplasmic contents such as hydrolytic enzymes and pneumolysin from the pneumococcus (Dowson *et al.*, 1997, Paton *et al.*, 1993). Furthermore, intranasal administration of autolysin-negative pneumococci resulted in no inflammation in the

lungs contrary to the inflammation observed with the wild-type pneumococci (Canvin *et al.*, 1995).

The enzyme neuraminidase, produced by all pneumococcal clinical isolates (Kelly *et al.*, 1967, O'Toole *et al.*, 1971), is toxic to animals and cleaves terminal sialic acid residues from glycoproteins and glycolipids (Lock *et al.*, 1988, Scanlon *et al.*, 1989). This enzyme enhances the colonisation of the pneumococcus in the lung, possibly by exposing cell surface receptors thus helping the bacteria to colonise (Andersson *et al.*, 1983, Scanlon *et al.*, 1989). To date, two genetically unrelated neuraminidases are known to be produced by the pneumococcus: NanA associated with the cell surface of the bacteria (Cámara *et al.*, 1991, 1994) and NanB (Berry *et al.*, 1988, 1996). *In vivo* experiments showed that the mutants NanA and NanB had significantly reduced virulence compared to the wild-type pneumococci (F. Hernon, Dept. of Microbiology and Immunology, personal communication).

The hydrolytic enzyme hyaluronidase also is produced by the pneumococcus and is thought to hydrolyse hyaluronic acid in connective tissue thus allowing spread and invasion of the pneumococcus in the host (Berry *et al.*, 1994, Mitchell *et al.*, 1997). Intranasal infection with mutant pneumococci deficient in hyalurodinase survived longer in lungs of mice when compared with wild-type pneumococci (Mitchell *et al.*, 1997).

All the pneumococcal strains produce hydrogen peroxide ($H_2O_2$) (Duane *et al.*, 1993). In addition to pneumolysin, $H_2O_2$ is cytotoxic to alveolar epithelium and responsible for the reduction of ciliary beating on rat brain ependyma by pneumococci (Hirst *et al.*, 2000).

A recent study reported that superoxide dismutase (SOD) is an important pneumococcal virulence factor (Yesilkaya *et al.*, 2000). A SOD-A inactivated mutant showed low virulence, delayed growth in lungs and slower appearance in blood compared to the wild-type pneumococci (Yesilkaya *et al.*, 2000).

### 1.5.1 Pneumolysin

Pneumolysin, the most extensively studied proven virulence factor of *S. pneumoniae*, is a multifunctional haemolytic toxin produced by nearly all clinical isolates (Kanclerski and Höllby 1987). Pneumolysin, a polypeptide of 470 amino acids having a molecular weight of 53 KDa, belongs to a group of pore forming toxins known as thiol-activated

cytolysins that share similar structural and antigenic characteristics (Walker *et al.*, 1987, Alouf and Geoffroy 1991). Unlike other members of the family, pneumolysin is a cytosolic protein and is released when the bacteria autolyse under the influence of autolysin or lytic antibiotic (Johnson 1972). The cytolytic activity of pneumolysin involves binding to the cholesterol-containing membrane and insertion into the lipid bilayer, followed by oligomerization to form transmembrane pores, which results in cell lysis (Bhakdi and Tranun-Jensen 1988, Paton *et al.*, 1993). *In vitro* studies showed that at sublytic concentrations, highly purified pneumolysin has detrimental effects on a wide range of cells and tissues (Paton *et al.*, 1993, Paton 1996). Electron microscopy studies showed that pneumolysin monomer is an asymmetric molecule composed of four globular domains that are equal in size (Morgan *et al.*, 1994). Structure-function analysis of pneumolysin demonstrated that a domain towards the C terminus of the protein that includes the unique cysteine residue (amino acids 427 to 437) is crucial for cytolytic activity of the toxin. A number of single amino acid substitutions within this region reduce dramatically the cytolytic activity of pneumolysin by up to 99,9% (Boulnois *et al.*, 1991, Saunders *et al.*, 1989).

Pneumolysin, in addition to its cytolytic activity, has a wide range of biological activities that make it able to inhibit mechanisms of host defence as well as subvert complement and immune responses.
*In vitro* studies showed that pneumolysin, due to its cytolytic activity, is able to injure and disrupt the integrity of a range of eukaryotic cells, including type II rat alveolar epithelial cells involved in the lung-capillary barrier (Rubins *et al.*, 1993), nasal and tracheo-bronchial ciliated epithelial cells (Steinfort *et al.*, 1989, Feldman *et al.*, 1990) and pulmonary arterial endothelial cells (Rubins *et al.*, 1992). It has been suggested that these injuring effects of pneumolysin will result in leakage of serum into the alveoli, thus facilitating growth and spread of pneumococci (Rubins *et al.*, 1993, Wood 1941).

Pneumolysin also activates phospholipase A in pulmonary artery endothelial cells, thus resulting in production of inflammatory lipid mediators promoting further cellular damage and induction of inflammatory responses (Rubins *et al.*, 1994). Furthermore, at sublytic concentration, pneumolysin slows ciliary beating of respiratory epithelial cells (Feldman *et al.*, 1990). It has also been reported that pneumolysin is involved in the

separation of epithelial cell tight junctions thus facilitating adherence of the bacteria to host cells and subsequently invasion of the host (Rayner *et al.*, 1995).

Investigations of the interaction between pneumolysin and immune cells showed that the toxin has dramatic, complex effects by stimulating or inhibiting their cellular functions without affecting their viability. At sublytic concentrations (1 ng/ml), pneumolysin is able to inhibit the respiratory burst, the ability to kill pneumococci, chemotaxis and random migration of human polymorphonuclear (PMN) leukocytes (Paton and Ferrante 1983) and these effects could be blocked in the presence of cholesterol. Pneumolysin also inhibits the respiratory burst and release of lysosomal enzymes by monocytes (Nandoskar *et al.*, 1986). However, pneumolysin stimulates production of pro-inflammatory cytokines IL-1 (β) and TNF-α by human monocytes (Houldsworth *et al.*, 1994) and production of nitric oxide as well as IL-6 and TNF-α by human macrophages (Braun *et al.*, 1999), thus indicating that pneumolysin has a direct role in cytokine-mediated inflammation. Also, it has been reported that the toxin markedly inhibits the proliferative response of lymphocytes to several mitogens and decreases the ability of these cells to produce immunoglobulins and lymphokines (Ferrante *et al.*, 1984).

Interestingly, *in vitro* studies also reported that pneumolysin is capable of activating the classical complement pathway in the absence of specific anti-toxin antibody. Initial study demonstrated that highly purified pneumolysin (10 μg/ml) causes significant activation of human complement as measured by C3 conversion (Paton *et al.*, 1984). It was concluded that complement activation by pneumolysin occurs through the classical pathway since that activation was not detected in the presence of EDTA and resulted in the reduction of the human serum opsonic activity (Paton *et al.*, 1984). Subsequent studies showed that even lower levels of pneumolysin (1 μg/ml) activate complement, due at least in part to non-specific binding of the toxin to the Fc region of human immunoglobulin G (IgG) (Mitchell *et al.*, 1991). Furthermore, two distinct regions of the pneumolysin amino acid sequence showed some homology with a contiguous sequence of human acute-phase protein CRP, which also activates the classical complement pathway in the absence of antibody by binding C1q (Volanakis and Kaplan 1974). Codons for some of these residues in pneumolysin were modified by site-directed mutagenesis. Changing the acidic residue at position 385 of the pneumolysin gene to an uncharged residue (Asp385>Asn) inhibits IgG binding and abolishes complement activation but has no effect on the toxin haemolytic activity (Mitchell *et al.*, 1991).

Interestingly, several mutations in the pneumolysin gene that reduced its cytolytic activity had no effect on the ability of pneumolysin to activate complement thus demonstrating the two functions of the toxin are mediated by different parts of the molecule (Mitchell *et al.*, 1991).

Structural analysis showed no similarity between pneumolysin and CRP whereas similarity was detected between domain four of pneumolysin and Fc portion of antibody thus suggesting several mechanisms by which pneumolysin could be activated. For instance, pneumolysin can bind directly to C1q in a similar manner to Fc or alternatively the toxin may form mixed aggregates with IgG and subsequently activate complement. However, further investigations are required to understand the molecular mechanism of complement activation by pneumolysin.

*In vivo* effects of the cytolytic activity and complement activation activity of pneumolysin were investigated. Administration of purified pneumolysin into the lungs of rats generated pathological lesions identical to that of pneumococcal pneumonia (Feldman *et al.*, 1991). A recent study demonstrated that intranasal instillation with purified pneumolysin is associated with a dose-dependent influx of polymorphonuclear leukocytes (PMN) in bronchoalveolar lavage fluid (BALF) and increased concentration of IL-6 and macrophage inflammatory protein MIP-2 in BALF (Rijneveld *et al.*, 2002).

Interestingly, the toxicity of pneumolysin is not only limited to the respiratory tract. Ciliated ependymal cells from the brain of rats were sensitive to the dramatic effects of pneumolysin more than respiratory cilia, thus highlighting the perturbation of this non-specific host defence of cilia function could be important during pneumococcal meningitis (Mohammed *et al.*, 1999, Hirst *et al.*, 2000). Also, cytotoxic effects of pneumolysin on hair cells of guinea pig cochlea have been demonstrated thus indicating that deafness following pneumococcal otitis media could be due to a certain extent by pneumolysin (Comis *et al.*, 1993). Subsequent study showed that this damage resulted from the stimulation of nitric oxide production by pneumolysin (Amaee *et al.*, 1995). In addition, pneumolysin alone caused inflammation in a model of ocular infection similar to the pathology observed with the whole organism (Johnson and Allen 1975).

In conclusion, highly purified pneumolysin used in all these different systems demonstrated that the toxin modulates host defence mechanisms and is a potent pneumococcal virulence factor causing tissue damage and promoting inflammation.

An initial study demonstrated that immunization of mice with pneumolysin could partially protect mice from subsequent challenge with *S. pneumoniae*, thus strongly indicating that pneumolysin plays a crucial role in pneumococcal diseases (Paton *et al.*, 1983). The availability of the cloned pneumolysin gene and sequence (Paton *et al.*, 1986, Walker *et al.*, 1987) has permitted the evaluation of the contribution of the whole pneumolysin and its separate cytotoxic and complement activation properties to pathogenesis in several models of pneumococcal infections.

**Studies with pneumolysin-negative pneumococci**

Isogenic mutants of type 2 (Berry *et al.*, 1989) and type 3 *S. pneumoniae* (Berry *et al.*, 1992) deficient in the production of pneumolysin, termed (PLN-A), were made by insertion-duplication mutagenesis thus disrupting the pneumolysin gene. The mutant strain PLN-A was used in different models of pulmonary, ocular and systemic infections and demonstrated that the toxin pneumolysin is crucially involved in the pathogenesis of the pneumococcus.

Intranasal and intraperitoneal administration of PLN-A resulted in virulence reduction by 10-fold and 100-fold respectively compared to the wild-type organism (Berry *et al.*, 1989). After an intranasal challenge, PLN-A pneumococci had slower growth in the lung and induced much less inflammation as well as a delayed development of associated septicaemia compared to the wild-type organism (Canvin *et al.*, 1995). Furthermore, the production of pneumolysin affected the pattern of inflammatory cell influx to the site of inflammation. During bronchopneumonia, the infection with PLN-A strain resulted in slower, delayed and less intense accumulation of neutrophils, T and B lymphocytes compared to the influx observed with the wild-type pneumococci (Kadioglu *et al.*, 2000). A study by Benton *et al.* (1995) showed that when PLN-A is co-infected intravenously with wild-type pneumococci, the mutant exhibits the same pattern of growth as the wild-type in the blood thus suggesting that pneumolysin exerts its effects at a distance. From this model, investigators suggested that the toxin plays a critical role in the early hours after infection in preventing the generation of inflammation and facilitating the maximal growth of pneumococci thus leading to the establishment of acute sepsis rather than chronic bacteraemia in mice (Benton *et al.*, 1995).

In addition, the isogenic mutant PLN-A was used in a model of lobar pneumonia and was less virulent than wild-type, had reduced ability in multiplication within the lung and invasion of the bloodstream compared to the wild-type pneumococci (Rubins *et al.*, 1995). Interestingly, the addition of exogenous purified pneumolysin promoted the growth of PLN-A in the lung (Rubins *et al.*, 1995). The use of mice deficient in the fifth complement component C5 (C5-/-) showed that interactions between complement and pneumolysin are significant. After intratracheal instillation, the growth of PLN-A and wild-type bacteria was enhanced by 2-fold in the lung of C5-deficient mice whereas PLN-A bacteria were cleared in C5 sufficient mice (Rubins *et al.*, 1995).

PLN-A did not induce separation of tight junctions of epithelial cells or the adherence of the bacterium when used to infect human respiratory mucosa grown in organ culture, thus suggesting that pneumolysin has a major role in the initial steps of pneumococcal invasion (Rayner *et al.*, 1995). When used in a model of experimental meningitis in guinea pig, PLN-A infection resulted in an inflammatory response in CSF similar to the wild-type infection but there was a remarkable structural and physiological damage of the cochlea in the presence of pneumolysin (Winter *et al.*, 1996).

Furthermore, a deletion mutant of *S. pneumoniae* unable to produce pneumolysin showed a greatly reduced virulence in a rabbit model of ocular infection (Johnson *et al.*, 1990). Conversely, infection with a pneumolysin-negative mutant in a rabbit model of meningitis and a model of otitis media suggested that pneumolysin has a minimal role in the generation of inflammation in both models (Friedland *et al.*, 1995, Sato *et al.*, 1996).

**Studies with isogenic mutants expressing modified versions of pneumolysin**

Site-directed mutagenesis manipulation of the primary amino sequence of pneumolysin gene identified the involvement of different domains of the toxin in its different functions. Molecular studies demonstrated that domain 4 of pneumolysin is implicated in binding of the toxin to the membrane surface and also in activating complement (Rossjohn *et al.*, 1998).

In order to determine the relative individual contribution of pneumolysin's activities in the virulence of *S. pneumoniae, in vivo* experiments were done using pneumococci mutant strains lacking either or both cytolytic or complement activation properties. Instillation of mutated forms of the toxin into the rat lung showed that both activities play a role in the inflammation and resulted in less severe pathological changes

compared to the wild-type protein effects (Feldman *et al.*, 1991). Recently reported, intranasal inoculation with pneumolysin mutant with reduced cytolytic activity resulted in a reduction in the influx of PMN into the lung of mice thus indicating that the cytolytic activity is important for the inflammatory response (Rijneveld *et al.*, 2002). In a model of lobar pneumonia, two mutated forms of the toxin lacking either complement activation activity (Asp385>Asn) or cytolytic activity (Trp433>Phe) were co-instilled with PLN-A pneumococci and showed that pneumolysin cytolytic activity increased bacterial growth and invasion into lung tissue during the early period after infection whereas its ability to activate complement appeared important for the bacterial growth in the lung at later times (Rubins *et al.*, 1995). Furthermore, complement-activation negative mutant was constructed from a type 3 *S. pneumoniae* and showed a reduced virulence in a rabbit intra-corneal model, thus indicating a significant role for complement activation by pneumolysin in this model of corneal infection (Johnson *et al.*, 1995).

Isogenic mutant strains of type 2 *S. pneumoniae* strain D39 were used in several models of pneumococcal infections. The determination of the individual role of pneumolysin's activities varied between the *in vivo* studies and mostly was associated with the different routes and sites of infection used.

In a model of systemic infection, the overall survival rate was not different between mice challenged intraperitoneally with pneumococci wild-type D39 or those expressing pneumolysin gene (H+/C-) with (Asp385>Asn) which abolishes only the complement activation property. However, mice challenged with isogenic strains (H2-/C+, His367> Arg) or (H3-/C+, Trp433>Phe and Cys428>Gly) having a reduced haemolytic activity to approximately 0.02 and 0.0001% of the wild-type level, had a greater overall survival rate than mice challenged with wild-type bacteria (Berry *et al.*, 1995). Thus, the investigators concluded that pneumolysin's cytolytic rather than its complement activation properties affected bacterial growth in this model of intraperitoneal challenge. They suggested that this is probably due to the infection taking place via the systemic route where complement components are present in excess (Berry *et al.*, 1995).

In a model of lobar pneumonia, both pneumolysin's activities contributed specifically to the early pathogenesis. The haemolytic activity of the toxin correlated with acute lung injury and bacterial growth at 3 and 6h after endotracheal instillation, while in contrast,

pneumolysin's complement activating activity correlated with bacterial growth and bacteraemia at 24h after pulmonary infection and was not associated with the degree of alveolar-capillary injury or recruitment of leukocytes during the initial stage of infection (Rubins *et al.,* 1996). Furthermore, it has been shown that pneumolysin's complement activating activity inhibits killing of mutant bacteria in an *in vitro* complement-dependent neutrophil killing assay (Rubins *et al.,* 1996).

In a model of bronchopneumonia, the isogenic strains lacking either the cytotoxic activity or the complement activation activity by pneumolysin had reduced virulence compared with wild-type strain D39 (Alexander *et al.,* 1998). Furthermore, the ability to activate complement remarkably affected the growth of pneumococci in the lungs and associated bacteraemia in the first 24h after intranasal infection while the reduction of cytolytic activity affected only bacterial growth only at 6h after infection (Alexander *et al.,* 1998). Intriguingly, an unexpected finding was that after intranasal, intraperitoneal or intravenous challenge, the isogenic mutant strain H3-/C- carrying three point mutations in the pneumolysin gene thus abolishing both activities (Asp385>Asn, Cys428>Cly, Trp433>Phe) was more virulent than the PLN-A gene disruption mutant indicating that pneumolysin may possess some other unidentified activity that is not abolished by these three mutations (Berry *et al.,* 1999, Alexander *et al.,* 1998, Benton *et al.,* 1997).

In addition, interesting recent studies investigated the role of pneumolysin's complement activating activity during *S. pneumoniae* bacteraemia using hypocomplementemic cirrhotic rats. After intravenous infection with wild-type *S. pneumoniae* type 3 (H+/C+) there was a decrease in bacterial number and 55% mortality in control rats whereas an increase in bacterial number and 100% mortality were observed with cirrhotic rats (Alcantara *et al.,* 1999). Both control and cirrhotic rats cleared effectively isogenic mutant strains (H+/C- and PLN-A) from their bloodstream (Alcantara *et al.,* 1999). Thus these experiments demonstrated that production of pneumolysin decreased pneumococcal clearance from the bloodstream and increased the rate of mortality in both control and cirrhotic rats. Subsequent investigations detected low levels of complement C3 and decrease in the opsonisation activity of *S. pneumoniae* in sera of cirrhotic rats infected with wild-type strain (H+/C+) when compared with sera from (H+/C+)-infected control rats (Alcantra *et al.,* 2001). Using this model of pneumococcal

bacteraemia, the investigators concluded that pneumolysin's complement activating activity has a significant negative, detrimental effect on serum complement levels and reduces the serum opsonic activity in cirrhotic host thus, exclusively enhancing pneumococcal virulence in the hypocomplementic animals. Conversely, pneumolysin's complement activating activity exerts only minimal influence on host defence in a host with intact complement-generating system (Alcantra *et al.*, 1999, 2001).

## 1.6 Aims of the project

The contribution of complement and lung surfactant protein D to the innate immunity against the respiratory pathogen *S. pneumoniae* was investigated in this project. A mouse model of bronchopneumonia and septicaemia was used in all the *in vivo* studies.

### 1.6.1 Complement studies

The *in vivo* effects of pneumolysin's complement activation activity on bacterial growth kinetics in lungs and blood, severity of inflammation and recruitment of leukocytes into the sites of infection were studied in mice infected with wild-type *S. pneumoniae* type 2 strain D39 and its isogenic mutant strain (H+/C-) producing pneumolysin which lacks the ability to activate complement. The effect of pneumolysin's complement activation on the extent of the total complement C3 activation in the mouse lung was investigated in mice infected with wild-type pneumococci and the isogenic mutant strain PLN-A unable to produce pneumolysin.

Furthermore, this thesis reports an investigation on the overall contribution of the classical complement pathway and the effect of the ability of pneumolysin to activate complement on the innate immunity during pneumococcal bronchopneumonia using C1q knockout mice. The outcome of pneumococcal growth in lungs and blood, histological changes, recruitment of inflammatory leukocytes and the extent of activation of local complement C3 were compared between C1q deficient and sufficient mice.

### 1.6.2 Surfactant protein D studies

As discussed in section 1.4.3, lung surfactant protein D appears to play an important role in the innate immunity against several pathogens invading the respiratory tract. In this

thesis, data from *in* vitro and *in vivo* studies were combined in order to understand the role of Sp-D in the innate immunity to the pneumococcus.

The interaction between Sp-D and the pneumococci was examined. *In vitro* binding and agglutination of different clinical isolates of *S. pneumoniae* having different capsule type and multi-locus sequence type by lung surfactant protein D is reported. Whether Sp-D could be an opsonin for the pneumococcus with human neutrophils was also investigated.

Sp-D knockout mice were used to investigate the *in vivo* role of Sp-D in the immunity to the pneumococcus during the initial phase of bronchopneumonia. Pneumococcal growth in nasopharynx, trachea, lungs and blood, lung inflammation and influx of inflammatory leukocytes are reported in Sp-D sufficient and deficient mice.

Furthermore, interaction between the Sp-D receptor (gp-340) and *S. pneumoniae* was investigated. *In vitro* binding and agglutination of the pneumococcus by gp-340 was tested. Finally, upregulation of Sp-D and CRP-ductin (gp-340 mouse homologous) in the mouse lung during bronchopneumonia is reported in two mice strains that have been infected with *S. pneumoniae.*

# Chapter 2: Materials and Methods

## 2.1 Materials, growth media and buffers

All chemicals were supplied by Sigma and BDH, bacteriological growth media by Oxoid and tissue culture media and solutions by Gibco Technologies unless otherwise mentioned.

Culture media for *S. pneumoniae* were made as follows:

Blood agar: 16 g of blood agar base dissolved into 400 ml of distilled water and then autoclaved. When cooled to around 45°C, 20 ml of horse blood (Oxoid, Basingstoke, Hampshire, UK) was added.

Brain Heart Infusion broth: 14.8 g was dissolved in 400 ml of distilled water and then autoclaved.

The saline buffers used were:

Tris-buffered saline (25mM Tris): 8g NaCl, 0.2g KCl and 3g Tris-base were made up to 1 litre of distilled water, pH adjusted to 7.4 by HCl and then autoclaved.

Phosphate-buffered saline: 8g NaCl, 0.2g KCl, 1.44g $Na_2HPO_4$ and 0.24g $KH_2PO_4$ were made up to 1 litre of distilled water and pH adjusted to PH 7.4 using HCl and then autoclaved.

## 2.2 Mouse strains

Outbred MF1 and inbred C57 BL/6j mice were obtained from Harlan Olac (Bicester, UK). Sp-D knockout mice, produced by targeted gene inactivation (Botas *et al.*, 1998), were kindly provided by Dr Howard Clark (Department of Biochemistry, University of Oxford, UK). Four pairs of C1q knockout mice were kindly obtained from Dr Marina Botto (Imperial College, London, UK) and were bred in the Biomedical Services Department of Leicester University (Section 2.1.1). The C1q deficient mice were generated by gene targeting as described previously (Botto *et al.*, 1998). Sp-D and C1q knockout mice are on a C57 BL/6j genetic background. Mice used in all experiments were at least 9 weeks old. Before experimental use, all non-immune mice were allowed to acclimatise by keeping them for one week in an isolator, under standard conditions in Biomedical Services, with free access to water and food. Subsequent to pneumococcal infection, the mice were shifted to an isolator (MDH) for the remainder of the experiment.

### 2.2.1 Production of C1q knockout mice

Founder stock consisting of four pairs was transferred to Biomedical Services one week prior to mating. After acclimatization, a breeding program was followed to maintain the pedigree line and to further supply study groups. Concisely, after initial pairing, the males were removed when the females were visibly pregnant and were not replaced until the females had given birth and weaned the litter. The first litter was used as a reserve. The second litter provided the future breeding stock (FBS) for the next generation. Mice were coupled randomly in batch matings to produce additional study cohorts. In the Biomedical Services Department of Leicester University, four groups generated approximately 60 progeny staggered in age that were used in the study.

## 2.3 Pneumococcal strains

Strain D39 type 2 was obtained from the National Collection of Type Culture (NCTC 7466, London, UK) and two isogenic mutant strains: H+/C- (Pn385) made by site-directed mutagenesis of the pneumolysin gene followed by homologous recombination to replace the wild-type gene (Mitchell *et al.*, 1991) and PLN-A made by insertion duplication mutagenesis (Berry *et al.*, 1989) were used in the *in vivo* studies.

Ten clinical isolates of *S. pneumoniae,* from different countries were obtained from Dr Mark Enright, the Wellcome Trust Centre of the Epidemiology of Infectious Diseases (WTCEID, University of Oxford and now based at the University of Bath, UK). Molecular characterization by multi-locus sequence typing (MLST) was available for these clinical isolates (Table 2.1). Switched serotypes of pneumococci were kindly provided by Dr Francesco Ianelli (University of Siena, Italy) (Table 2.2).

Other encapsulated clinical pneumococcal strain 1N, 2 (strain D39), 3, 8, 14, 18C and the non-encapsulated strain RX1 were obtained from the culture collection of the Department of Microbiology and Immunology at Leicester University.

### 2.3.1 Bacterial culture

Pneumococci were grown on blood agar plates supplemented with 5 % (v/v) horse blood (TCS Microbiology), and incubated overnight at 37 °C in anaerobic jars. The following day, a loop of culture was inoculated into 10 ml brain heart infusion broth for overnight culture at 37 °C. For pneumococcal strains that were difficult to grow, BHI was supplemented with 20 % (v/v) foetal bovine serum. For the mutant strains, blood agar and BHI medium were supplemented with 1 μg/ml erythromycin.

| Strain | Serotype | MLST | Country | Year of isolation | Diagnosis |
|---|---|---|---|---|---|
| M393 | 3 | 260 | Spain | 1998 | Meningitis |
| M110 | 3 | 180 | UK | 1997 | Pneumonia |
| M290 | 3 | 49 | Uruguay | 1997 | Meningitis |
| M9 | 18C | 102 | Sweden | 1995 | Bacteraemia |
| M241 | 18C | 113 | Netherlands | 1980 | Meningitis |
| M208 | 18C | 119 | UK | 1996 | Meningitis |
| GM70 | 19A | 81 | Spain | 1990 | Bacteraemia |
| M41 | 23F | 81 | UK | 1997 | Unspecified |
| M322 | 14A | 156 | Spain | 1997 | Meningitis |
| M15 | 9V | 156 | UK | 1997 | Bacteraemia |

**Table 2.1.** Characteristics of *S. pneumoniae* strains obtained from WTCEID. (http://www.mlst.zoo.ox.ac.uk)

| Strain | Capsule type | Mutation |
|---|---|---|
| G54 | 19F | Parent strain of Ps3221 |
| Ps3221 | 3 | G54 with capsule switched from type 19F to 3 |
| HB 565 | 3 | Type 3 strain |
| Ps3205 | 3 | D39-type 2 with capsule switched to type 3 |

**Table 2.2.** *Streptococcus pneumoniae* strains obtained from the University of Siena.

### 2.3.2. Viable counting

In a microtitre plate, a serial dilution was performed by adding 20 µl of bacterial suspension to 180 µl of nanopure water and diluting to $10^{-6}$. A blood agar plate was divided into 6 sectors corresponding to different dilutions. This was done in duplicate. 3 x 20 µl of each bacterial dilution was dispensed into each sector. The plates were incubated overnight at 37 °C in anaerobic jars. Bacterial colonies were counted the following day.

## 2.4. Human Sp-D, rSp-D, gp-340 and antisera

Full-length molecule Sp-D, rSp-D and anti-human Sp-D antibody were graciously provided by Dr Paul Eggleton (Department of Biochemistry, University of Oxford, UK). Native human Sp-D was purified by affinity chromatography from bronchoalveolar lavage fluid from patients with alveolar proteinosis and anti-human Sp-D antibody was raised in the rabbit against the purified protein (Madan *et al.*, 1997). The recombinant protein Sp-D (rSp-D), consisting of 3 CRD and the α helical neck region of the molecule, was over expressed in *E. coli* (Kishore *et al.*, 1996) and *Pichia pastoris*. Glycoprotein gp-340 and anti-human gp-340 monoclonal antibody (213-06) were provided by Prof Uffe Holmskov (Department of Microbiology and Immunology, University of Southern Denmark, Denmark). Gp-340 was purified from bronchoalveolar lavage fluid from patients with alveolar proteinosis and anti-gp340 antibody used in ELISA assay was produced in mice (Holmskov *et al.*, 1997).

## 2.5 Bacterial solid phase enzyme-linked immunosorbent assay (ELISA)

### 2.5.1 Measurement of total protein concentration in bacterial suspension

Before coating the ELISA plates, the total protein concentration of each bacterial suspension was determined using the Bio-Rad protein assay that is based on the Bradford protein assay (Bradford *et al*, 1976). Briefly 50 µl of the bacterial suspension was added to 40 µl of nanopure water, 10 µl 10 %(w/v) SDS and boiled for 10 min. Different dilutions were made in 1 ml cuvettes and 200 µl Bio-Rad dye concentrate were added. After 15 min, the absorbance was measured at wavelength 595 nm. The unknown total protein concentration was determined from a standard curve. The standard protein used was bovine serum albumin (1-25 µg/ml).

### 2.5.2 Bacterial coating

*Streptococcus pneumoniae* were grown overnight in brain heart infusion broth to OD of ~1.5 measured at wavelength of 500nm. The suspension was centrifuged at 1000 xg for 15 minutes. The pellet was resuspended in 0.9 % (w/v) NaCl supplemented with 0.5% (v/v) formalin to kill the bacteria, for 1h with shaking as described previously by Barka *et al.* (1985). Bacteria were subsequently washed 3 times in Tris-buffered saline and resuspended in 2ml carbonate-bicarbonate coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH adjusted to 9.6 prior to use).

50 µl of the pneumococcal suspension were dispensed into Nunc polystyrene flat-bottomed 96-microtitre plates and bacterial adherence to wells was allowed at 4°C overnight. A protein concentrations of 8-11 µg per well was used (Section 2.4.1).

### 2.5.3 Binding assays

Binding of Sp-D to pneumococci was tested using a modification of the procedure of Hartshorn *et al.* (1998). After coating, the plates were washed with TBS and blocked with 3% (w/v) BSA in TBS for 1h at 37°C. 100 µl of recombinant human Sp-D (0, 5 or 10 µg/ml) in TBS containing 5mM calcium chloride, 100 µl polyclonal rabbit anti-human Sp-D (1 in 5000) and 100 µl goat anti-rabbit IgG antisera conjugated to horseradish peroxidase (1 in 2000) were added sequentially with washing between each step. The plates were washed with TBS-0.05% (v/v) tween 20(Polyoxyethylene-sorbitan Monolaurate) after each step and for each step, plates were incubated for 2h at 37°C. Binding of rSp-D to plates was detected with TMB peroxidase substrate (Bio-Rad). Reactions were developed for 15 min at 37°C and were stopped by adding 50 µl concentrated $H_2SO_4$. Absorbance was measured with an ELISA plate reader (Dynatech) at 450nm.

Binding of gp-340 to *S. pneumoniae* was done during a visit to the Department of Microbiology and Immunology, University of Southern Denmark, Denmark with the technical help of Ida Tornoe. The plates were coated with pneumococci, washed and blocked. 100 µl (0-5 µg/ml) human gp-340, 100 µl mouse monoclonal anti-human gp-340 (Hyb-213-6) and 100 µl goat anti-mouse IgG antisera (1 in 200) coupled to alkaline phosphatase were added sequentially with washing between each step with TBS-0.05% (v/v) tween 20. Plates were incubated for 2h at 37°C in each step. Binding of gp-340 to pneumococci was detected by addition of 100 µl PNPP alkaline phosphatase substrate (1mg/ml) in diethanol-amine to each well. Reactions were developed at 37°C for 15 min and the absorbance at 405nm was read in an ELISA plate reader.

## 2.6. Agglutination assays

### 2.6.1 Preparation of bacteria

*Streptococcus pneumoniae* were grown in brain heart infusion broth. The suspension was centrifuged at 1000 xg for 15 minutes, washed and then resuspended in 2 ml Tris-buffered saline, pH 7.4.

### 2.6.2. Microscopic aggregation

As described by Madan *et al.* (1997), in 50µl final volume, 30µl of the pneumococcal suspension were incubated with Sp-D (10µg/ml) for 90 min at 37°C in the presence and absence of calcium chloride (5 mM), in the presence of EDTA (10 mM) or maltose (100 mM). Following the incubation, microscopic observation was done under phase-contrast for the samples and the negative control in the absence of Sp-D (magnification x500). The average of the area of agglutinated bacteria ($\mu m^2$) and the number of clumps per field of view were determined for each pneumococcal strain.

### 2.6.3. Bacterial sedimentation

As described by Ligtenberg *et al.* (2001), 350 µl of the bacterial suspension was added to 350 µl TBS containing 5mM calcium chloride and gp-340 (10 µg/ml) in the absence and presence of EDTA (10 mM) into a 1ml cuvette. Initial OD at 500 nm of the suspension was ~ 1. Agglutination was measured by a fall in OD at 500 nm in a spectrophotometer at 37 °C. Each sample was measured at 30 min intervals for 3 hours.

## 2.7 Killing assay

### 2.7.1 Isolation of polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (PMN) were isolated from whole blood of healthy adult donors by a one step isolation procedure using Polymorphprep (Nycomed Pharma, Birmingham, UK) according to the manufacturer's instructions. Fifteen ml of blood were collected into a centrifuge tube containing heparin as an anticoagulant (1 unit/ml). Blood was diluted 1 in 1 using culture medium RPMI containing 5 mM L-glutamine. Carefully, 5-7 ml of anti-coagulated diluted blood were layered over 5 ml of Polymorphprep in a 15ml centrifuge tube. Care was taken to avoid mixing of the blood with the separation fluid. Samples were centrifuged at 500 xg for 35 minutes at 18-22 °C. After centrifugation, two leukocyte bands were visible. The top band consisted of

mononuclear cells and the lower band of polymorphonuclear cells; the erythrocytes were pelleted. The lower band containing PMNs of different tubes was harvested into one tube using a Pasteur pipette and 5 ml RPMI was added. The suspension of PMN cells was centrifuged for 8 minutes at 500 xg at 18-22 °C. Cells were washed twice using RPMI. Finally, leukocytes were resuspended in 1ml of RPMI. Using trypan blue dye exclusion assay (Curd *et al.*, 1978) at a 1:4 ratio of stain to cells, the % of viable cells and the number of PMNs was determined with a haemocytometer.

Before adding PMN cells to bacteria, the cells were centrifuged, resuspended in Hank's Balanced Salt Solution (1.2 mM $Ca^{2+}$) (HBSS) pH 7.4 at a final concentration of $10^7$cells /ml. The viability of PMN cells was checked at the beginning and end of the experiments. More than 98% of PMNs were viable and were used within 2 hours of isolation.

### 2.7.2. Preparation of autologous serum

Autologous serum, obtained from volunteers who had received pneumococcal polysaccharide vaccine (including type 2 capsule) was used as an opsonin source. Five ml of blood was collected into a centrifuge tube. To speed up the sedimentation of red blood cells, the tube was placed in a water bath at 37 °C for 30 minutes. Serum was collected and stored at – 70 °C.

### 2.7.3. Preparation of bacteria

One ml of an overnight culture of encapsulated *S. pneumoniae* type 2 (strain D39) ($10^7$ CFU/ml) and non-encapsulated strain RX1 ($10^9$ CFU/ml) were centrifuged at maximum speed in a microfuge for 2 min. The bacteria were washed twice with HBSS (1.2 mM $Ca^{2+}$). Bacterial pellets were resuspended in 1 ml of HBSS pH 7.4 and were diluted to make a suspension of 2 x $10^5$ CFU *S. pneumoniae*/ml for each strain. 100 µl of each pneumococcal strain were pre-opsonized by incubation at 37°C for 30 min under rotation with 20% (v/v) autologous serum, native Sp-D (10µg/ml) or rSp-D (20µg/ml).

### 2.7.4. Killing of pneumococci by PMN cells

The killing of pneumococci by human polymorphonuclear leukocytes was estimated by measuring the decrease in number of viable bacteria, in a micromethod modified from Braconier *et al.* (1982). PMN cells ($5x10^6$) were incubated with $5x10^5$ pre- or non-opsonised live bacteria/ml in a final volume of 250 µl in HBSS pH 7.4 at 37°C under

rotation. Samples were taken at 0, 30, 60 and 120 min, serially diluted in water, and plated onto blood agar plates to determine the number of viable bacteria.

### 2.8 Infection studies

A murine bronchopneumonia model was used in all the *in vivo* studies, as described previously (Kadioglu *et al.*, 2000).

#### 2.8.1 Animal passage of pneumococci

From plates streaked with *S. pneumoniae*, 10 ml of BHI were inoculated overnight. On the second day, bacteria were pelleted and resuspended in 5-10 ml of phosphate-buffered saline (PBS, pH =7.4). 200 µl of bacterial suspension was injected intravenously into the peritoneal cavity of female MF1 mice. At 24h post-infection, mice were deeply anaesthetised with 5 % (v/v) Fluothane (Astra-Zeneca, Macclesfield, UK) with 1 Litre $O_2$/min using a Fluotec 3 calibrated vaporiser (Cyprane). Cardiac puncture was performed to collect the blood and 200 µl was inoculated to 10 ml BHI and incubated statically overnight at 37 °C. The following day, bacteria were pelleted. 200 µl of the sedimented bacteria was added to 3 ml BHI supplemented with 20 % (v/v) foetal bovine serum and incubated for 5 hours at 37°C. When OD at 500 nm of 0.6 was reached, 0.5 ml aliquots of bacterial culture were placed into cryotubes and stored at –70°C. The next day, the viable counting was determined to check viability of bacteria. Passaged pneumococci were available to use for the next 3 months.

#### 2.8.2 Preparation of the infectious dose

An aliquot of passaged pneumococci was thawed at room temperature. 400 µl of the bacterial suspension was taken out of the cryotube, pelleted, resuspended in 400 µl of sterile PBS and was considered as the bacterial inoculum. Depending on the previously determined viable count (section 2.8.1), a dilution was done in order to administer $10^6$ pneumococcal CFU in 50 µl of PBS. The inoculum concentration was confirmed subsequently by quantitative culture on blood agar plates.

#### 2.8.3 Intranasal challenge of mice

All the infections were done by Prof. PW Andrew or Dr. Aras Kadioglu. Mice were first anaesthetised in a cabinet under 5% Fluothane with 1 Litre $O_2$/min. When the mouse was anaesthetised, 50 µl of PBS containing $10^6$ CFU of *S. pneumoniae* were

administered into the nostrils of the mouse. The mouse then was laid on its back, thus allowing the maximum amount of inoculum to reach the mouse lung, until recovery. Following infection, mice were transferred to an isolator dedicated to the purpose. Symptoms of disease scored by their severity from hunched, starry coat, lethargic through to moribund was recorded for up to 48h postinfection.

### 2.8.4 Determination of bacterial number in tissues and blood

At prechosen intervals following the infection, pre-selected groups of mice were deeply anaesthetised as before (section 2.8.1). Blood was collected by cardiac puncture and was kept on ice. Subsequently, the mice were killed by cervical dislocation without allowing recovery from anaesthesia. The nasopharyngeal tissue was uncovered, cartilage and associated soft tissue within the nasopharynx were removed into 10 ml of sterile distilled water and weighed. The trachea and lungs also were removed separately into 10 ml of sterile distilled water and weighed. All tissues were kept on ice during processing and were homogenized in a Stomacher-Lab blender (Seward Medical, London, UK) for 2 min. Viable counts in tissue homogenates and blood were determined as described previously (Section 2.3.2).

## 2.9 Histology

### 2.9.1 Preparation of frozen lung tissue

As described previously (Kadioglu *et al.*, 2000), at pre-selected times following infection, animals were killed and whole-lung samples were collected in chilled sterile distilled water and were dissected into 2 lobes. Aluminium foil moulded around a bijoux tube was used as a container for embedding the lungs in Tissue-Tec OCT (Sakura, Finetek, The Netherlands). In order to prevent snap freezing and tissue damage, the aluminium cylinder containing the lung and the OCT compound was placed in a beaker of isopentane heat buffer that was lying on the surface of liquid nitrogen. Lungs samples were stored at –70 °C. A few days before sectioning, the lungs were removed to –20 °C.

### 2.9.2 Cryostat sectioning

Sections (10-20 μm) were taken at –18 °C to –25 °C on a Bright cryostat and then allowed to dry at room temperature. Glass slides (Super-premium microscope slides) from BDH were used.

### 2.9.3 Haematoxylin and Eosin tissue sections staining

The protocol used for Haematoxylin and Eosin tissue staining was described in Wheater's Functional Histology, 3rd edition, 1993. Once dried, lung sections were subsequently stained for 25 sec with Meyer's Haematoxylin, washed with tap water, stained with Eosin for 25 sec and followed by a second wash under tap water. Both stains were filtered through tissue paper prior to use. Sections were dried out in a series of alcohol washes (70 % v/v, 90 % v/v and 100 % v/v solutions) for a period of 30 seconds in each solution and finally placed into xylene. Lung sections were left to dry, mounted in synthetic resin DPX solution, covered with a coverslip and examined under light microscopy. Haematoxylin stains nuclear detail blue to black and Eosin is used as a counterstain that stains the cytoplasm, connective tissue fibres and red blood cells orange to red.

## 2.10 Immunohistochemistry

All the incubation steps performed in all the techniques took place in a humid box at room temperature and care was taken to ensure that the whole surface area of the tissue sections was covered with liquid and not allowed to dry out.

### 2.10.1 APAAP staining technique

Leukocyte recruitment into lung tissue was analysed by an alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method, as described by Kadioglu *et al.* (2000). First, lung sections were fixed with acetone for 10 min at 4 °C. Slides were air dried, followed by a wash in PBS for 5 min. Normal rabbit serum (1 in 5) was added onto each slide for 5 min. Excess serum was tapped off and the primary antibody (rat anti-mouse monoclonal antibodies (10 μg/ml) to T cells (CD3), B cells (CD19), macrophages (F/480) or neutrophils (7/4) (Serotec, Oxford, UK) was added for 60 min, one antibody was added onto one section. Next, sections were washed in PBS for 5 min. Secondary antibody rabbit anti-rat antibody (1 in 25) (Dako, Denmark) was added to tissue sections for 30 min. Sections were washed with PBS for 5 min. A third antibody, APAAP rat monoclonal (1 in 50) was added for 30 min. Sections were washed in PBS for 5 min. Finally, Fast Red TR/Naphtol AS-MX (Sigma F4523) was added (to prepare this reagent, a Tris buffer tablet (Sigma) pH 7.4 was dissolved in 10 ml distilled water and a Fast Red tablet was then added). To inhibit endogeneous phosphatase, 10 μl 1M levamisole was added to the prepared Fast Red solution after being filtered through a

microdisc. At the last stage, the Fast Red substrate was generously added to each slide for 20 min. Subsequently, sections were washed with distilled water and counterstained briefly in filtered Haematoxylin. Finally, slides were washed with tap water, dried and fixed with Aquamount mounting medium (BDH). Positively red stained cells were enumerated in the inflamed areas of the lung. At least 10 fields/section were counted and the number of cells per $mm^2$ of inflamed lung tissue was calculated:
Number of cells/$mm^2$ = Mean of the number of cells x magnification / 100.

### 2.10.2 Peroxidase staining for complement C3

A peroxidase staining technique was modified from a protocol for frozen mucosa sections (Wang *et al.,* 1998). As described in the above section, lung sections were fixed in acetone followed by air-drying. The endogeneous peroxidase was blocked by applying freshly prepared 2 % hydrogen peroxide in methanol for 10-15 min at room temperature, followed by a wash in TBS for 5 min. 2 % v/v Foetal Calf Serum in TBS was applied for 10 min and then drained. Goat polyclonal anti-mouse complement C3 conjugated to horseradish peroxidase (ICN Biomedicals, Inc., Aurora, OH) diluted 1 in 50 was applied for 30 min. This was followed by washes of slides in TBS and distilled water for 5 min each. Peroxidase DAB substrate (Sigma) was prepared according to the manufacturer's instructions (1 tablet dissolved in 15 ml of TBS pH 7.6, filtered and 12 µl of $H_2O_2$ added prior to use) and applied to sections for 10 min. This substrate produces an insoluble brown product. Sections were then washed in running tap water, counterstained briefly with Meyers Haematoxylin, washed with tap water and finally mounted in Aquamount mounting medium. The specificity of the immunostaining was verified by replacing the anti-C3 antibody with unrelated Ab of the same subclass (goat anti-rabbit IgG peroxidase conjugated), as well as by omitting the primary antibody as a conventional staining control.

### 2.10.3 Biotin-Streptavidin for gp-340 (Indirect peroxidase staining)

A biotin-streptavidin immunoperoxidase technique was used on frozen lung tissue sections, adapted from a protocol for paraffin-embedded lung sections (Madsen *et al,* 2000). Briefly and as described above, sections were fixed in acetone, air dried, blocked for endogeneous peroxidase expression with 2 % $H_2O_2$ in methanol, washed in TBS, preincubated with 2 % (v/v) FCS in TBS for 10 min, incubated for 30 min with rabbit polyclonal IgG antibody against human gp-340 (40 µg/ml of 1.7 mg/ml stock) in TBS

containing 1 % (v/v) FCS, washed in TBS, incubated for 30 min with biotin-labelled goat anti-rabbit IgG (Dako) diluted (1 in 100) in TBS, washed with TBS, incubated with HRP-coupled streptavidin (Dako) diluted (1 in 300) in TBS, washed with TBS and distilled water, incubated for 10 min with DAB peroxidase substrate prepared as described previously (section 2.10.2), washed with tap water, counterstained with haematoxylin, washed in water and finally mounted in Aquamount medium. The specificity of the immunostaining was confirmed by omitting the primary antibody.

## 2.11. Western blotting of lung homogenates

### 2.11.1 Collection of lung tissue

At pre-chosen time points following the intranasal infection, groups of mice were sacrificed by cervical dislocation. For each animal, the chest was opened and the lungs were exposed. 1ml of sterile PBS was slowly injected into the right ventricle of the heart. Once they were perfused and fully extended, the lungs were collected into 3 ml of sterile PBS containing 2 μg/ml each of the protease inhibitors leupeptin, pepstatin A and 2 mM phenylmethylsulfonylfluoride (PMSF). Lung tissue was homogenized using Mixer Mill 300 (Ultra-Turrax T8 IKA, Labortechnik) and then centrifuged at 8000 xg for 30 min in the cold room at 4 °C. Supernatants of lung homogenates were aliquoted and stored at – 70 °C until required.

### 2.11.2 Measurement of total protein concentration in lung homogenate

Before separating lung homogenate samples by SDS-PAGE, the total protein concentration of each sample was determined using the Bio-Rad protein assay. Briefly, in a 1ml cuvette, 2 μl of the sample was mixed well with 798 μl distilled water and 200 μl Bio-Rad dye concentrate. After 15 min, the $A_{595nm}$ was measured (Section 2.5.1).

### 2.11.3 SDS-PAGE and electroblotting

Lung homogenate samples were analysed by the Laemmli (1970) system of discontinous SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting as described by Sambrook *et al.* (1989). A mini protein 2-cell modular mini-electrophoresis system (Bio-Rad) was used according to the manufacturer's instructions.

The SDS-PAGE solutions:

Resolving gel (10 %): Distilled water 1.9ml, 30 % acrylamide 1.7 ml, 1.5 M Tris-HCl (pH8.8) 1.3 ml, 10 % (w/v) SDS 0.05 ml, 10 % (w/v) ammonium persulfate (APS) 0.05 ml, TEMED (N, N, N', N'-tetramethylethylenediamine) 0.002 ml.

Stacking gel (5 %): Distilled water 2.1ml, 30 % acrylamide 0.5 ml, 1 M Tris-HCl (pH6.8) 0.38 ml, 10 % (w/v) SDS 0.03 ml, 10 % (w/v) ammonium persulfate (APS) 0.03 ml, TEMED 0.003 ml.

SDS- PAGE loading buffer pH 6.8: Tris-HCl 100mM, Dithiothreitol (DTT) 200mM, SDS 4 % (w/v), Bromophenol blue 0.2 % (w/v), Glycerol 20 % (v/v).

SDS-PAGE electrophoresis buffer pH 8.3: Tris base 25mM, glycine 250mM, SDS 0.1 % (w/v).

SDS transfer buffer pH 8.3 : Tris base 48mM, Glycine 49mM, SDS 0.03 %, Methanol 100 % (v/v).

Following the addition of TEMED and ammonium persulphate (APS) to the resolving solution described above, the mixture was poured between the plates and overlaid with water. After polymerisation, TEMED and APS were added to the stacking solution, which was poured above the resolving gel. A comb was inserted to create wells. Lung homogenate samples were appropriately diluted in loading buffer in order to load 40 μg of total protein per well in a total volume of 20 μl and were boiled for 5 min prior to loading on the gel along side a well containing prestained protein marker (Bio-Rad) of molecular weight ranging from 203 KDa-7.2 KDa.

Electrophoresis was performed through 10 % (w/v) polyacrylamide gels at 70 V for 2 hours. After electrophoresis, proteins were transferred onto a nitrocellulose blotting membrane (Electran-BDH) at 100 V for 50 min. After transfer, proteins were visualised and fixed to the nitrocellulose membrane by staining with Ponceau S solution (0.5% w/v in 5% w/v TCA).

All incubations and washings took place at room temperature on a rotating platform. Following the transfer, the membrane was incubated overnight in a blocking buffer (5 % w/v skimmed milk from Difco in PBS). Next day, membrane was washed 4 x 10 min in PBS–0.05 % Tween 20, incubated with HRP conjugated goat polyclonal anti-mouse complement C3 (ICN Pharmaceuticals, Inc.) diluted 1 in 2000 in fresh blocking buffer for 2 hours, washed 3x 10 min in PBS-Tween 20 each followed by a 1x 10 min wash in

PBS without tween. Finally, using the ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK), protein bands were visualised according to the manufacturer's instructions. The ECL system utilises the oxidation of luminol by peroxidase to produce light which was enhanced by the second reagent. Equal volumes of reagents 1 and 2 (supplied with the kit; 0.125ml/cm$^2$) were mixed and poured onto the blot and incubated for 1 min at room temperature. The blot was drained, wrapped in Saran Wrap and exposed for 3 min to Kodak X-Ray films.

Protein bands of interest were quantitated by densitometry using the Bio-Rad documentation system. The software used for image analysis was the Muli analyst, Bio-Rad version 1.1.

### 2.12. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR for mouse Sp-D and CRP-ductin was done in the Department of Microbiology and Immunology, University of Southern Denmark, with the help of Dr. Jens Madsen. RT-PCR consists of two parts: synthesis of cDNA from mRNA by reverse transcription (RT) and amplification of a specific cDNA by polymerase chain reaction (PCR).

#### 2.12.1 Collection of lungs

At different pre-chosen time points and immediately after death of mice, the lungs were removed, dissected out into four pieces, aliquoted separately into cryotubes, snap frozen in liquid nitrogen and finally stored at -70°C until required.

#### 2.12.2 Isolation of total RNA from lung tissue

Total RNA from lung tissue was isolated using RNeasy Mini Kit (Quiagen). Prior to use, 10 µl of β–Mercaptoethanol was added to 1 ml RLT buffer and the concentrated RPE buffer was diluted by adding 4 volumes of 100% ethanol.

Two pieces of the same lung tissue were removed from storage at –70 °C, immediately placed into 3 ml of RLT buffer and homogenised using a Mixer Mill 300 (Ultra-Turrax T8 IKA, Labortechnik). The homogeniser and all materials used were autoclaved at 120 °C overnight.

Afterwards, the tissue lysate was centrifuged for 3 min at maximum speed in a microcentrifuge and the supernatant was carefully transferred into a new microcentrifuge tube and diluted (1in 1) with 70 % ethanol. Without delay, 700 µl aliquots were loaded successively onto the RNeasy column in a 2 ml collection tube and centrifuged for 20

sec at 8000 xg and the flow-through was discarded. To wash the column, 700 µl RW1 buffer was added to the column and centrifuged 20 sec at 8000 xg. The RNeasy column was transferred into a new 2 ml collection tube. The column was further washed by 500 µl of buffer RPE and centrifuged for 20 sec at 8000 xg. To dry the RNeasy silica-gel membrane, 500 µl of RPE buffer were added and centrifuged for 2 min at 8000 xg. Finally to elute, the RNeasy column was transferred to a new collection tube. 50 µl of RNase-free water was pipetted directly onto the membrane, centrifuged for 1 min at 8000 xg. The total RNA obtained was kept on ice and the concentration was measured by the absorbance at 260 nm and 280 nm (RNA concentration in µg/ml = $A_{260}$ /0.025 considering that $A_{260}$ of 1 indicates RNA concentration of 40 µg/ml). The quality of the RNA was checked by running 1 µl through a 1 % agarose gel.

### 2.12.3 Agarose gel electrophoresis

Nucleic acid fragments can be separated according to size by agarose gel electrophoresis (Sambrook *et al.*, 1989). 1 % agarose was dissolved in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH adjusted to 7.7 with glacial acetic acid). Electrophoresis was performed in TAE buffer containing 0.5 µg/ml ethidium bromide. Before loading the gel, diluted samples (1 µl sample in 10 µl of RNase free water) were added to 2 µl of 6 x gel loading buffer (0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 15 % (w/v) Ficoll type 400 (Pharmacia) in nanopure water. 1 Kb DNA size markers (Gibco, BRL) in gel loading buffer were also loaded into the gel. Samples were electrophoresed at 70 volts for 1-2 hours.

DNA or RNA fragments were visible by exposure of the agarose gel to UV light from a long wave UV transilluminator.

### 2.12.4 Synthesis of cDNA from RNA

Complementary DNA (cDNA) was synthesised from total RNA of lung tissue using a reverse primer. In a microcentrifuge tube, a total volume of 11 µl containing 2 µg of the total RNA, distilled water and 1 µl oligo dT (500 µg/ml) (Gibco) was heated to 65 °C for 5 min and quickly chilled on ice.

9 µl of reaction mix was added to the microcentrifuge tube and it contained:

1 µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH)

2 µl 0.1 M DTT

4 µl 5 X First-strand buffer (250 mM Tris-HCl pH 8.3, 375mM KCl, 15mM $MgCl_2$)

1 µl RNase OUT Recombinant Ribonuclease inhibitor (40 units/ µ l)

1 µl (200 units) Superscript II RNase H⁻ reverse transcriptase (Gibco, BRL).

The cDNA was synthesised at 42°C for 50 min and the reaction was stopped by heating at 70 °C for 15 minutes. The first-strand cDNA was used as a template for amplification in PCR.

### 2.12.5 Polymerase chain reaction (PCR)

PCR reactions were set up on ice, to a final volume of 30 µl including:

5 µl template (cDNA)

3 µl of 10 X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$)

3 µl of dNTP (2 mM of each)

2 µl of of primers (10-25 pmol/µl) (1 µl of each primer, forward and reverse)

16.8 µl of distilled water

0.2 µl of *Taq* polymerase (5 units/µl) (Sigma)

The tubes were placed in a Perkin Elmer Gene Amp PCR System 2400 and PCR was performed as follows: 1 min at 94 °C, 25 cycles for mouse Sp-D and 31 cycles for CRP-ductin, each cycle consisting of: 94 °C for 30 sec (DNA denaturation), 55 °C for 30 sec (DNA annealing) and 72 °C for 90 sec for Sp-D and 30 sec for CRP-ductin (DNA synthesis), followed by a 72 °C for 7 min.

For mouse Sp-D, the forward primer was Sp-D f1 (5'-CAG ACA GTG CTG CTC TG-3') and the reverse primer was mSp-D r1 (5'-TCA GAA CTC ACA GAT AAC AAG-3'). This primer pairs spans the neck and CRD of Sp-D and should give rise to a single product of 459 bp.

For mouse gp-340 (CRP-ductin), the primer pair Sca8 f1 (5'-GGA AAT GAT TCT TCA TTG GCG-3') and PST r1 (5'-TAC ATA GGA AGG ACT TGT AGTC-3') was used. These span the 8$^{th}$ SRCR domain and the following linker region rich in proline, serine and threonine residues and should give rise to a product of 381 bp.

As an internal standard for the amount of RNA used in PCR, a primer pair for the mouse transferrin receptor mRNA was used. The primers m TFR f1 (5'-TTG GAT TCA TGA GTG GCT ACC-3') and m TFR r1(5'-CCA TGT TTT GAC CAA TGC TG-3') were used as described above for mouse CRP-ductin in PCR of 25 cycles. This primer pair gives rise to a product of 371 bp. Reaction products were analysed by agarose gel electrophoresis (Section 2.12.3).

## 2.13. Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was done by the analysis of variance (ANOVA) followed by the Bonferroni correction. The INSTAT program on Macintosh was used. Significance was considered as P values $<0.05$.

# Chapter 3: Results

## 3.1 Contribution of the complement activation activity of pneumolysin to pneumococcal virulence *in vivo*

Site directed mutagenesis showed that distinct parts of the pneumolysin molecule are responsible for its cytolytic and complement activation activities (Boulnois *et al.*, 1991). Using homologous recombination, isogenic pneumococcal strain derivatives of the virulent strain *S. pneumoniae* type 2 strain D39 were made, producing pneumolysin lacking one or other of its wild-type activities (Berry *et al.*, 1995). A defined point mutation (Asp385 > Asn) was made in the chromosomal pneumolysin gene that resulted in the abolition of pneumolysin's ability to activate complement but with a complete haemolytic activity (H+/ C-) (Mitchell *et al.*, 1991).

The strain H+/ C- was used in different models of pneumococcal infection and these studies showed that complement-activating activity of pneumolysin has a specific contribution to virulence of the pneumococci. In a model of lobar pneumonia in mice, complement-activating activity was associated with bacterial growth and bacteraemia at 24 h after pulmonary infection (Rubins *et al.*, 1995). Following an intranasal challenge of mice, the mutant H+/ C- was less virulent than the wild-type and the ability of pneumolysin to activate complement affected the behaviour of pneumococci in lungs and blood in the first 24 h after infection (Alexander *et al.*, 1998).

To further investigate the mechanisms by which complement-activating activity of pneumolysin individually influences *in vivo* events during bronchopneumonia and septicaemia, mice were infected intranasally with either the wild-type *S. pneumoniae* D39 or the isogenic mutant strain H+/ C-. Bacterial growth kinetics in lungs and blood, histological changes and the host immune response to pneumococci producing pneumolysin lacking complement activation property were investigated and compared to the parental wild-type strain.

In this chapter, data for the wild-type strain were provided by Dr Aras Kadioglu, Dept. of Microbiology and Immunology, University of Leicester.

### 3.1.1 Growth of wild-type and H+/ C- mutant in lung tissue and blood

There were clear differences in the growth curves of the wild-type and mutant strains (H+/ C-) in the lungs (Fig 3.1) and the blood (Fig 3.2).

The number of H+/ C- pneumococci in the lungs remained constant over 24 h post-infection, contrary to the wild-type pneumococci which showed a sharp decline in number over the first 12 h post-infection ($P < 0.01$, compared to t zero) (Fig 3.1).

Thereafter, the wild-type levels increased at 24 and 48 h post-infection, numbers of the wild-type and H+/ C- pneumococci were not significantly different ($P > 0.05$).

The ability of pneumolysin to activate complement influenced the timing of bacteraemia after intranasal infection (Fig 3.2). The wild-type pneumococci were detected in blood at 12h after infection, while H+/ C- pneumococci was detected earlier at 6 h after infection, but by 24 and 48 h there was no difference ($P > 0.05$) in the number of wild-type and H+/ C- bacteria in the blood.

### 3.1.2 Histological analysis of wild-type and H+/ C- infected lung tissue

At intervals following the infection, histological examination of lung tissue sections from mice infected with the wild-type and the mutant H+/ C- was done following haematoxylin and eosin staining of the sections (Fig 3.3, 3.4 and 3.5).

Generally, the inflammation in lung tissue sections was seen as a hypertrophy of the bronchiole wall (enlargement of alveolar cells due to fluid accumulation) and heavy cellular infiltration to the inflamed areas. It is worth mentioning that in bronchopneumonia some areas of the lungs are inflamed while others look healthy.

At 24 h after infection, the H+/ C- lung sections exhibited less severe inflammation as compared to the wild-type tissue sections at the same time point. The wild-type sections showed inflammation and heavy cellular infiltration centered around bronchioles and perivascular areas. By 24h after infection, the H+/ C- sections showed heavy cellular infiltrate restricted to areas tightly associated with and within infected bronchioles (Fig 3.4) but no infiltration of perivascular areas. The parenchyma was not involved in inflammation (Fig 3.4) and interstitial alveolitis was not detected at 24 h after infection. The hypertrophy of the cell wall was slighter when compared to the wild-type tissue sections at 24 h.

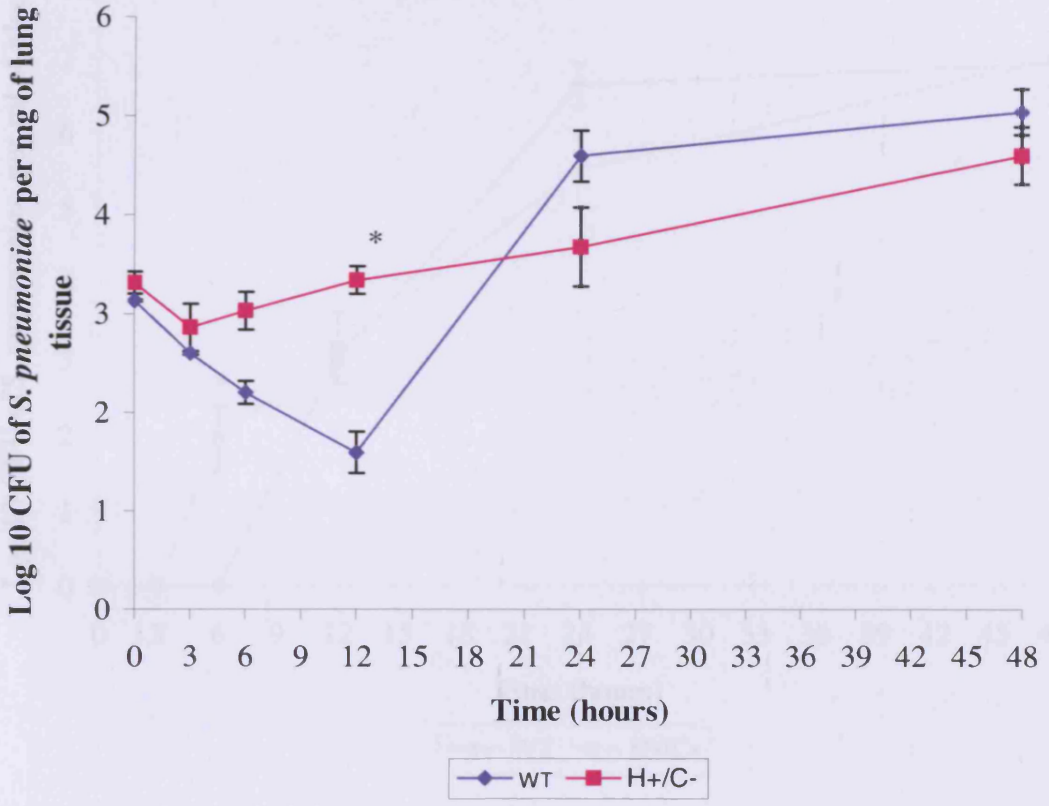



**Figure 3.1** Time course of change in numbers of *S. pneumoniae* wild-type and H+/ C- in the lungs of MF1 mice infected intranasally with $10^6$ CFU (n = 10 for each time point, error bars indicate SEM).

* P <0.05 for H+/ C- at 12h compared to wild-type.

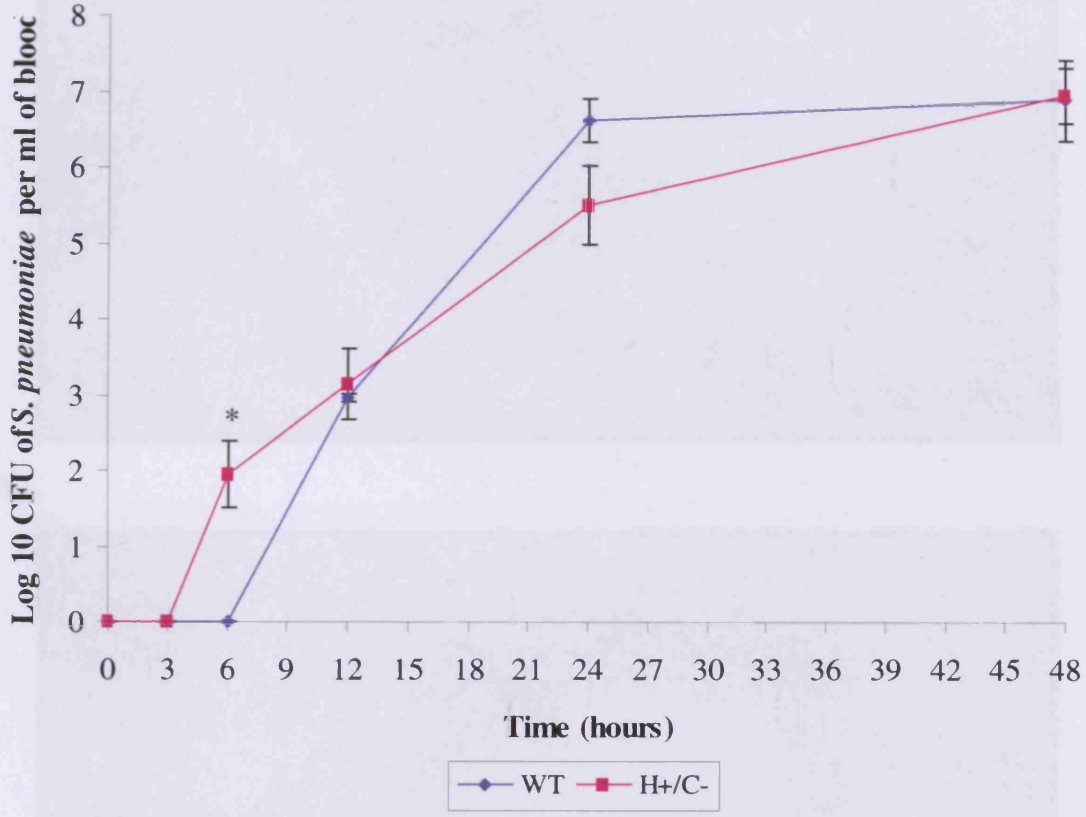
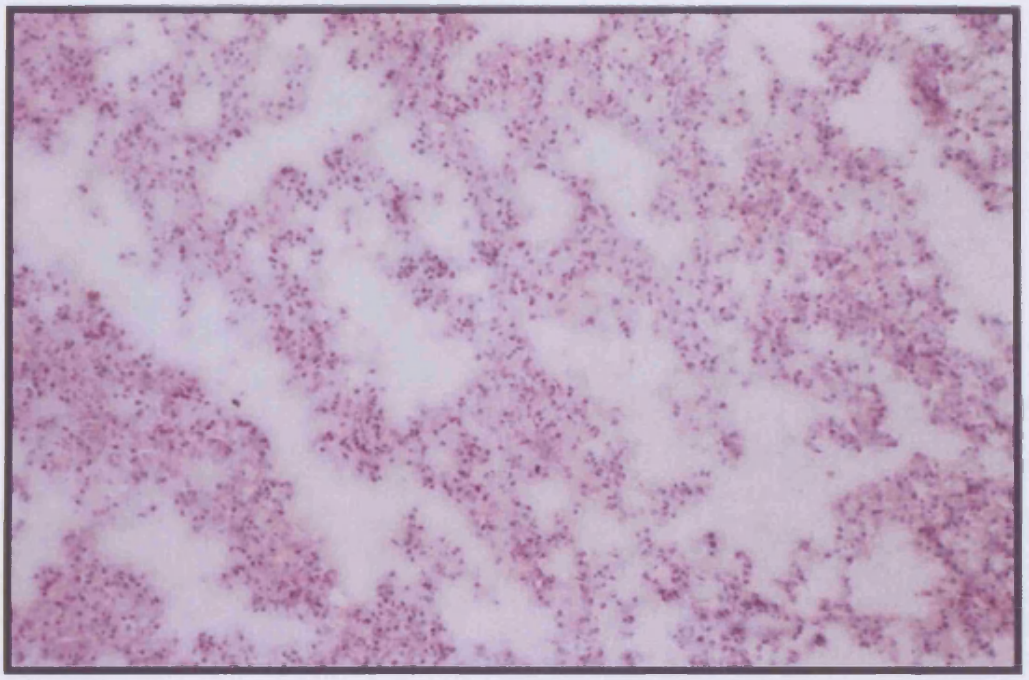



**Fig 3.2** Time course of change in numbers of *S. pneumoniae* witld-type and H+/ C- in the blood of MF1 mice infected intranasally with $10^6$ CFU (n = 10 for each time point, error bars indicate SEM).

* P <0.05 for H+/ C- at 6 h compared to the wild-type.

(A)



(B)

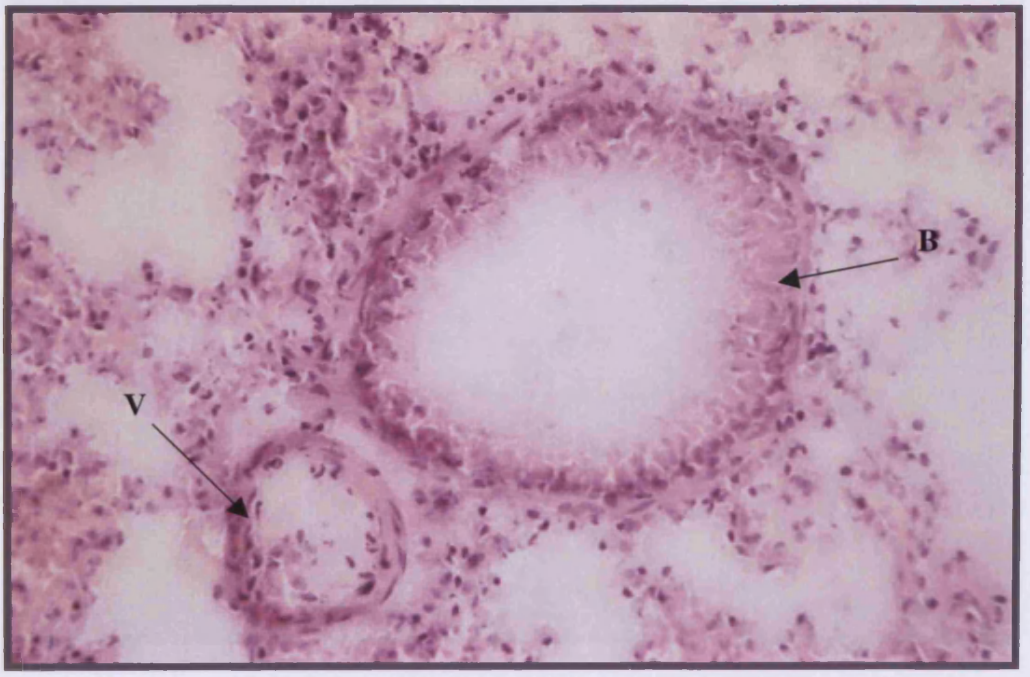
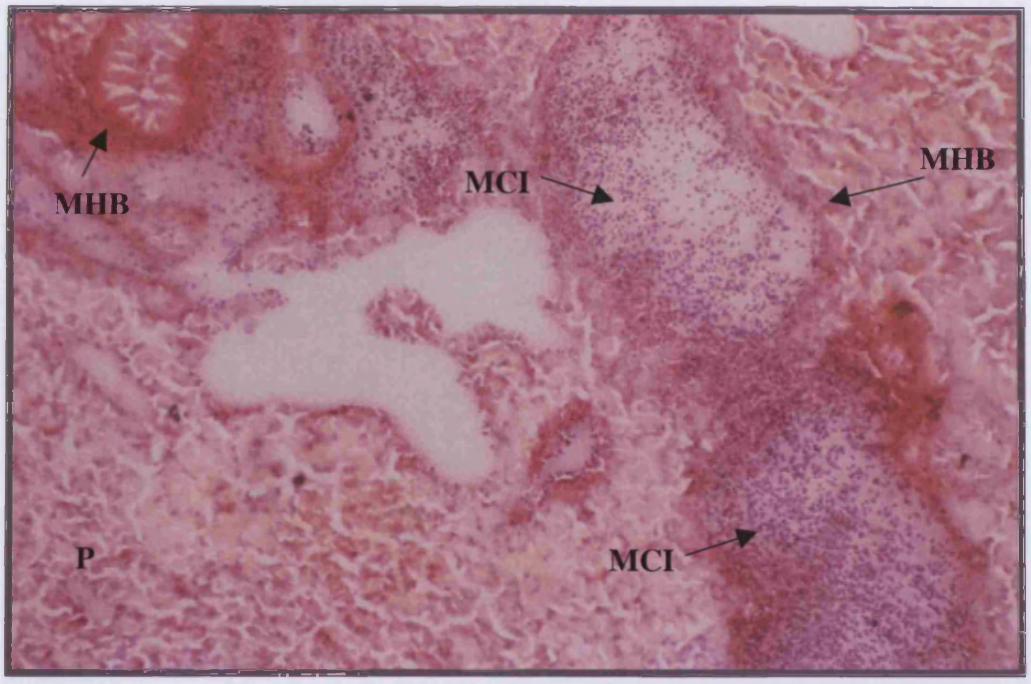



**Fig 3.3** Light microscopy of lung tissue from MF1 mice infected with $10^6$ CFU of *S. pneumoniae* H+/ C- at (A) 0h and (B) 12h post-infection. Magnification (A) x250, (B) X400. B= bronchiole, V= venule.

(A)




(B)

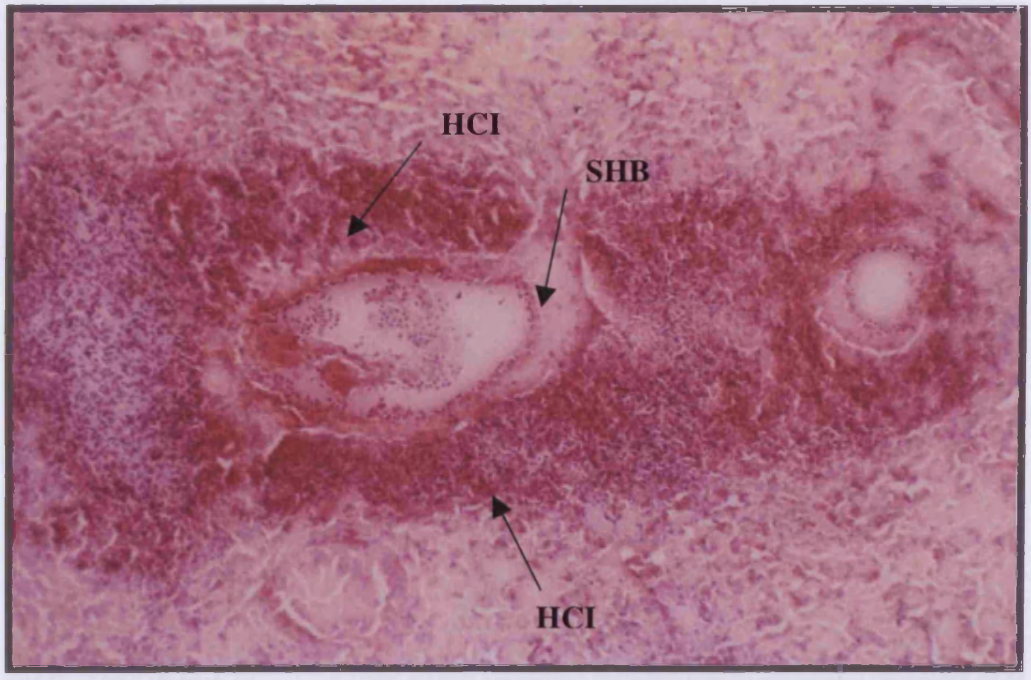
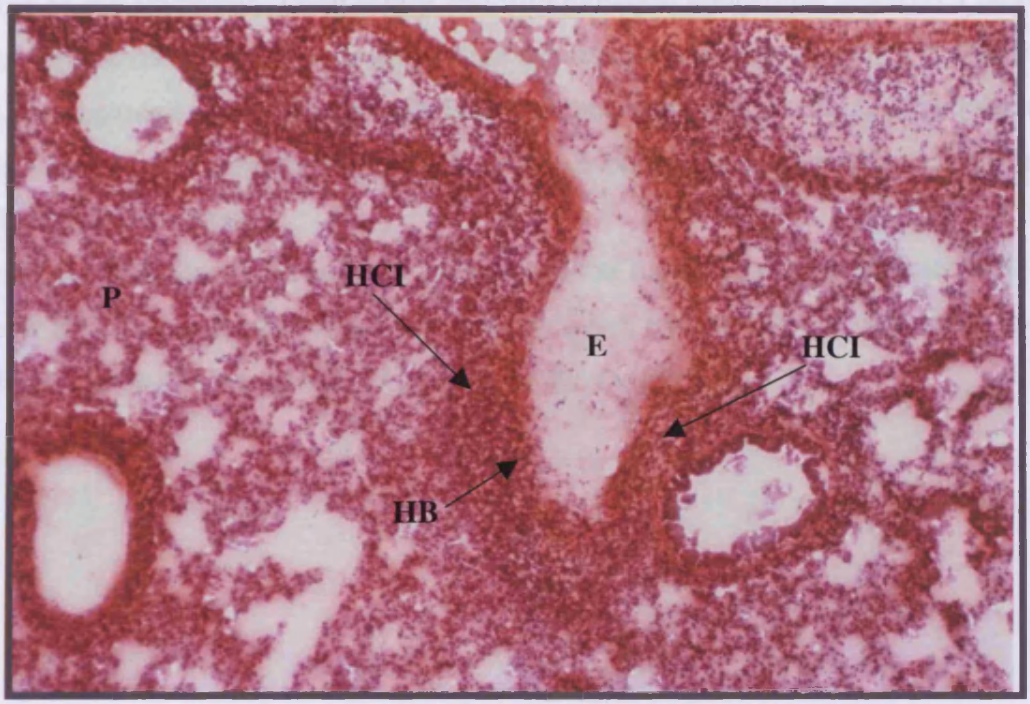



**Fig 3.4** Light microscopy of lung tissue from mice infected with $10^6$ CFU of *S. pneumoniae* (A) H+/ C- and (B) wild-type at 24 h post-infection. Magnification x300. MCI and HCI= mild and heavy cellular infiltration, MHB and SHB= medium and severe hypertrophy of the bronchiole wall, P= parenchyma not involved in inflammation.

(A)




(B)

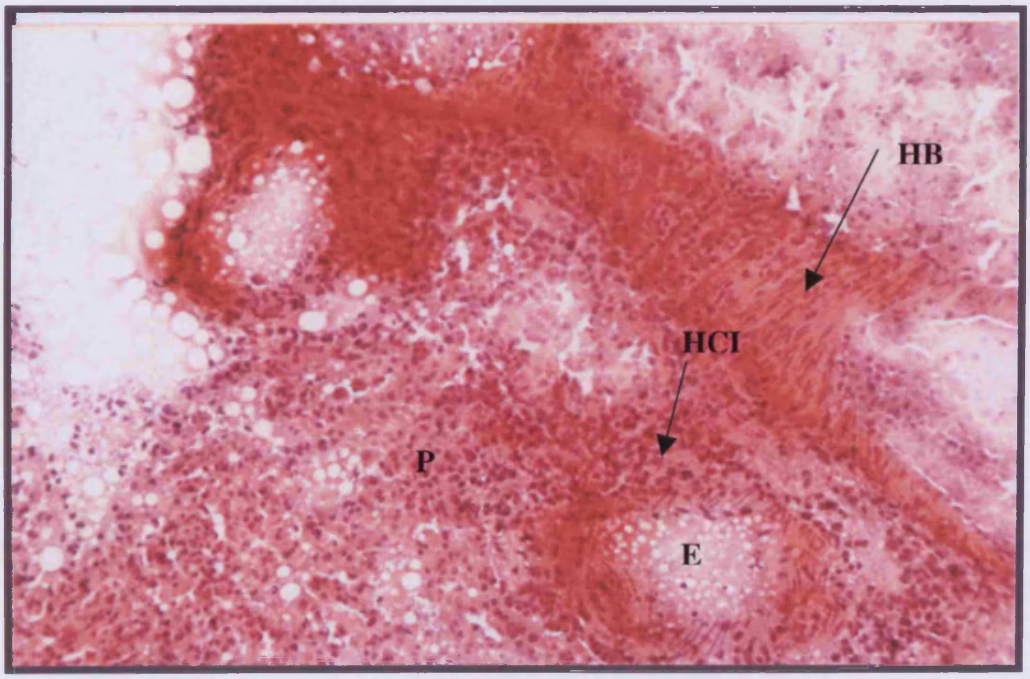



**Fig 3.5** Light microscopy of lung tissue from mice infected with $10^6$ CFU of *S. pneumoniae* (A) H+/ C- and (B) wild-type at 48 h post-infection. Magnification x300. HB= hypertrophy of the bronchiole wall, HCI= heavy cellular infiltration, E= exudate in bronchiole and P= parenchyma extensively infiltrated.

By 48 h post-infection, H+/C- and wild-type lung tissue sections showed the same degree of inflammation. Lung injury and inflammation almost had covered a large area of the lung surface. The hypertrophy of the bronchiole wall was a distinctive feature (Fig 3.5) accompanied with a severe multifocal peribronchial, perivascular and intrabronchial infiltration of inflammatory cells (Fig 3.5). The bronchioles and lung alveoli appeared to be filled with exudates.

Overall, at 48 h after infection, the majority of the lungs from mice infected with wild-type or H+/ C- pneumococci showed consolidation, hypertrophy of infected bronchiole walls and extensive infiltration of lung parenchyma (Fig 3.5).

### 3.1.3 Immunohistochemical analysis of inflammatory cell infiltrates into the lung

Immunohistochemistry was done to identify leukocytes and to analyse their infiltration into lung tissue over the time course after intranasal infection with the wild-type and H+/ C- pneumococci. Antibodies to T cells (CD3), B cells (CD19), macrophages (F4/80) and neutrophils (7/4) were used. Positively stained cells were enumerated in inflamed areas of sectioned lung tissue only.

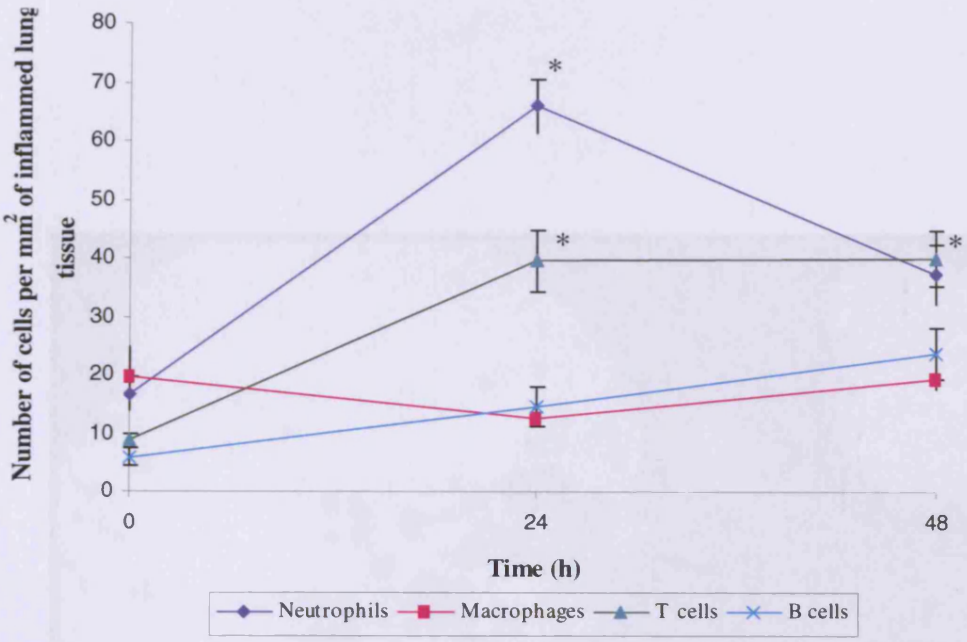
In the inflamed areas of H+/ C- infected lung tissue, neutrophils showed the same pattern of recruitment as in the wild-type infected lung tissue. Infiltration of neutrophils was detected within inflamed bronchioles, in bronchiole walls, perivascular areas nearby inflamed bronchioles, as well as the alveolar spaces (Fig 3.7).

The number of neutrophils showed a significant increase in H+/ C- lung tissue sections ($P < 0.05$) at 24 h, compared to time zero (Fig 3.6). The number of neutrophils then decreased significantly ($P < 0.05$) by 48 h post-infection. The same picture was seen with in lungs infected with wild-type pneumococci ($P > 0.05$ between wild-type and H+/ C- at each time point). Overall, neutrophils were much more abundant than macrophages or lymphocytes.

The number of T cells in lung tissue sections remained constant over the 48 h following infection with H+/ C- pneumococci ($P > 0.05$) (Fig 3.6 B). In contrast, in lungs from mice infected with wild-type pneumococci, numbers of T lymphocytes showed a significant increase at 24 and 48 h post-infection ($P < 0.001$) (Fig 3.6 A). T cells were heavily detected in tissue surrounding inflamed bronchioles and slightly in close proximity to the bronchiole walls themselves by 24 h (Fig 3.8).

The total number of macrophages in inflamed areas of wild-type or H+/ C- pneumococci infected lung tissue sections remained constant ($P > 0.05$) at 0, 24 and 48 h post-infection (Fig 3.6 A and B). Macrophages were seen inside inflamed bronchioles, in perivascular tissue areas surrounding such bronchioles, and in alveolar spaces (Fig 3.8).

(A)




(B)

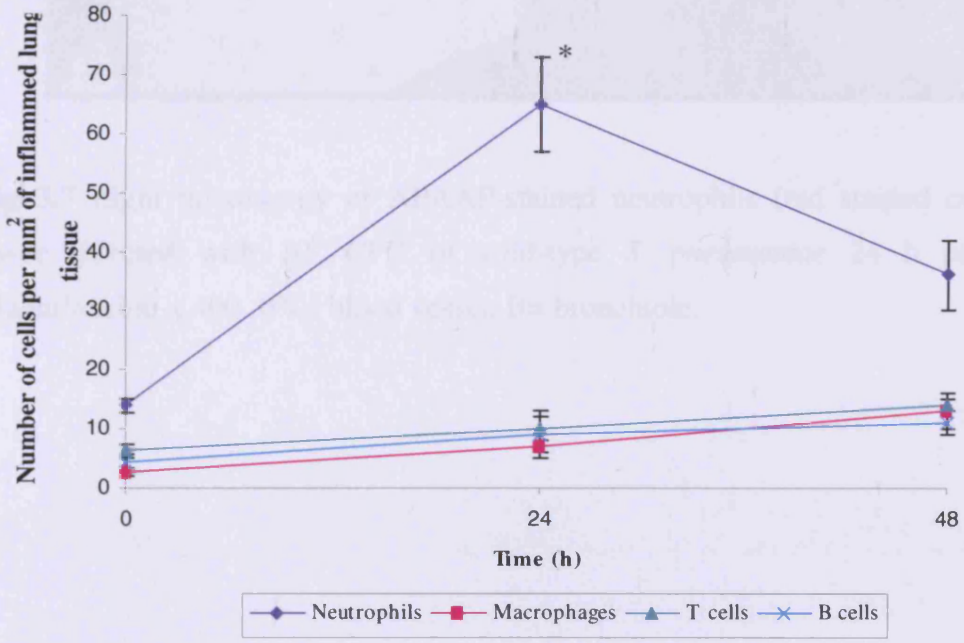



**Fig 3.6** Numbers of neutrophils, macrophages, T cells and B cells in tissue sections from lung sections of MF1 mice infected intranasally with $10^6$ CFU of (A) wild-type and (B) H+/ C- *S. pneumoniae.* (n = 4 for each time point, error bars indicate SEM).

* P <0.05 significantly different compared to time zero.

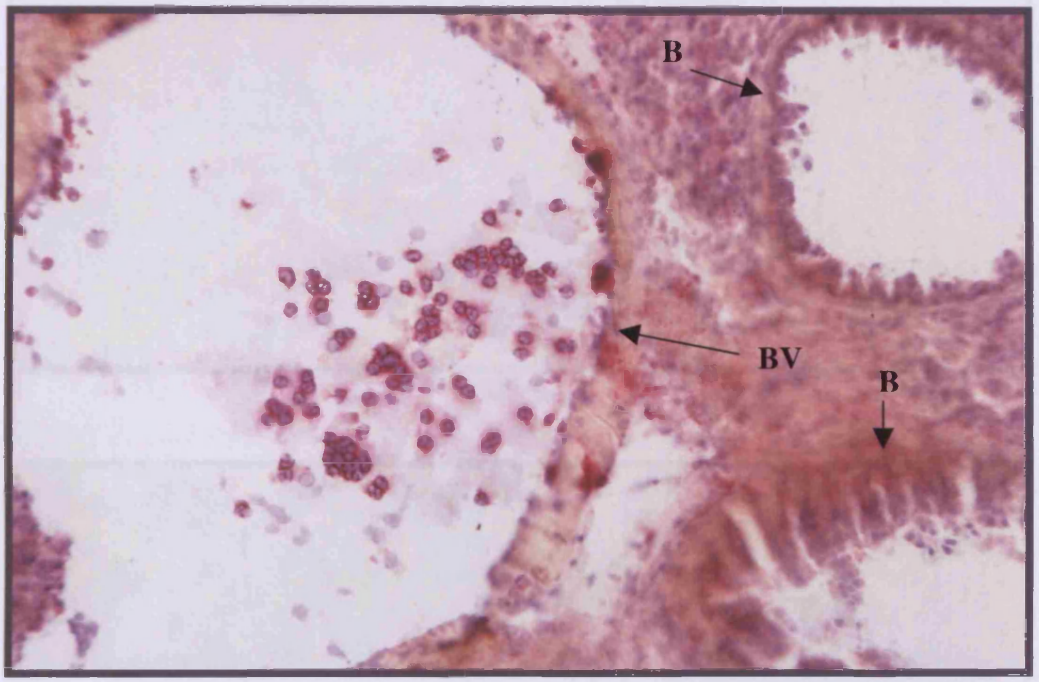
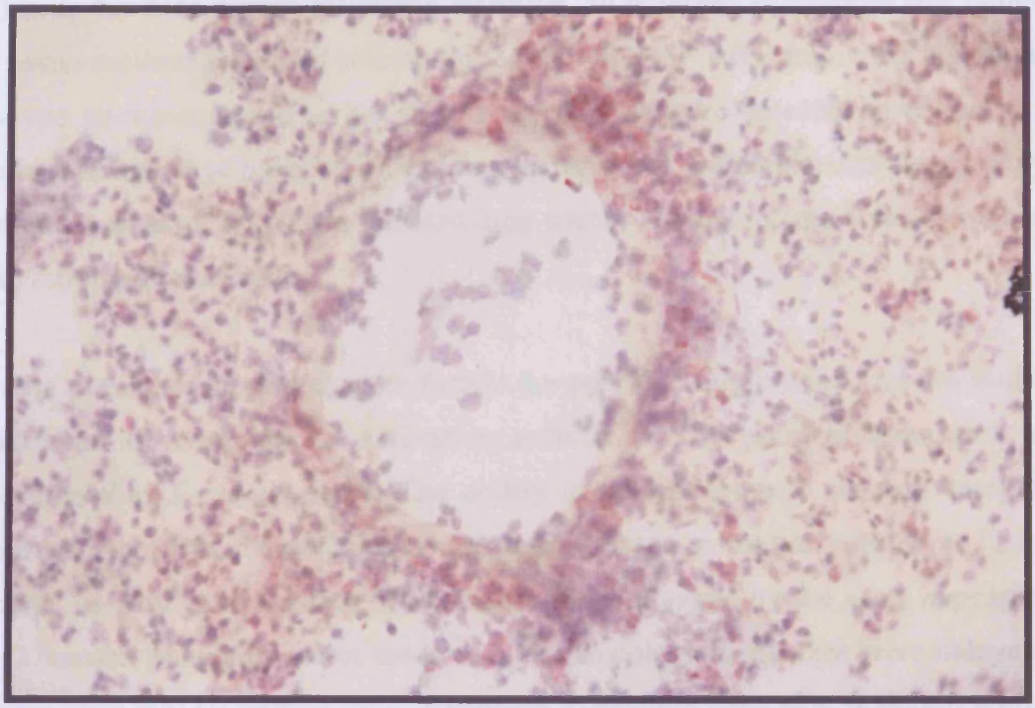



**Fig 3.7** Light microscopy of APAAP-stained neutrophils (red stained cells) in lung tissue infected with $10^6$ CFU of wild-type *S. pneumoniae* 24 h post-infection. Magnification x 400. BV= blood vessel, B= bronchiole.

(A)



(B)

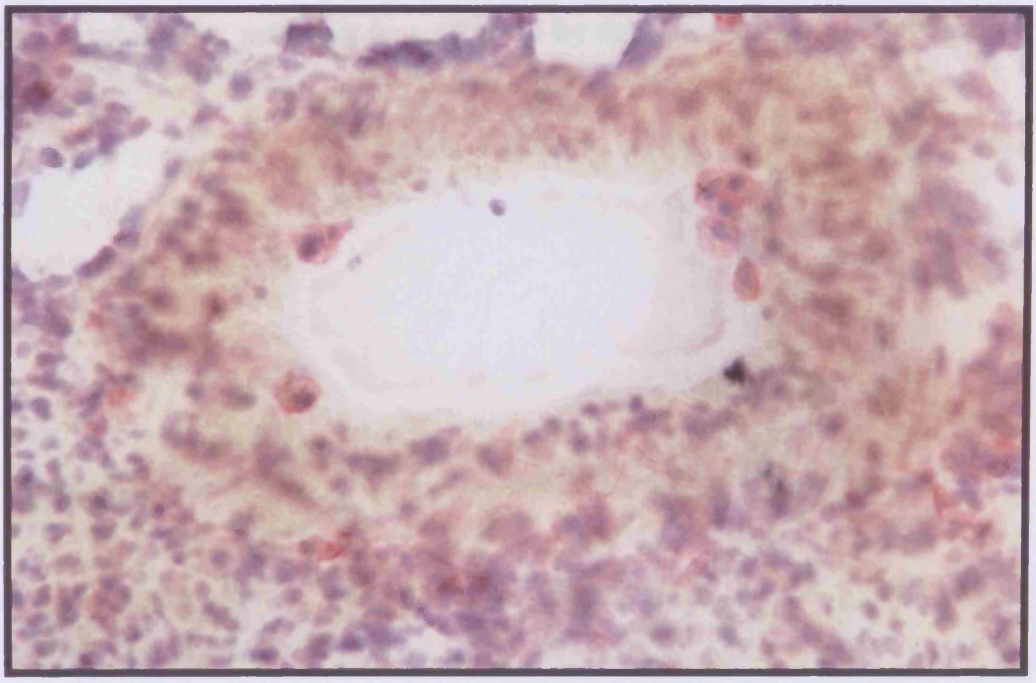


**Fig 3.8** Light microscopy of APAAP-stained (A) T lymphocytes and (B) macrophages (red stained cells) in lung tissue infected with $10^6$ CFU of wild-type *S. pneumoniae* 24 h post-infection. Magnification x 400.

The number of B cells in H+/ C- infected lung tissue sections remained unchanged ($P > 0.05$) over the time course following infection. In contrast, B lymphocytes number in lung tissue sections increased steadily by 24 and 48 h ($P < 0.05$) following infection with wild-type pneumococci (Fig 3.6 A and B). B cells were detected in tissue in close proximity of inflamed bronchioles and to a lesser extent within alveolar spaces. By 48 h, in inflamed areas of wild-type infected lung tissue, B lymphocytes were also observed within inflamed bronchioles.

The data presented in this chapter fortify the previous observations on the individual contribution of complement activation activity of the toxin pneumolysin to the pathogenesis of *S. pneumoniae*. The ability of pneumolysin to activate complement influenced the pneumococcal behaviour in lung tissue in the early hours at 12 h after infection, as well as the timing of the onset of bacteraemia by the early appearance of H+/ C- mutant pneumococci in the blood. The histological changes were delayed after infection with H+/ C- mutant strain compared to changes seen after infection with wild-type pneumococci. Additionally, the infiltration of T lymphocytes into lung tissue over the time course after infection was affected by the complement activation activity of pneumolysin.

## 3.2 Activation of complement C3 in mouse lungs by the pneumococcal toxin pneumolysin

Previous studies, involving the instillation of exogenous pneumolysin directly into the lung, suggested that the ability of the toxin to activate the complement system was responsible to a large extent for the pronounced inflammation characterizing pneumococcal infections (Feldman *et al.*, 1991). Subsequent studies showed that mutant pneumococci unable to produce pneumolysin generated less inflammation in lung tissue when compared with the wild-type (Canvin *et al.*, 1995, Kadioglu *et al.*, 2000).

As reported in 3.1, the complement activation activity of pneumolysin made a contribution to the pathogenesis of *S. pneumoniae* that was distinct from the contribution of the anticellular activity of the toxin.

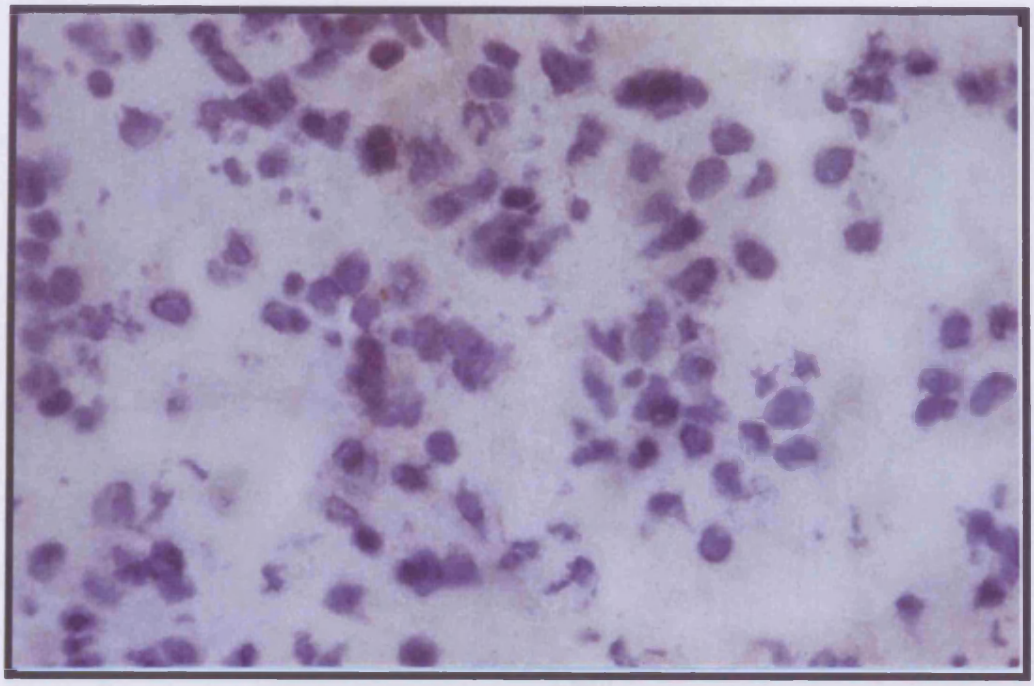
The present study reports an investigation of the local effect of pneumococci expressing variants of pneumolysin on the activation of complement C3 in mouse lungs during pneumococcal bronchopneumonia. Mice were infected intranasally with either wild-type *S. pneumoniae* D39 or the isogenic mutant strain PLN-A unable to produce pneumolysin. Immunohistochemistry and Western blotting were done on infected mice lungs to determine C3 activation.

### 3.2.1 Immunohistological detection of complement C3 in lung tissue

Immunoperoxidase staining using antibody against complement C3 was performed on non-infected, wild-type and PLN-A infected lung tissue sections to detect complement C3 and its activation products.

In non-infected lungs, only a light staining for complement C3 was detected on the surface of some bronchial epithelial cells (Fig 3.9). At 48 h after infection, the sections of lungs infected with wild-type exhibited strong staining surrounding the areas of inflamed bronchioles while the sections of lungs after PLN-A infection showed less staining when compared to the wild-type sections at the same time point (Fig 3.10). When the anti-C3 antibody was omitted or an unrelated antibody was used, no staining was seen. Since the anti-mouse C3 antibody recognized C3, C3b and iC3b (Wang *et al.*, 1998), immunohistochemistry reflected the presence of complement C3 as well as its cleavage products. However, from the above experiment, the total amounts of C3 and its cleavage products were found to a much higher extent in wild-type than PLN-A infected lung tissue at 48h after infection.

(A)



(B)

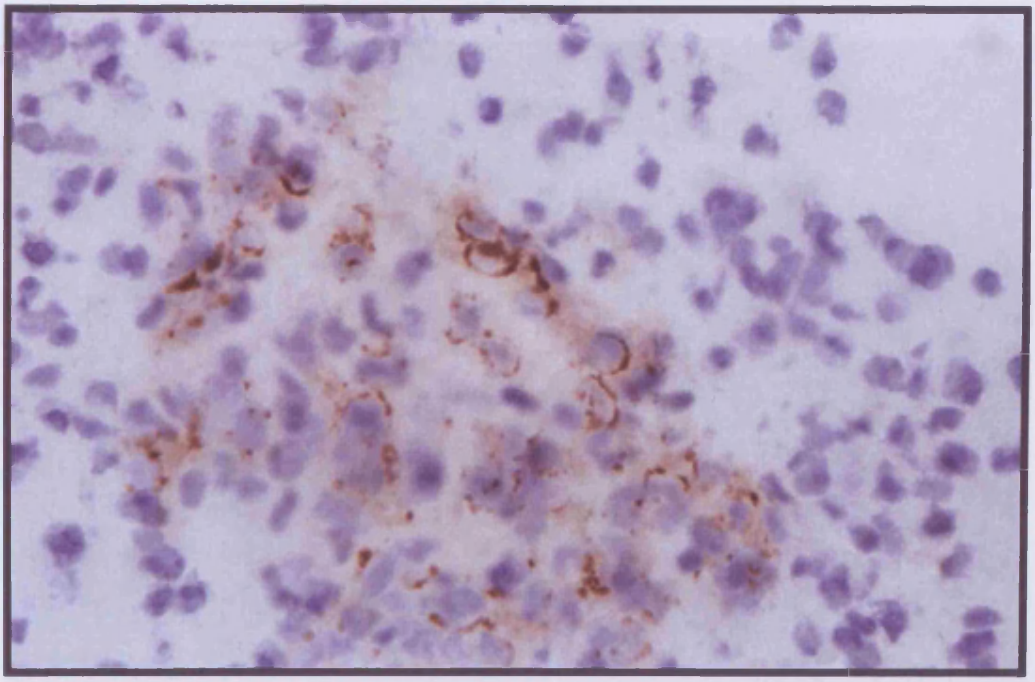
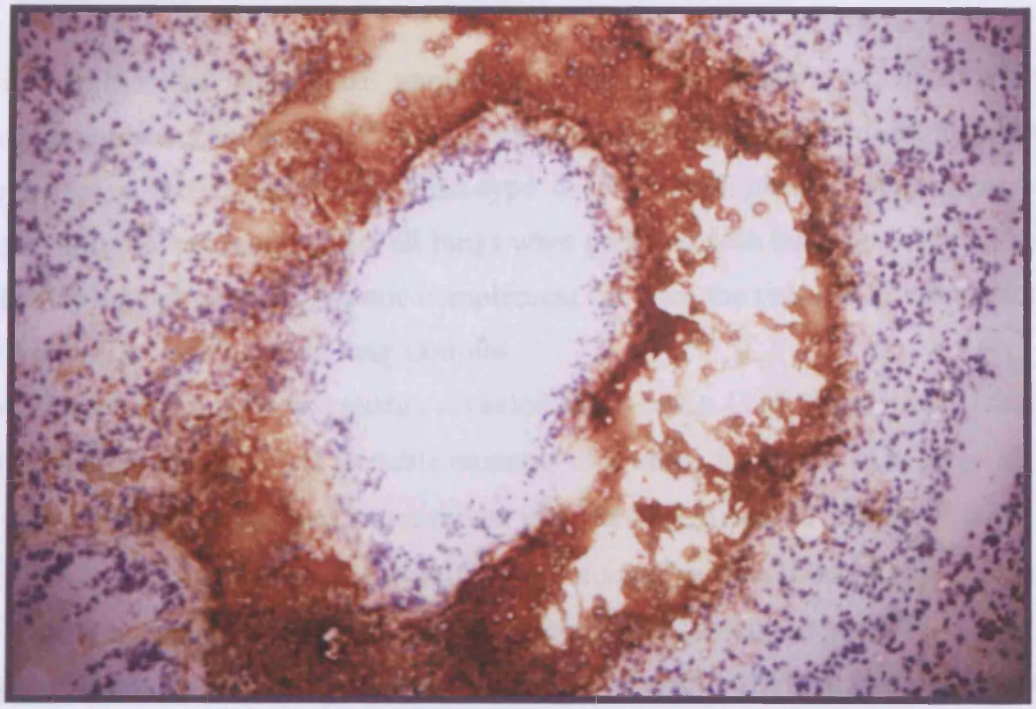


**Fig 3.9** Immunohistochemistry of non-infected frozen lung sections in (A) absence and (B) presence of anti-mouse C3 antibody. Brown deposit indicates signal for complement C3. Magnification x500.

(A)



(B)

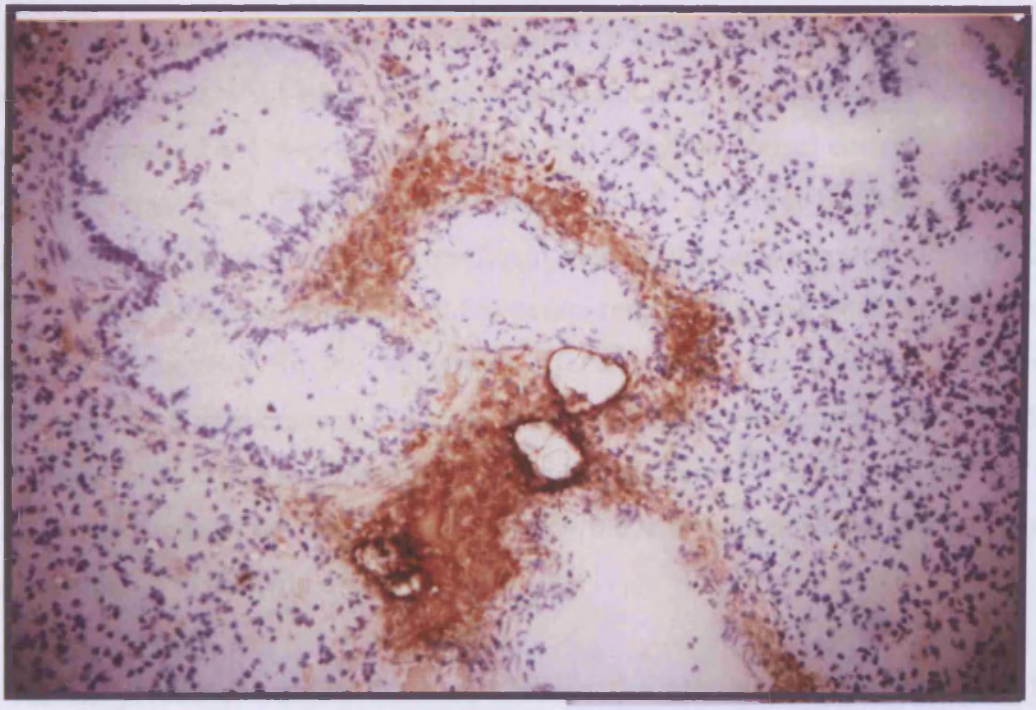


**Fig 3.10** Immunohistochemistry of (A) wild-type *S. pneumoniae* or (B) PLN-A infected frozen lung sections. Brown deposit indicates signal for C3 and its cleavage products. Magnification x250-300.

### 3.2.2 Activation of complement C3 in lungs infected with wild-type or PLN-A pneumococci

To determine the effect of pneumolysin on complement activation during bronchopneumonia, amounts of the cleavage product (iC3b) in lung homogenates of mice infected intranasally with wild-type or PLN-A *S. pneumoniae* mutant were compared. It is worth noting that all lungs were perfused with buffer prior to extraction, to alleviate any effect that systemic complement C3 from the pulmonary circulation may have had on C3 within whole lung samples.

Immunoblots of lung homogenates revealed the α chain (115 KDa) and β chain (75 KDa) of complement C3 and variable amounts of α chain fragments of iC3b (~ 39 KDa) that were constantly detected. However, as indicated by increased signals of the α chain fragments of iC3b, more complement C3 was activated in lung tissue coming from wild-type infected mice compared to PLN-A infected lung tissue at time 24 h (Fig 3.11. compare lanes 5 and 7 with 6 and 8) and 48 h post-infection (Fig 3.11. compare lanes 9 and 10 with 11 and 12). Using densitometry, the relative intensities of the α chain fragments of iC3b detected in different lung homogenates were compared. The results indicated a substantial difference in the extent of complement C3 activation between lungs infected with wild-type or PLN-A pneumococci. By 24 h after infection, the cleavage product of C3 was increased by 4-fold (relative optical density: 2.67 ± 0.64) compared to time zero (Relative optical density: 0.51 ± 0.16) ($P < 0.05$) in wild-type infected lungs while there was no significant increase (Relative optical density: 0.85 ± 0.20) in PLN-A infected tissue ($P > 0.05$, compared to t 0 h and $P < 0.05$, compared to wild-type). Thereafter, in the wild-type infected lungs, the level of activation of complement C3 was increased to 8-fold at time 48 h post-infection (4.99 ± 0.46) ($P < 0.05$, compared to t 24 h), while there was no increase (1.82 ± 0.68) detected in PLN-A infected lung tissue at the same time point ($P > 0.05$, compared to t 0 and 24 h and $P < 0.001$, compared to the wild-type).

The results presented in this section, showed the presence of pneumolysin had a significant impact on complement C3 activation in mouse lung tissue.

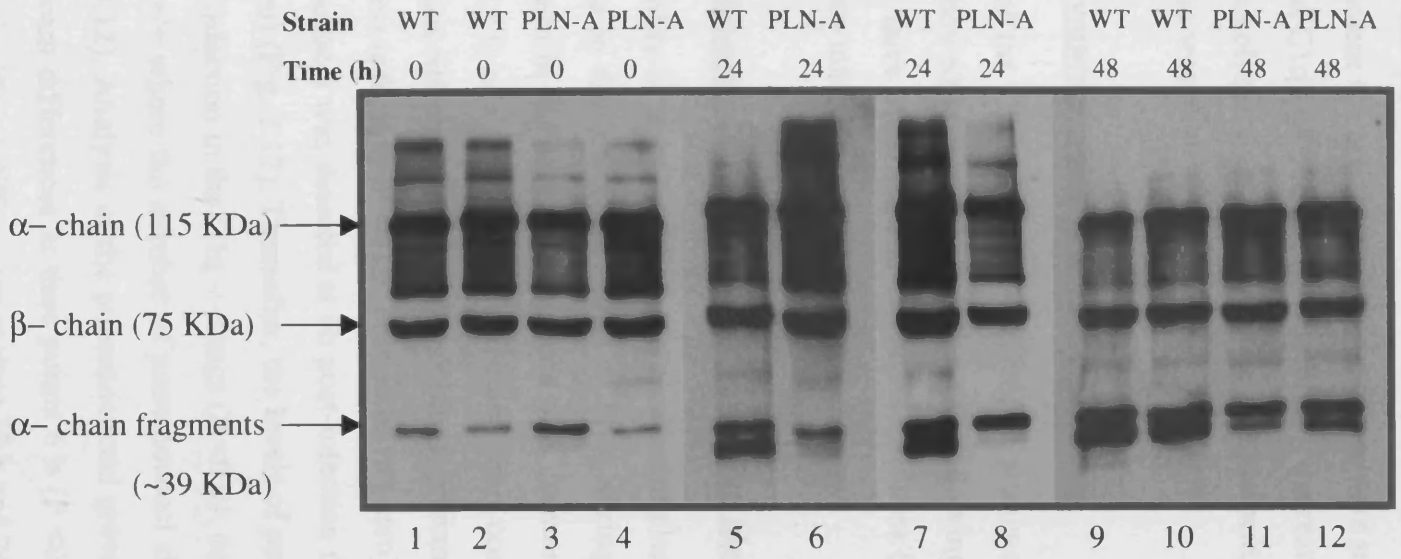



**Fig 3.11** Analysis of C3 activation in wild-type and PLN-A infected mouse lung tissue. MF1 mice were infected intranasally with $10^6$ CFU of wild-type *S. pneumoniae* or the PLN-A mutant. Lung homogenates were collected at 0, 24 and 48 h after infection, homogenised and 40 µg of total protein from each lung were electrophoresed on 10 % SDS-PAGE gels under reducing conditions. Proteins were transferred to nitrocellulose and visualised with anti-C3 antibody and enhanced chemiluminescence. The blot shows the C3 cleavage in lung homogenates of mice infected with wild-type pneumococci collected at time 0 h (lanes 1 & 2), 24 h (lanes 5 & 7) and 48 h (lanes 9 & 10) or with PLN-A pneumococci at time 0 h (lanes 3 & 4), 24 h (lanes 6 & 8) and 48 h (lanes 11 & 12). (n = 4 lungs for each time point).

### 3.3 Complement activation during bronchopneumonia in C1q deficient mice

To further investigate the overall role of the classical pathway and in particular the effect of the ability of pneumolysin to activate the classical complement pathway during pneumococcal bronchopneumonia, non-immune C1q deficient mice (C1q -/-) and C57 Bl/6j control mice (C1q +/+) were infected intranasally with wild-type *S. pneumoniae* D39. Bacterial growth kinetics in lungs and blood, histological changes seen in infected lungs, the pattern of inflammatory cell recruitment, in addition to analysis of complement C3 activation in lung tissue were investigated and compared between C1q (-/-) and C1q (+/+) mice. By combining the results, a fuller picture of the overall effect of pneumolysin's ability to activate complement on *in vivo* events during pneumococcal infection would be obtained.

#### 3.3.1 Symptoms following infection

None of the C1q +/+ mice challenged intranasally with $10^6$ wild-type *S. pneumoniae* CFU showed symptoms over the 48h following the infection. In contrast, 5 of the 10 C1q -/- mice observed showed signs of illness (starry coat and hunched appearance) at 48 h post-infection.

#### 3.3.2 Growth of pneumococci in lungs and blood of C1q -/- and C1q +/+ mice

The ability of pneumococci to activate complement critically influenced the growth of bacteria in the lungs and blood of mice during bronchopneumonia. Clear differences were seen in the patterns of growth of *S. pneumoniae* in the lungs (Fig. 3.12) and blood (Fig. 3.13) of C1q deficient mice and their correspondent C1q +/+ control mice after intranasal infection. In the C1q -/- lungs, pneumococci showed a decrease in number by 12 h post-infection ($P < 0.05$, compared to t zero), while a sharp decline in the number of pneumococci was detected at 6h post-infection in the C1q +/+ lungs ($P < 0.05$, compared to t zero) (Fig. 3.12). Thereafter, the levels of pneumococci increased substantially by 48 h post-infection in the C1q -/- lungs ($P < 0.01$, compared to t 24 h), in contrast to the C1q lungs +/+ where the number of pneumococci showed a decrease by 48 h postinfection (Fig. 3.12). Analysis of the pneumococcal growth in C1q -/- and C1q +/+ mice showed significant differences at time points 6 h ($P < 0.05$) and 48h ($P < 0.001$) post-infection, but no significant differences at time 12 h and 24 h after infection ($P > 0.05$).

By 24h after intranasal infection, *S. pneumoniae* appeared in blood of C1q -/- and C1q +/+ mice with no difference in the numbers of pneumococci between the two strains ($P > 0.05$). Thereafter, a notable bacterial increase was detected in C1q -/- blood ($P < 0.01$, 48h versus 24h) while in contrast no bacteria were recovered from C1q +/+ blood at 48h post-infection (Fig. 3.13).

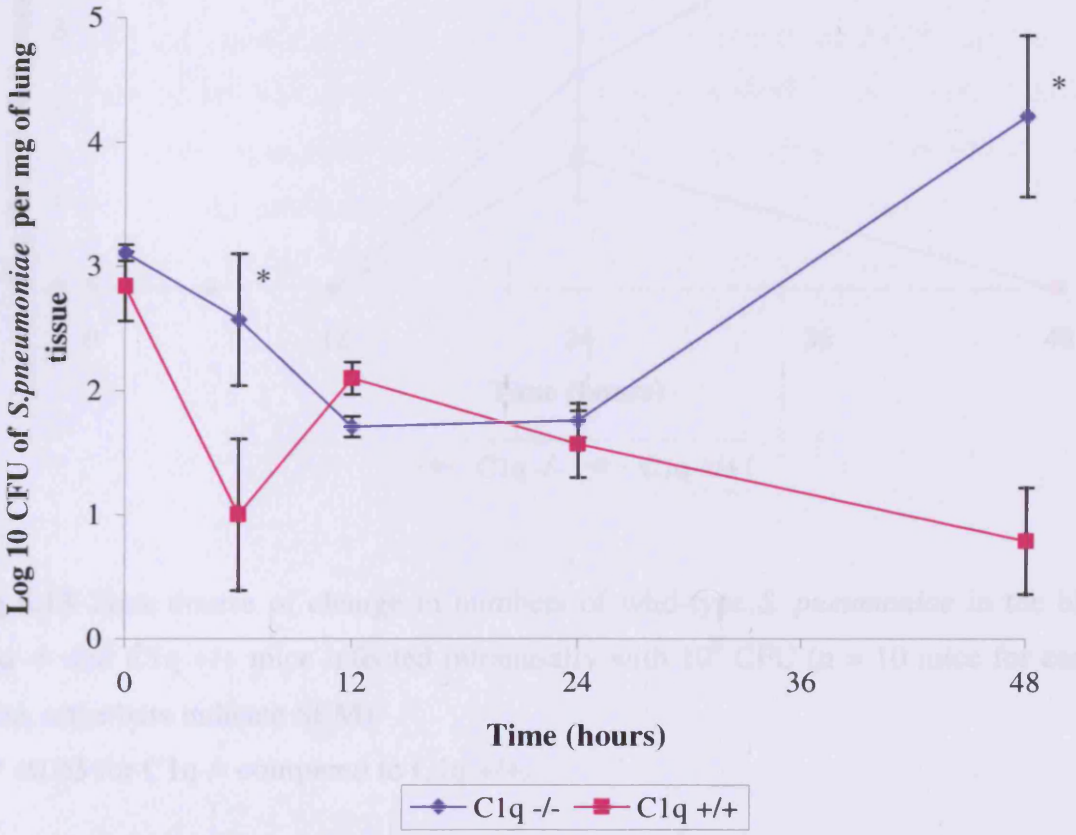



**Fig 3.12.** Time course of change in numbers of wild-type *S. pneumoniae* in the lungs of C1q -/- and C1q +/+ mice infected intranasally with $10^6$ CFU (n = 10 mice for each time point, error bars indicate SEM).

*P <0.05 for C1q-/- compared to C1q +/+

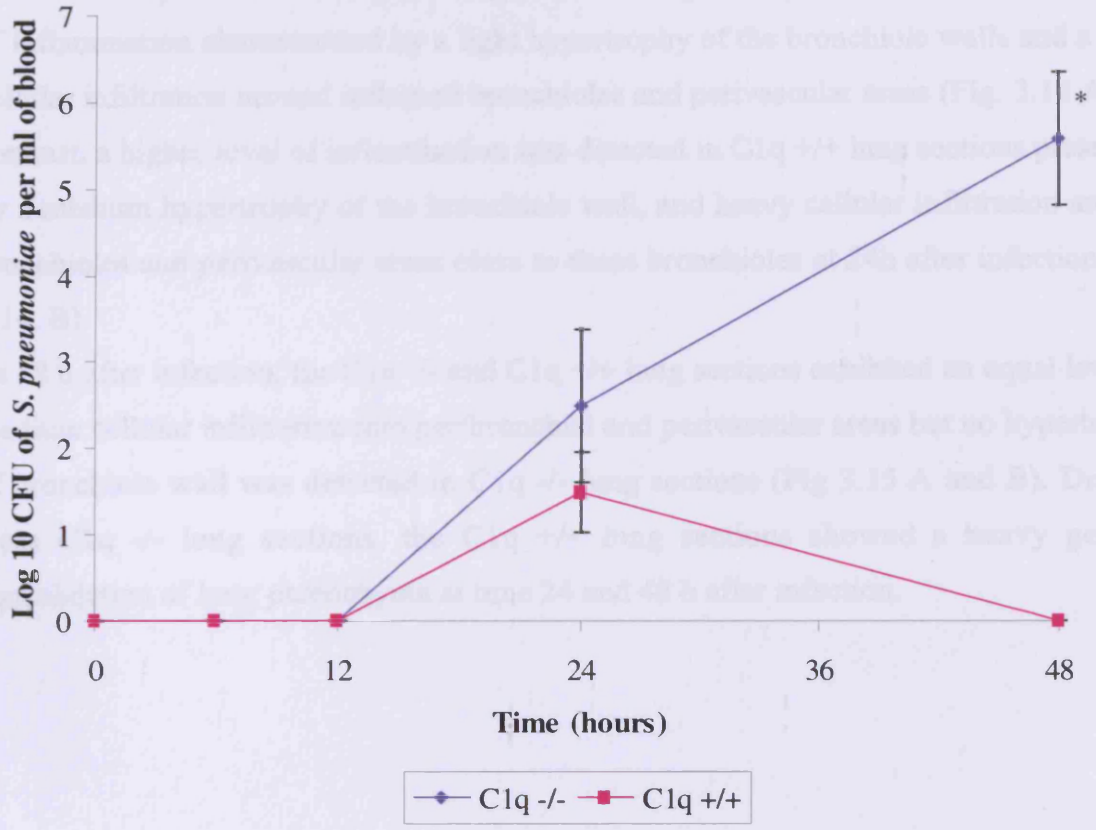



**Fig 3.13** Time course of change in numbers of wild-type *S. pneumoniae* in the blood of C1q -/- and C1q +/+ mice infected intranasally with $10^6$ CFU (n = 10 mice for each time point, error bars indicate SEM)

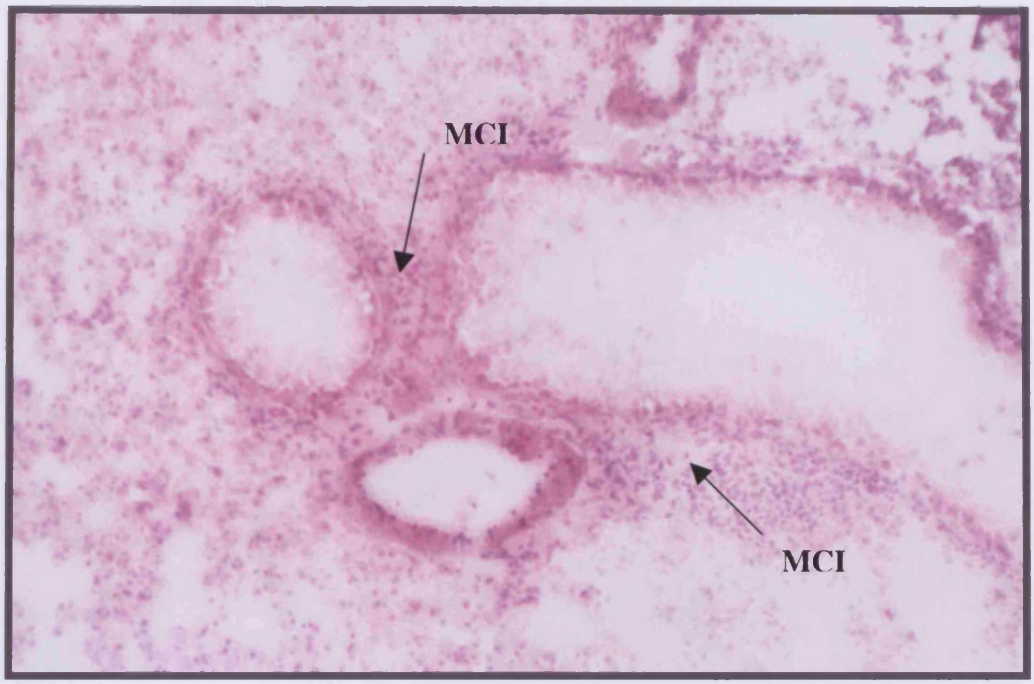
* P <0.05 for C1q-/- compared to C1q +/+.

### 3.3.3 Histology of C1q -/- and C1q +/+ mice infected with pneumococci

Histological examination of lung tissue sections of C1q -/- and C1q +/+ mice infected with *S. pneumoniae* was done at time 24 and 48 h after infection. The histological changes observed in the C1q -/- lung sections were less severe at time 24 h after infection compared to the C1q +/+ lung sections. C1q -/- lungs exhibited a minimal level of inflammation characterized by a light hypertrophy of the bronchiole walls and a light cellular infiltration around inflamed bronchioles and perivascular areas (Fig. 3.14 A). In contrast, a higher level of inflammation was detected in C1q +/+ lung sections presented by a medium hypertrophy of the bronchiole wall, and heavy cellular infiltration around bronchioles and perivascular areas close to these bronchioles at 24h after infection (Fig 3.14. B).

At 48 h after infection, the C1q -/- and C1q +/+ lung sections exhibited an equal level of medium cellular infiltration into peribronchial and perivascular areas but no hypertrophy of bronchiole wall was detected in C1q -/- lung sections (Fig 3.15 A and B). Distinct from C1q -/- lung sections, the C1q +/+ lung sections showed a heavy general consolidation of lung parenchyma at time 24 and 48 h after infection.

(A)




(B)

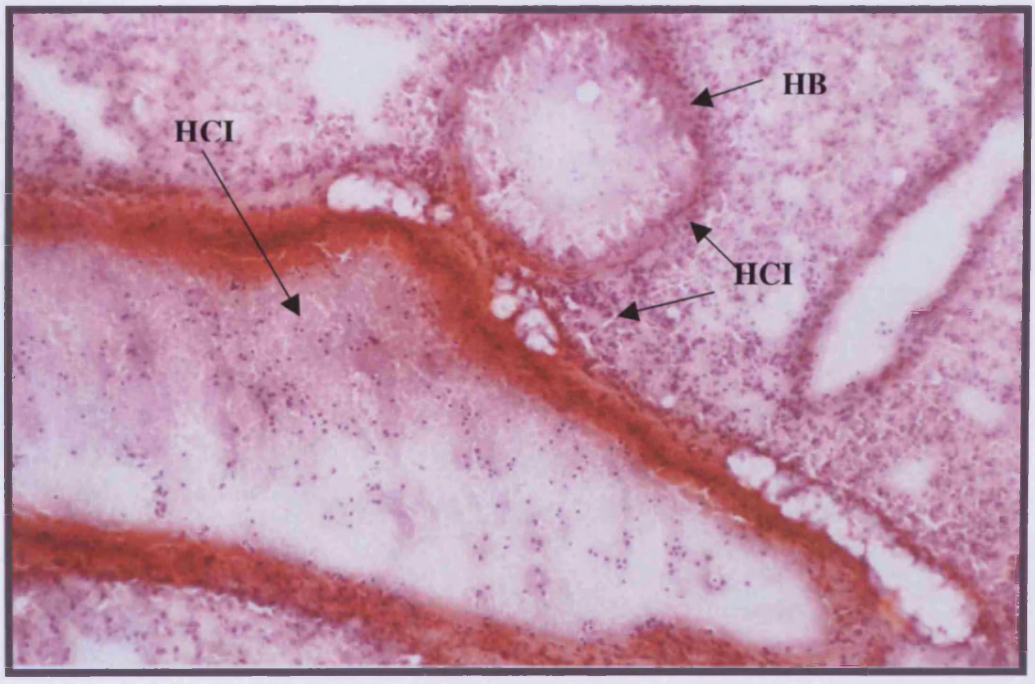
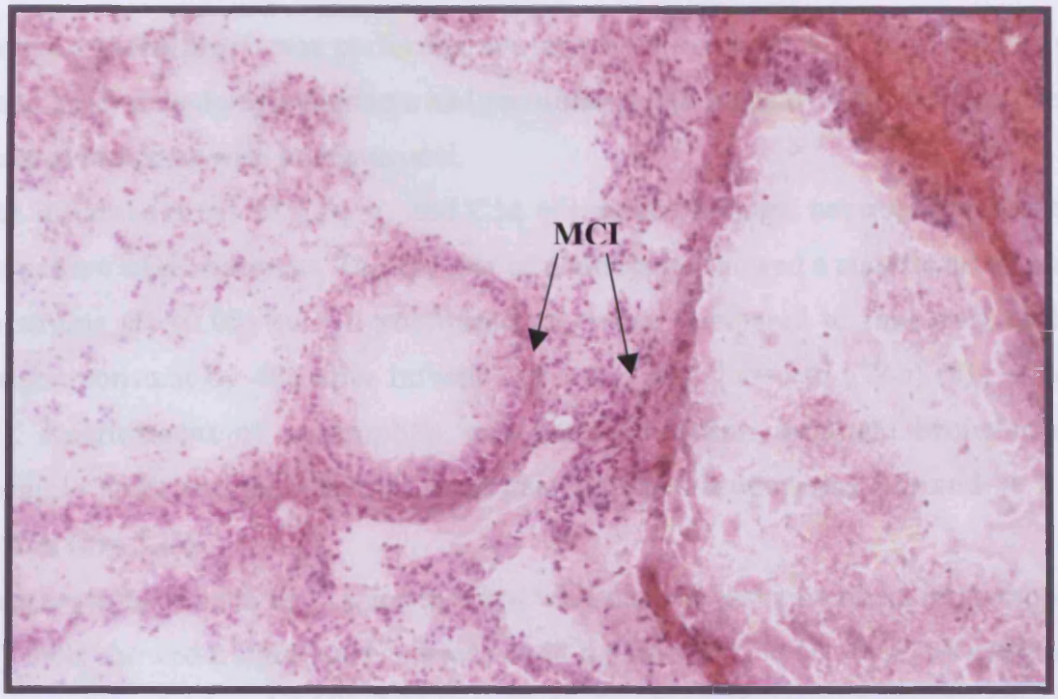



**Fig 3.14.** Light microscopy of lung tissue from (A) C1q -/- and (B) C1q +/+ mice infected with $10^6$ CFU of *S. pneumoniae* at 24h post-infection. Magnification x300. MCI and HCI= minimal and heavy cellular infiltration, HB= hypertrophy of the bronchiole wall.

(A)




(B)

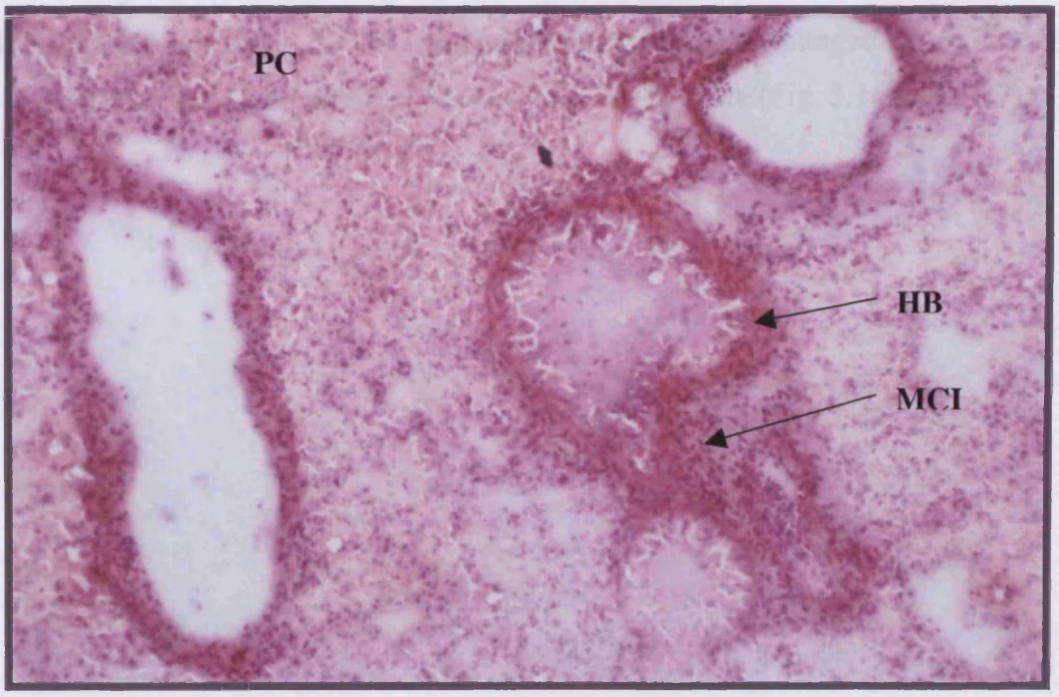



**Fig 3.15.** Light microscopy of lung tissue from (A) C1q -/- and (B) C1q +/+ mice infected with $10^6$ CFU of *S. pneumoniae* at 48h post-infection. Magnification x300. MCI= medium cellular infiltration, HB= hypertrophy of the bronchiole wall and PC= parenchyma consolidation.

### 3.3.4 Immunohistochemical analysis of inflammatory cell infiltrates

Immunohistochemistry was performed on lung tissue sections of C1q -/- and C1q +/+ mice to analyse leukocyte numbers and recruitment into lungs over the time course after intranasal infection with pneumococci.

In the inflamed areas of C1q -/- and C1q +/+ infected lungs, neutrophils showed the same pattern of recruitment. The number of neutrophils showed a significant increase in both strains ($P < 0.05$) at 24h post-infection, when compared to time zero and then remained constant by 48h after infection ($P > 0.05$, compared to t 24h) (Fig. 3.16 and 3.17). Recruitment of neutrophils was detected within inflamed bronchioles, in bronchiole walls and perivascular areas near inflamed bronchioles 24h and 48 h post-infection (Fig 3.18).

In lung sections from C1q -/- mice infected with pneumococci, numbers of macrophages and T cells showed a significant increase at 48 h post-infection ($P < 0.05$) as compared to time zero (Fig. 3.16 and 3.19). In contrast, the numbers of macrophages and T cells in C1q +/+ lung tissue sections remained constant and did not increase over the 48hrs following infection (Fig. 3.17). B cells numbers remained unchanged ($P > 0.05$) over the time course after infection of either C1q -/- or C1q +/+ mice (Fig. 3.16 and 3.17).

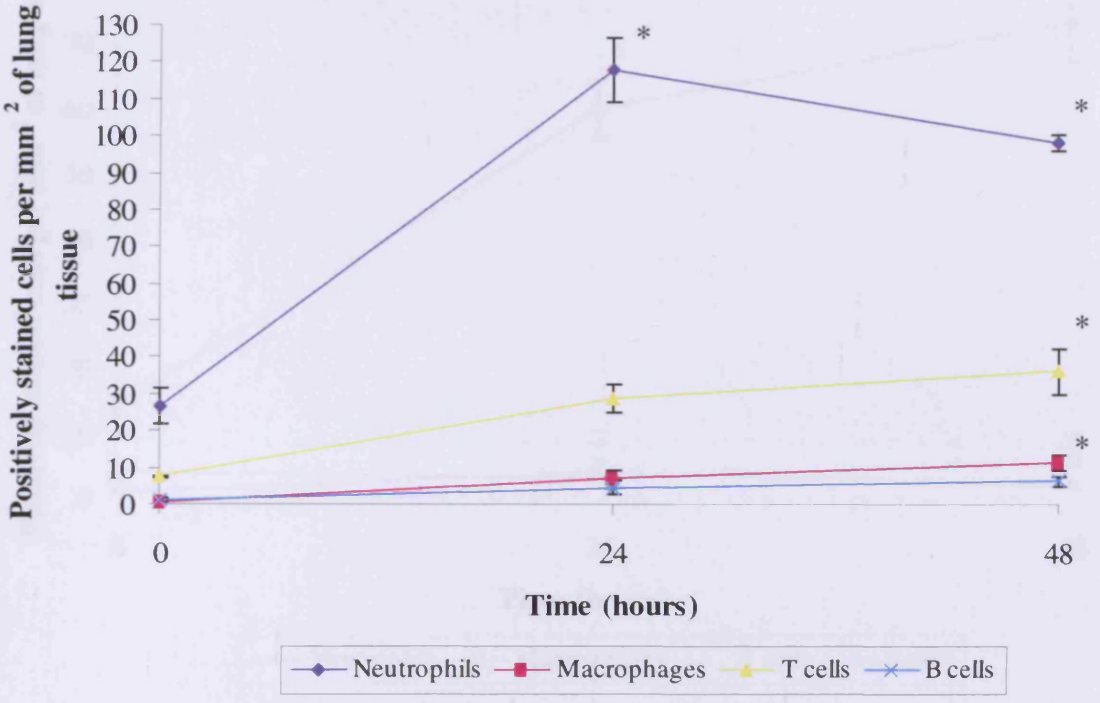



**Fig 3.16.** Numbers of neutrophils, macrophages, T cells and B cells in tissue sections from lungs of C1q -/- mice infected intranasally with $10^6$ CFU of *S. pneumoniae*. (n = 2 mice for each time point, error bars indicate SEM). * P <0.05 significantly different when compared to time zero.

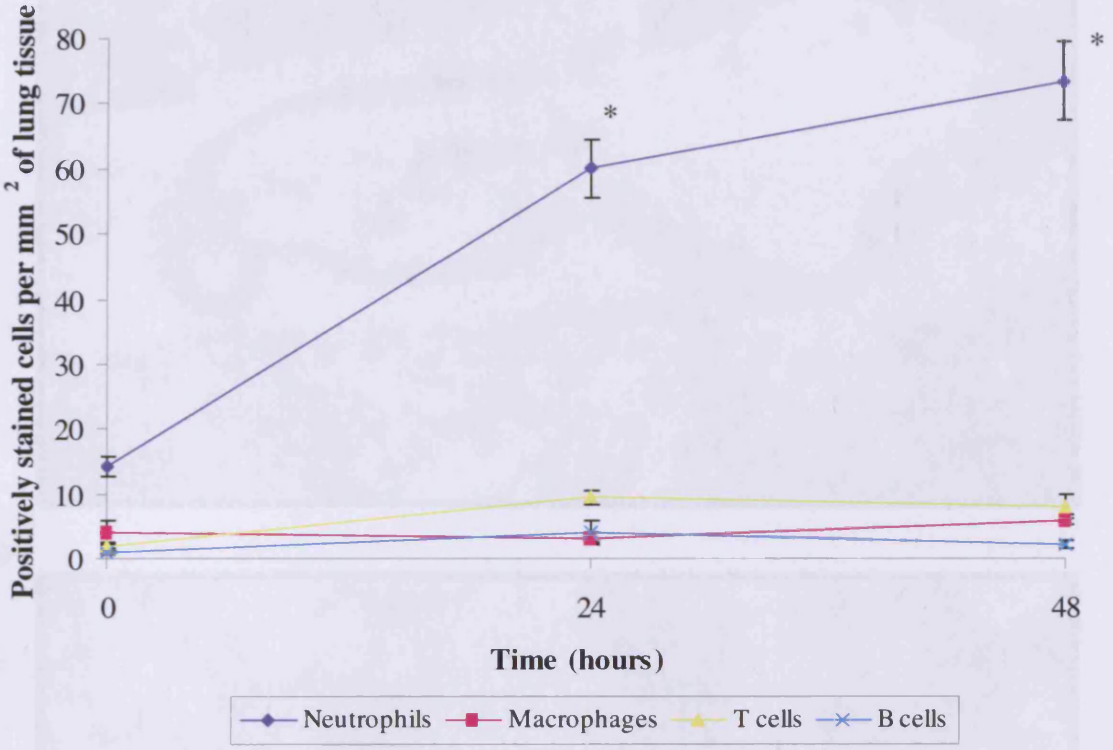
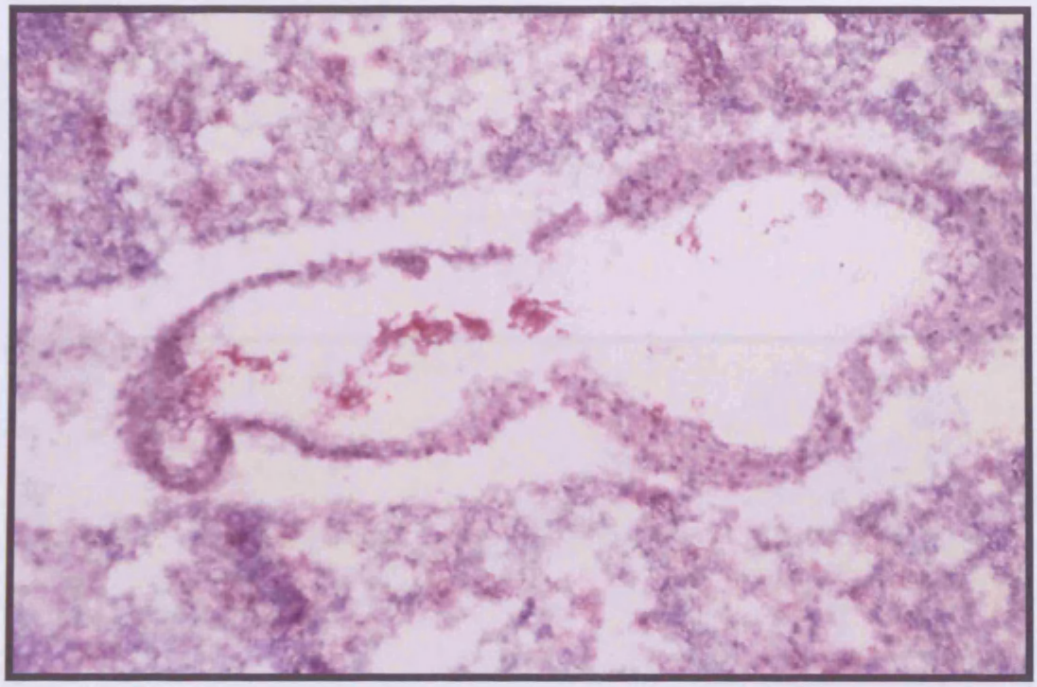



**Fig 3.17.** Numbers of neutrophils, macrophages, T cells and B cells in tissue sections from lungs of C1q +/+ mice infected intranasally with $10^6$ CFU of *S. pneumoniae*. (n = 2 mice for each time point, error bars indicate SEM). * P <0.05 significantly different when compared to time zero.

(A)



(B)

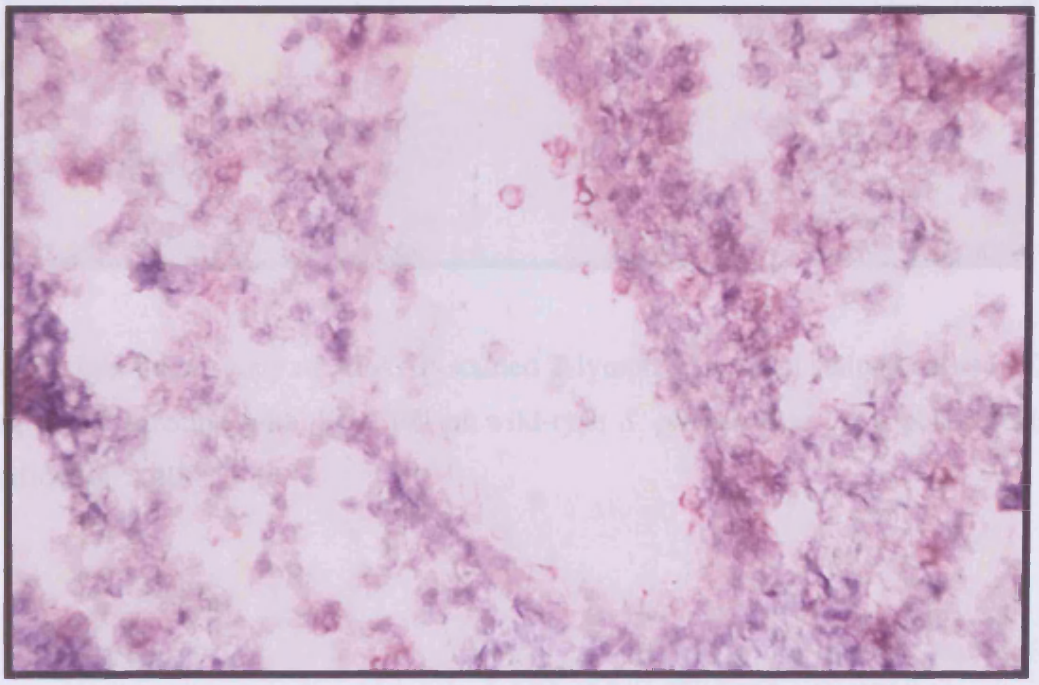


**Fig 3.18** Light microscopy of APAAP-stained neutrophils (red stained cells) inside and around a bronchiole in C1q +/+ (A) and C1q -/- (B) lung tissue infected with $10^6$ CFU of wild-type *S. pneumoniae* 24 h post-infection. Magnification x 400.

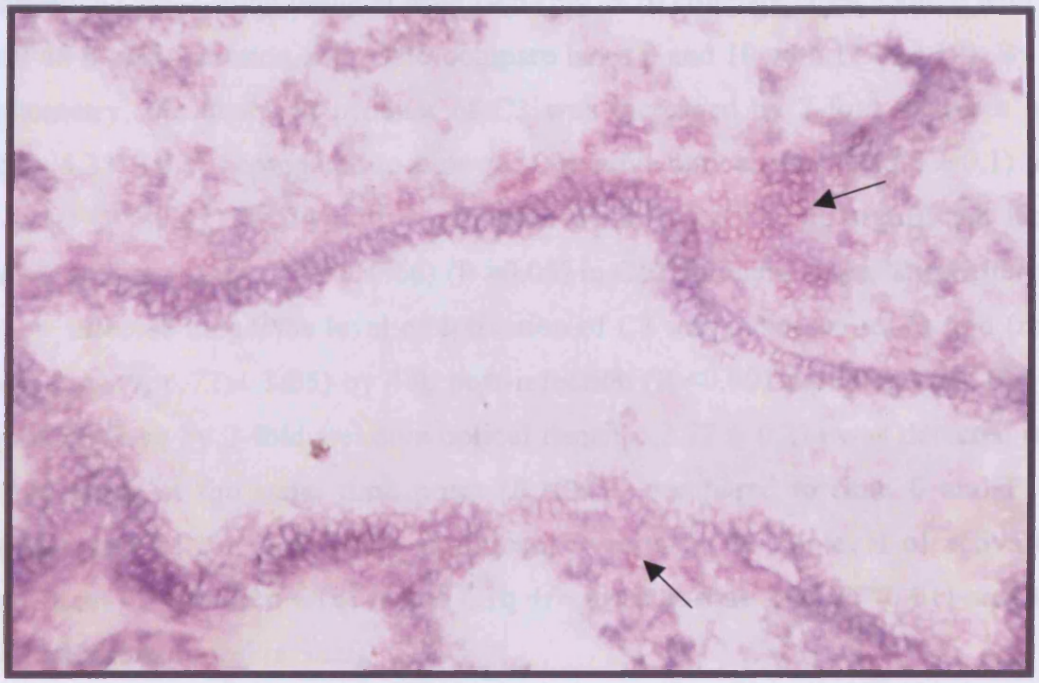


**Fig 3.19** Light microscopy of APAAP-stained T lymphocytes (red stained cells) in C1q-/- lung tissue infected with $10^6$ CFU of wild-type *S. pneumoniae* 24 h post-infection. Magnification x 400.

### 3.3.5 Comparative C3 activation in C1q -/- and C1q +/+ lungs infected with pneumococci

To investigate more directly how the ability of pneumolysin to activate the classical complement pathway affected the total activation of complement C3 by pneumococci during bronchopneumonia, we compared the amounts of the cleavage product (iC3b) in lung homogenates of C1q +/+ and C1q -/- mice infected intranasally with wild-type *S. pneumoniae*. A substantial amount of complement C3 was activated in C1q +/+ compared to C1q -/- lung tissue at time 24 h (Fig. 3.20 compare lanes 5 and 6 with 7 and 8) and 48 h post-infection (Fig. 3.20 compare lanes 9 and 10 with 11 and 12). By using densitometry, the cleavage product of C3 was increased by 7-fold (relative optical density: 5.33 ± 0.38) compared to time zero (relative optical density: 0.6 ± 0.1) in C1q +/+ lungs ($P < 0.01$) by 24 h after infection while there was no significant increase (relative optical density: 1.89 ± 0.66) ($P > 0.05$) in C1q deficient lungs. Thereafter, in the C1q +/+ infected lungs, the level of activation of C3 was increased to 10-fold (relative optical density: 6.72 ± 1.05) by 48h post-infection ($P < 0.001$ compared to t 0h) while only an increase by 3-fold (relative optical density: 2.77 ± 0.21) was detected in C1q infected lungs at the same time point ($P < 0.01$, compared to time 0 and $P > 0.05$ compared to t 24 h). There were significant differences in the level of activation of complement C3 between C1q -/- and C1q +/+ mice at time 24h ($P < 0.01$) and 48h ($P < 0.05$) after infection (Fig 3.20).

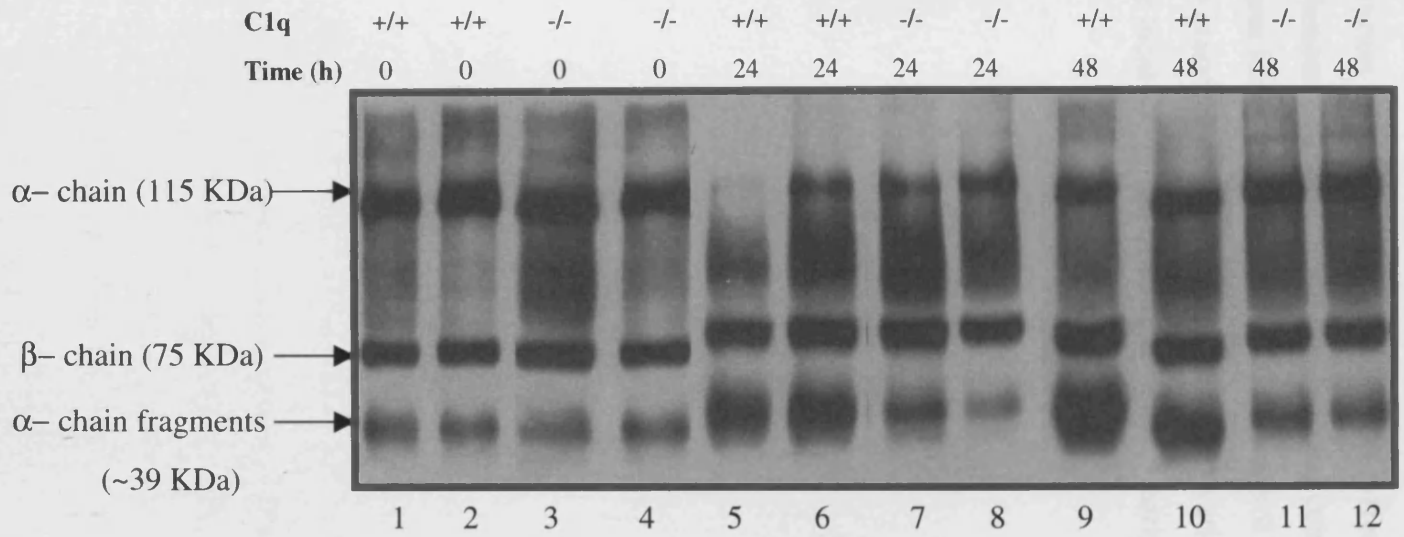



**Fig 3.20** Analysis of C3 activation in C1q +/+ and C1q -/- infected mouse lung tissue. C1q deficient mice and control mice were infected intranasally with $10^6$ CFU of wild-type *S. pneumoniae*. Lung homogenates were collected at 0, 24 and 48 h after infection, homogenised and 40 μg of total protein from each lung were electrophoresed on 10 % SDS-PAGE gels under reducing conditions. Proteins were transferred to nitrocellulose and visualised with anti-C3 antibody and enhanced chemiluminescence. The blot shows the C3 cleavage in lung homogenates of C1q +/+ mice infected with wild-type pneumococci collected at time 0 h (lanes 1 & 2), 24 h (lanes 5 & 6) and 48 h (lanes 9 & 10) and C1q -/- mice at time 0 h (lanes 3 & 4), 24 h (lanes 7 & 8) and 48 h (lanes 11 & 12). (n = 4 lungs for each time point).

The importance of the overall role of the classical complement pathway and the contribution effect of pneumolysin's ability to activate complement was evaluated in bronchopneumonia by using C1q deficient mice. The lack of the classical complement pathway activation by the pneumococci affected the outcome of bacterial growth in lung tissue as early as 6 h and later at 48h after infection, and as well as to the level of bacteraemia. Histological changes in C1q deficient mice showed less hypertrophy and cellular infiltration when compared to the control mice at 24 h after infection. T cell infiltration into the lung tissue was affected by the activation of the classical complement pathway. Additionally, local complement C3 was slightly activated in C1q deficient lungs when compared to the control lungs; thus emphasizing the important role of the classical complement pathway activation by the pneumococci, and pneumolysin, on the local activation of complement C3 early after infection.

# Chapter 4: Results

## 4.1 Interaction of lung surfactant protein D with different strains and serotypes of *S. pneumoniae*

An enormous number of studies have highlighted the important multifunctional role of lung surfactant protein D as a first line local defence molecule in the respiratory tract (Wright, 1997, Lawson and Reid 2000). Since the airway is the natural portal of entry of *S. pneumoniae*, lung surfactant protein D lining the epithelium of the alveoli, is a candidate to play an important role in innate defence against the pneumococcus. A previous study reported that highly multimerized Sp-D molecules bound to serotype 4, 19 and 23 *S. pneumoniae,* inducing their agglutination and enhancing their uptake by neutrophils (Hartshorn *et al.,* 1998).

Assuming that agglutination of *S. pneumoniae* will have an important role in collecting and limiting the spread of the pathogen through the lung tissue, in the present study, the interaction between lung surfactant protein D and different strains and serotypes of pneumococci was studied. Although carbohydrate was involved in the binding of Sp-D by Gram-negative bacteria (Restrepo *et al.,* 1999, Pikaar *et al.,* 1995), no investigation of its role in pneumococcal binding has been reported.

Whether the capsule type is a determinant of *S. pneumoniae* aggregation by lung surfactant protein D was investigated. Different strains of pneumococci having the same capsule-type or the same multilocus sequence type were assessed for their binding and agglutination by Sp-D. Also, strains of *S. pneumoniae* in which the capsule had been switched in the laboratory were tested for their agglutination by Sp-D. Moreover, encapsulated and non-encapsulated strains of *S. pneumoniae* were used to investigate whether Sp-D enhanced uptake of different strains of pneumococci by neutrophils.

### 4.1.1 Binding of recombinant Sp-D to *S. pneumoniae*

A solid-phase bacterial ELISA revealed that recombinant human Sp-D (5-10 μg/ml) bound to all strains of *S. pneumoniae* used in this study, whatever the serotype in the presence of calcium (Table 4.1). In the absence of calcium, no binding was detected. There was no significant difference in the extent of binding between strains of the same serotype ($P \geq 0.05$).

Strain M9 (serotype 18C) bound rSp-D to a higher extent than all the other serotypes used ($P \leq 0.001$) but the two other 18C strain were not significantly different from the

other serotypes. Two pneumococcal strains of the same multilocus sequence type showed differences in the extent of binding: Strain M41 (serotype 23 F) bound rSp-D (5 μg/ml) to a higher extent than GM70 (serotype 19A) ($P \leq 0.05$). However, two strains M322 and M15 of different serotypes but of the same multilocus sequence type were not different in binding rSp-D ($P \geq 0.05$).

A recombinant Sp-D synthesized in yeast did not bind any of the pneumococcal strains shown in Table 4.1, despite the recombinant fragment having been crystallized and shown to be structurally intact.

| | | | rhSp-D concentrations | | | |
|---|---|---|---|---|---|---|
| **Strain** | **Serotype** | **MLST** | **0 μg/ml** | **5 μg/ml** | **10 μg/ml** | **5 μg/ml+EDTA** |
| M393 | 3 | 260 | 0.18 ± 0.01 | 0.30 ± 0.02 | 0.34 ± 0.01 | 0.16 ± 0.03 |
| M110 | 3 | 180 | 0.19 ± 0.01 | 0.27 ± 0.01 | 0.37 ± 0.04 | 0.20 ± 0.01 |
| M290 | 3 | 49 | 0.13 ± 0.01 | 0.22 ± 0.02 | 0.25 ± 0.01 | 0.11 ± 0.02 |
| M9 | 18C | 102 | 0.20 ± 0.02 | 0.40 ± 0.02 | 0.47 ± 0.02 | 0.23 ± 0.03 |
| M241 | 18C | 113 | 0.21 ± 0.01 | 0.34 ± 0.03 | 0.46 ± 0.05 | 0.22 ± 0.01 |
| M208 | 18C | 119 | 0.21 ± 0.02 | 0.31 ± 0.02 | 0.38 ± 0.07 | 0.18 ± 0.04 |
| GM70 | 19A | 81 | 0.13 ± 0.09 | 0.22 ± 0.02 | 0.26 ± 0.01 | 0.15 ± 0.01 |
| M41 | 23F | 81 | 0.17 ± 0.01 | 0.29 ± 0.06 | 0.32 ± 0.01 | 0.15 ± 0.02 |
| M322 | 14A | 156 | 0.12 ± 0.08 | 0.19 ± 0.01 | 0.25 ± 0.01 | 0.13 ± 0.01 |
| M15 | 9V | 156 | 0.10 ± 0.04 | 0.17 ± 0.01 | 0.25 ± 0.007 | 0.12 ± 0.03 |

**Table 4.1** Binding of rhSp-D to different strains of *S. pneumoniae.*
Binding was assessed by bacterial solid-phase ELISA. Experiments were done in 5mM calcium or EDTA. Values are means ± SEM of optical density at 450nm from 3-4 replicate experiments. In all cases the OD was significantly increased when compared with control value (in absence of rhSp-D), $P < 0.05$ except when EDTA was used $P > 0.05$.

### 4.1.2 Agglutination of *S. pneumoniae* by full length Sp-D

To investigate the physical effect resulting from rSp-D binding to pneumococci, different serotypes were incubated with multimerized, full length Sp-D (10 μg/ml). There was no agglutination of any strain in the presence of 10mM EDTA, emphasizing that it took place in a calcium-dependent manner. No agglutination of any strain was detected when recombinant Sp-D was used indicating that full-length multimerized Sp-D molecules are required to aggregate *S. pneumoniae.* As shown in Table 4.2, several serotypes of *S. pneumoniae* were agglutinated by full length Sp-D. The average area of agglutinated bacteria and the number of clumps per field of view differed between the serotypes. For instance, four clumps of agglutinated bacteria per field of view were detected for the serotype 14 significantly different ($P \leq 0.001$) than the serotype 2 where only one clump per field was detected. As another example, the average area of agglutinated bacteria was significantly greater with the serotype 1N than the serotype 8 ($P \leq 0.001$).

The supposition that Sp-D binds to carbohydrate structures on the surface of the pneumococcus was tested by using maltose (100mM) as a competitive sugar. A significant inhibition of agglutination was observed with all strains ($P \leq 0.05$) as shown in Table 4.2.

To further explore the mechanism of agglutination, strains of *S. pneumoniae* of different capsule type and MLST were utilized. As shown in Table 4.3, Sp-D induced agglutination of several, but not all, strains of *S. pneumoniae* and the extent of agglutination differed between the agglutinated strains. Pneumococcal strains of the same serotype showed differences in the amount of agglutination by Sp-D. For instance, serotype 3 strain M110 was strongly agglutinated by Sp-D while the serotype 3 strain M393 showed no agglutination (Fig 4.1, 4.2 and Table 4.3). The same also was true for 18C strains (Fig 4.3 and Table 4.3). However, pneumococcal strains having the same multilocus sequence type but different capsules also were differently agglutinated by Sp-D. For example, strains GM70 and M41 are both multilocus sequence type 81 but only the latter strain was agglutinated by the surfactant protein.

Agglutination by Sp-D was also tested using strains of *S. pneumoniae* in which the serotypes had been switched in the laboratory. Strain HB565, serotype 3 was strongly agglutinated by Sp-D, whereas strain Ps3205, in which the capsule had been switched to

type 3 from type 2 in the strain D39 (D39 showed agglutination) was not agglutinated by Sp-D. Furthermore, strain Ps3221 in which capsule had been switched to type 3 from 19F showed agglutination by Sp-D, while strain G54, the type 19F parent of PS3221, did not show any agglutination (Table 4.3).

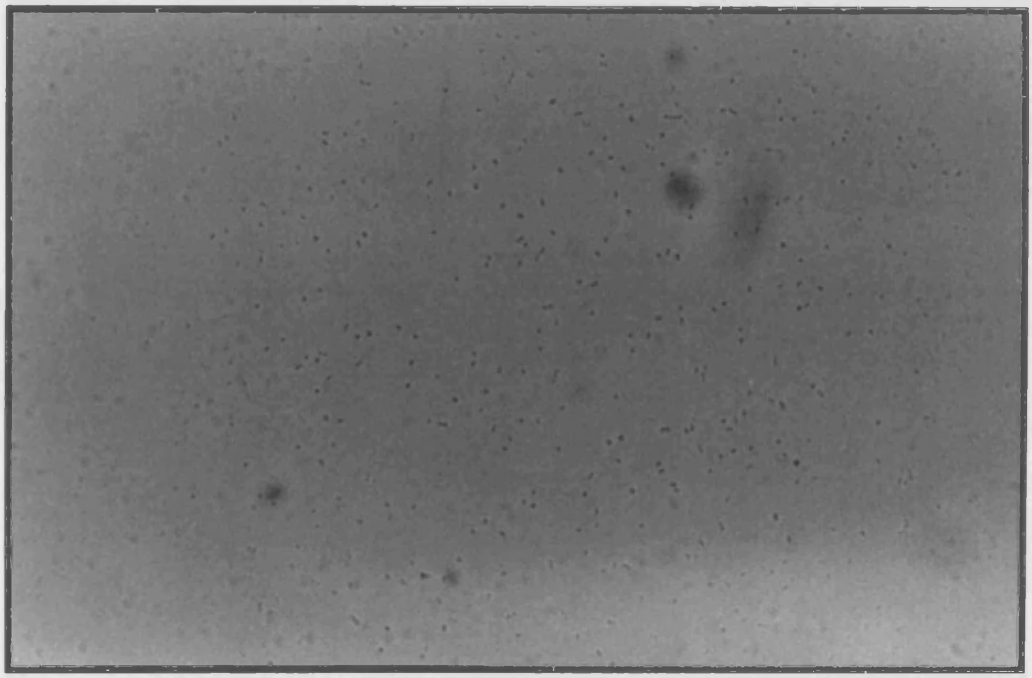
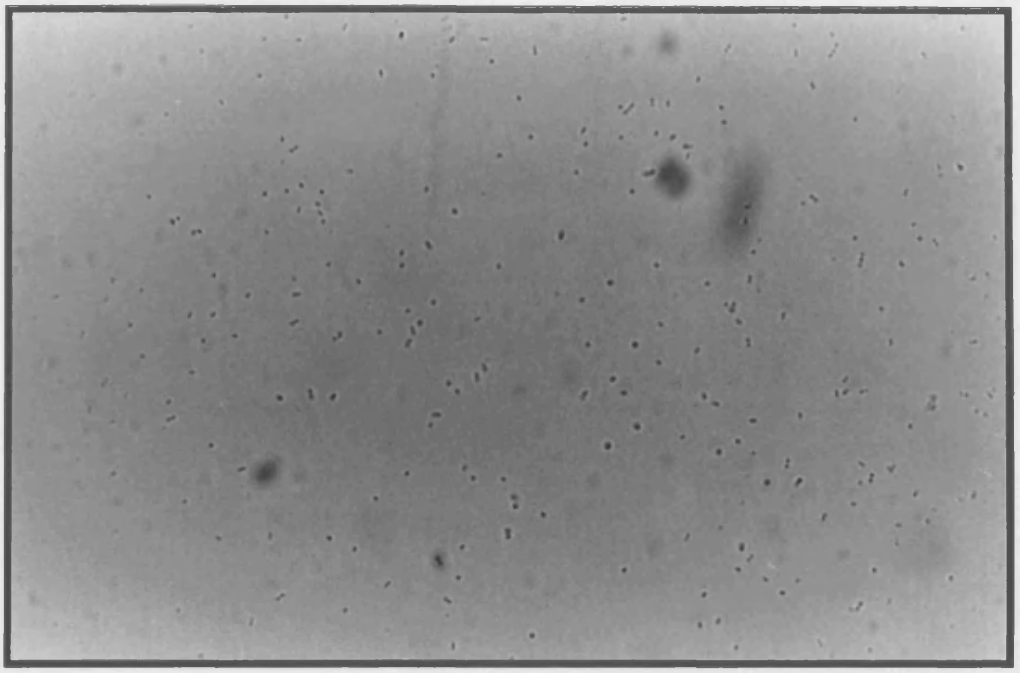


**Fig 4.1.** Agglutination of *S. pneumoniae* by lung surfactant protein D. Serotype 3 strain M393 was incubated with 10 μg/ml of full length Sp-D molecule for 90 min in the presence of calcium (5mM) and showed no agglutination after examination by phase-contrast microscopy (x500). Results are representative for at least four similar experiments.

(A)



(B)

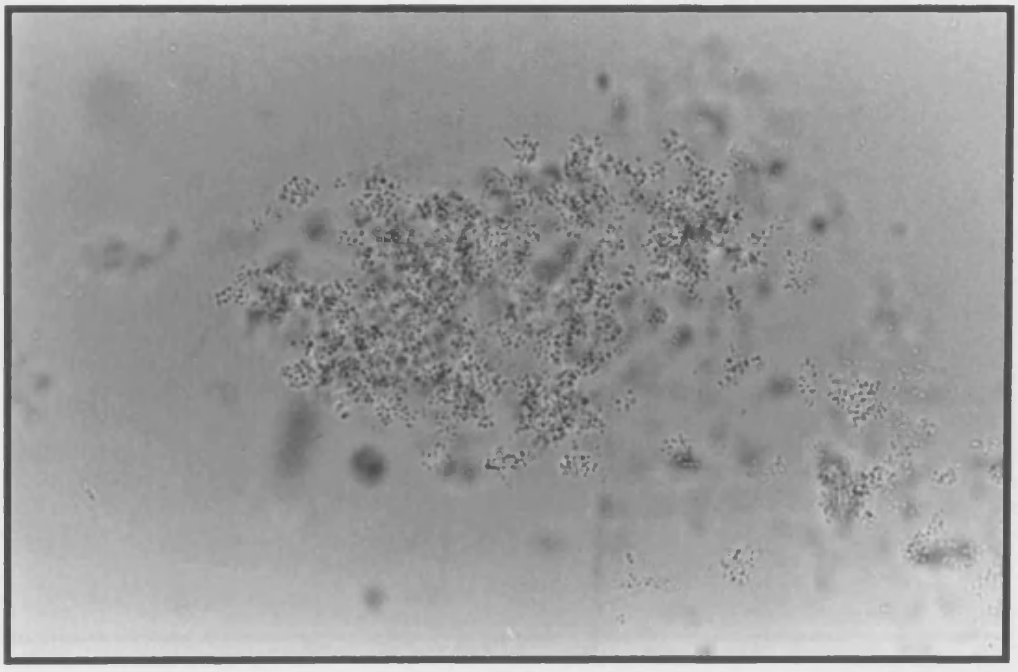
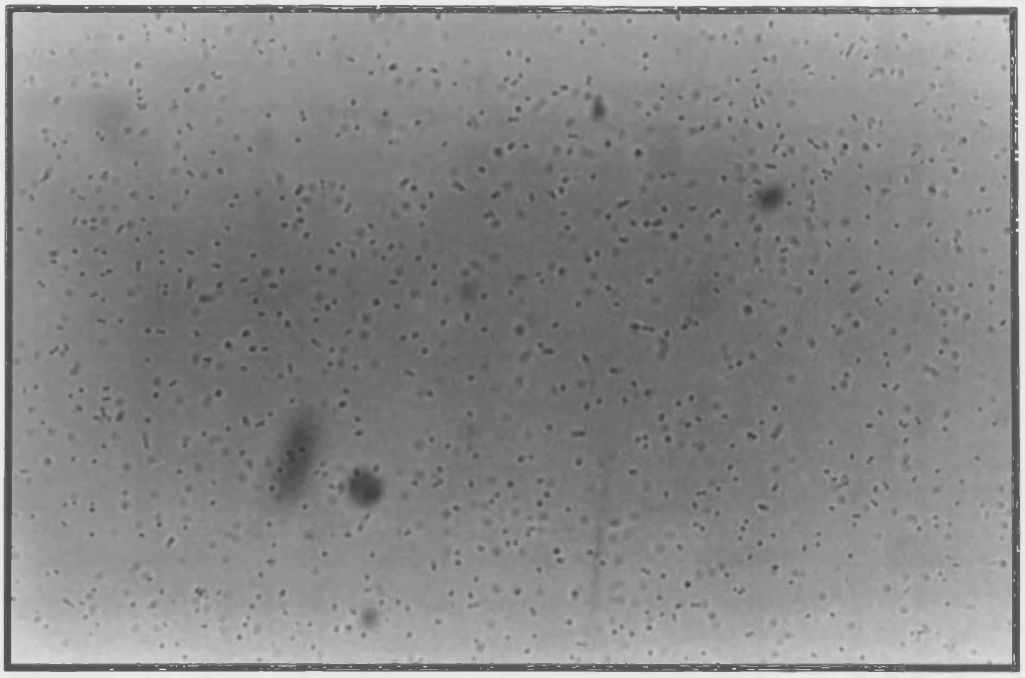


**Fig 4.2.** Agglutination of *S. pneumoniae* by lung surfactant protein D. Serotype 3, Strain M110 (A-B) was incubated respectively with 0 and 10 μg/ml of full length Sp-D molecule for 90 min in the presence of calcium (5mM) and was examined by phase contrast microscopy (x500). Results are representative for at least four similar experiments.

**(A)**



**(B)**

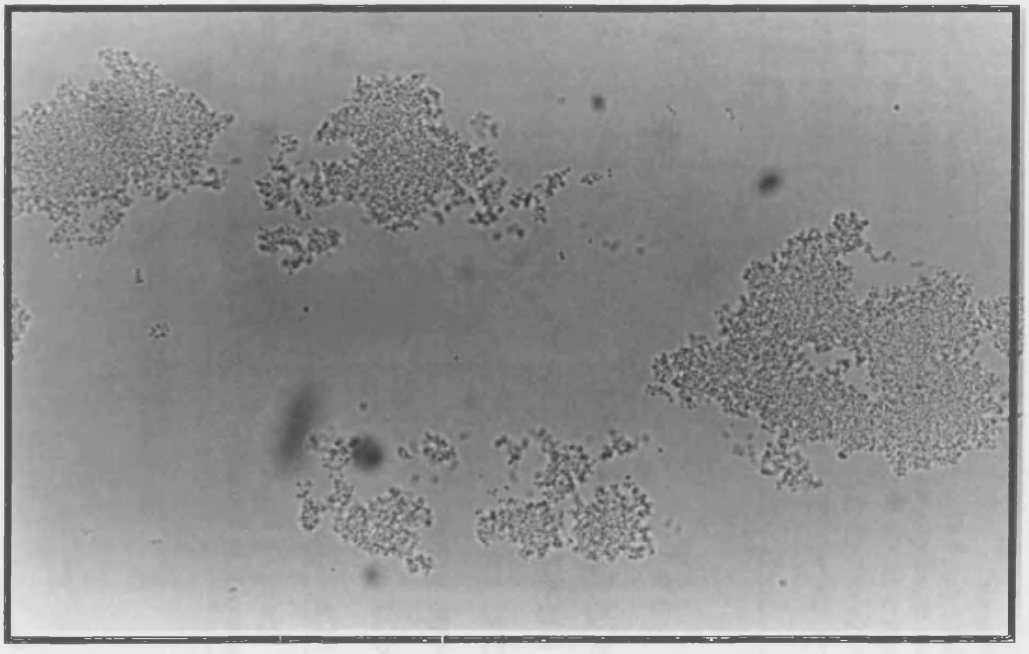


**Fig 4.3.** Agglutination of *S. pneumoniae* by lung surfactant protein D. Serotype 18C, strain M9 (A-B) was incubated respectively with 0 and 10 µg/ml of full length Sp-D molecule for 90 min in the presence of calcium (5mM) and was examined by phase-contrast microscopy (x500). Results are representative for at least four similar experiments.

| | Absence of maltose | | Presence of maltose (100mM) | |
|---|---|---|---|---|
| **Serotype** | **Number of clumps /field of view** | **Average area of agglutinated bacteria ($\mu m^2$)** | **Number of clumps /field of view** | **Average area of agglutinated bacteria ($\mu m^2$)** |
| 1N | $12.9 \pm 0.75^{\ddagger}$ | $96.68 \pm 0.83$ | $11.8 \pm 0.99^{\ddagger}$ | $36.35 \pm 0.42$* |
| 3 | $4.33 \pm 0.28$ | $27.64 \pm 0.67$ | $0.8 \pm 0.22$* | $12.6 \pm 0.41$* |
| 14 | $4.00 \pm 0.66$ | $25.48 \pm 0.78$ | $1.3 \pm 0.25$* | $11.93 \pm 0.38$* |
| 8 | $2.17 \pm 0.29$ | $17.78 \pm 0.45^{\dagger}$ | $1 \pm 0.21$* | $16.68 \pm 0.11$* |
| 2 | $1.11 \pm 0.35^{\dagger}$ | $128.62 \pm 0.87^{\ddagger}$ | NA | NA |
| RX1 | $4.00 \pm 0.25$ | $108 \pm 2.6$ | $1.7 \pm 0.26$ | $14.44 \pm 0.6$* |
| *E.coli* Y1088 (5 μg/ml) | $3.9 \pm 0.28$ | $97.2 \pm 1.11$ | $2.73 \pm 0.33$ | $21.99 \pm 0.51$* |

**Table 4.2.** Agglutination of different serotypes of *S. pneumoniae* by lung surfactant protein D.
Pneumococci were incubated with Sp-D (10 μg/ml) for 90 min in the presence of calcium (5 mM) with and without maltose (100 mM) and were examined by phase-contrast microscopy (X500). Average area of agglutinated bacteria was measured and the number of clumps per field of view were counted. Substantial difference in the extent of agglutination was detected when maltose was used as a competitive sugar. NA, no agglutination. Data are the mean ± SEM of 4-5 experiments. ‡ $P < 0.05$ significantly higher and † $P < 0.05$ significantly lower compared to the values of all other serotypes, * $P < 0.05$ significantly different when compared to the control values in the absence of maltose.

| Strain | Serotype | MLST | Number of clumps / field of view | Average area of agglutinated bacteria ($\mu m^2$) |
|---|---|---|---|---|
| M393 | 3 | 260 | NA | NA |
| M110 | 3 | 180 | 3.2 ± 0.38 | 179 ± 0.2 |
| M290 | 3 | 49 | 4.3 ± 0.53 | 124 ± 0.5 |
| M9 | 18C | 102 | 5.2 ± 0.50‡ | 156 ± 0.4 |
| M241 | 18C | 113 | NA | NA |
| M208 | 18C | 119 | NA | NA |
| GM70 | 19A | 81 | NA | NA |
| M41 | 23F | 81 | 4.2 ± 0.37 | 64 ± 0.1 |
| M322 | 14A | 156 | 0.7 ± 0.21† | 6.1 ± 0.2 |
| M15 | 9V | 156 | NA | NA |
| Ps3221 | 3 | 63 | 2.2 ± 0.29 | 14.2 ± 0.3 |
| G54 | 19F | 63 | NA | NA |
| HB565 | 3 | 378 | 3.1 ± 0.20 | 88 ± 0.6 |
| Ps3205 | 3 | ND | NA | NA |

**Table 4.3** Agglutination of different strains and serotypes of *S. pneumoniae* by lung surfactant protein D.
Pneumococci were incubated with Sp-D (10 μg/ml) for 90 min in the presence of calcium (5 mM) and were examined by phase-contrast microscopy (X500). The average area of agglutinated bacteria was measured $\mu m^2$ and was significantly different $P< 0.05$ between all the agglutinated strains ($P <0.05$) and the number of clumps per field of view was counted for agglutinated strains, ‡ $P< 0.05$ significantly higher and † $P< 0.05$ significantly lower compared to the values of other strains. NA, not agglutinated. Results are mean ± SEM of 3-5 experiments.

### 4.1.3 Killing of *S. pneumoniae* by neutrophils in the presence of Sp-D and rSp-D

A further investigation on the interaction between *S. pneumoniae* and lung surfactant protein D was done by testing if Sp-D would be an opsonin for pneumococci. Experiments were done with encapsulated serotype 2, non-encapsulated Rx1 *S. pneumoniae* and human neutrophils in the presence or absence of Sp-D (10 μg/ml), rSp-D (20 μg/ml) or autologous serum from vaccinated donors.

Data showed that killing by neutrophils was enhanced by the presence of serum. There was an approximately 3-Log decrease ($P \leq 0.01$ compared to time zero) in colony-forming units for both pneumococcal strains, over 120 minutes incubation with neutrophils (Fig 4.4 and 4.5). In contrast, in the presence of Sp-D or rSp-D and neutrophils the numbers of pneumococci remained constant over the 120 min for both strains ($P \geq 0.05$ versus time 0 min).

Remarkably, in the absence of any opsonin, the number of non-encaspulated pneumococci Rx1 showed a significant decrease after 60 min exposure to neutrophils ($P < 0.01$) compared to time zero (Fig 4.4). In contrast, for the encapsulated serotype 2, there was no decrease in number in the absence of serum ($P > 0.05$) indicating the anti-phagocytic activity of the pneumoccoccal capsule (Fig 4.5).

There was no change ($P > 0.05$) in number of pneumococci when incubated with serum in the absence of neutrophils (Fig 4.4 and 4.5).

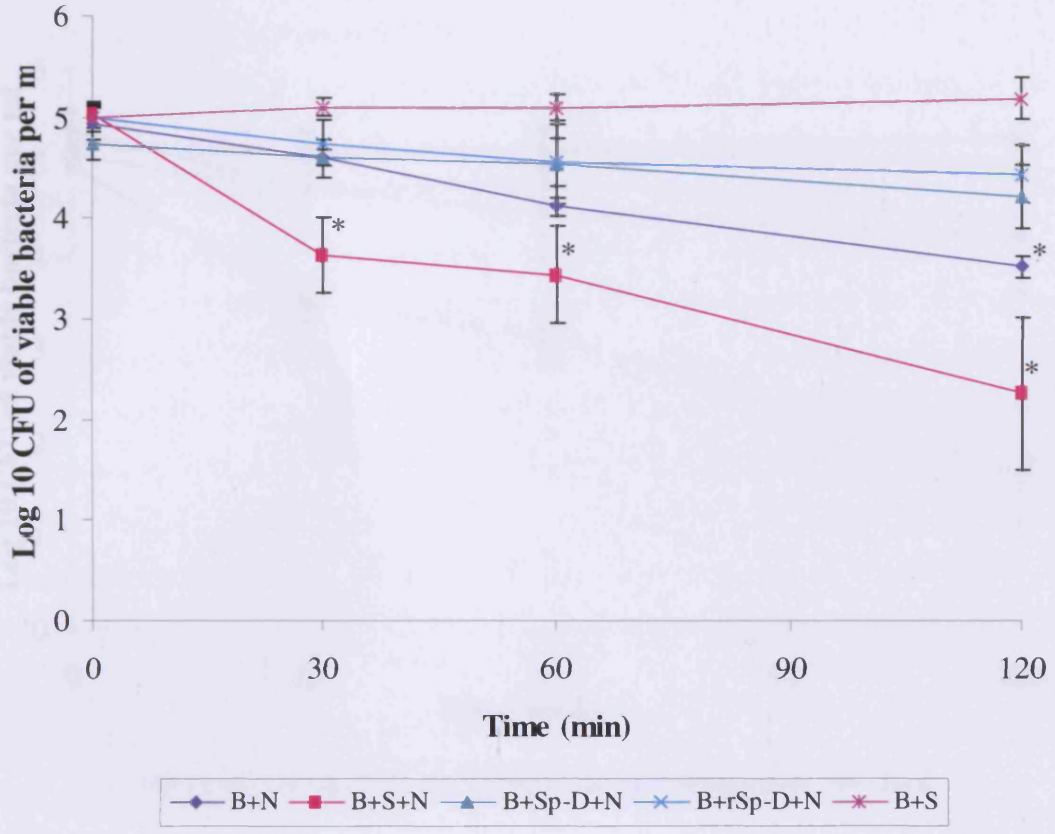



**Fig 4.4.** Killing of *S. pneumoniae* non-encapsulated RX1 strain by neutrophils. Pneumococci ($5x10^5$) (B) were incubated with neutrophils ($5x10^6$) (N) in the presence of autologous serum 20 % (v/v) (S), Sp-D (10 µg/ml), rSp-D (20 µg/ml), or serum alone (S) for 120 min. Samples were taken at 0, 30, 60 and 120 min to determine the number of viable bacteria. The results are the means ± SEM of four independent experiments. * $P < 0.05$ significantly different compared to time zero.

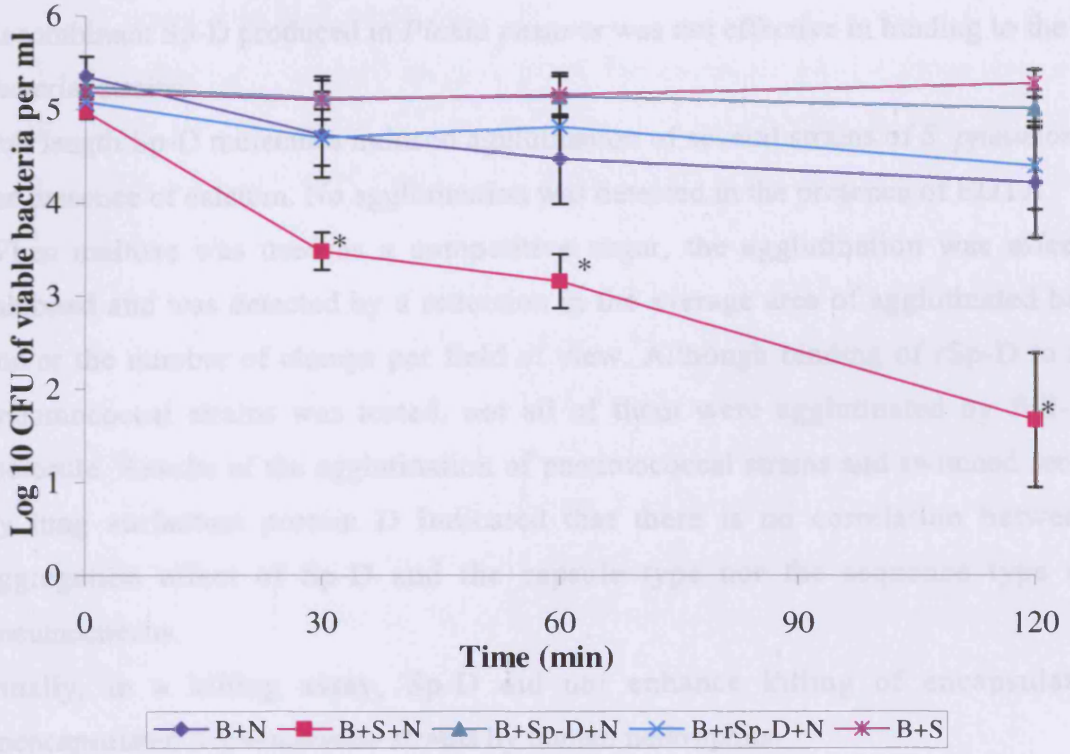



**Fig 4.5.** Killing of *S. pneumoniae* encapsulated D39 strain by neutrophils. Pneumococci ($5x10^5$) (B) were incubated with neutrophils ($5x10^6$) (N) in the presence of autologous serum 20 % (v/v) (S), Sp-D (10 µg/ml), rSp-D (20 µg/ml), or serum alone (S) for 120 min. Samples were taken at 0, 30, 60 and 120 min to determine the number of viable bacteria. The results are the means ± SEM of four independent experiments. * $P < 0.05$ significantly different compared to time zero.

In summary, data presented in this section show that recombinant human Sp-D, composed of the head/neck of the molecule and three carbohydrate recognition domains, bound to several strains and serotypes of *S. pneumoniae* in a calcium dependent manner. The extent of this binding was not affected in a simple manner by the capsule type or the sequence type of the pneumococcal strain.

Recombinant Sp-D produced in *Pichia pastoris* was not effective in binding to the tested bacterial strains.

Full length Sp-D molecules induced agglutination of several strains of *S. pneumoniae* in the presence of calcium. No agglutination was detected in the presence of EDTA.

When maltose was used as a competitive sugar, the agglutination was effectively inhibited and was detected by a reduction in the average area of agglutinated bacteria and/or the number of clumps per field of view. Although binding of rSp-D to all the pneumococcal strains was tested, not all of them were agglutinated by full-length molecule. Results of the agglutination of pneumococcal strains and switched serotypes by lung surfactant protein D indicated that there is no correlation between the aggregation effect of Sp-D and the capsule type nor the sequence type of the pneumococcus.

Finally, in a killing assay, Sp-D did not enhance killing of encapsulated or unencapsulated *S. pneumoniae* strains by human neutrophils.

## 4.2 *In vivo* contribution of lung surfactant protein D in the natural innate immunity to *S. pneumoniae*

In previous work, Sp-D knockout mice were infected with Group B streptococcus (LeVine *et al.*, 2000). Deficiency in Sp-D did not alter bacterial clearance from the lung but enhanced production of cytokines and lung inflammation. In contrast, when Sp-D deficient mice were infected with Influenza A virus, the clearance of the virus was deficient in knockout mice when compared to control mice and enhanced production of inflammatory cytokines and infiltration of neutrophils into the lung were associated with the absence of Sp-D (LeVine *et al.*, 2001).

Lung surfactant protein D is present in the trachea, bronchioles and alveolar spaces of normal mice (Crouch *et al.*, 1992, Wong *et al.*, 1996). It was of great interest to assess the *in vivo* contribution of Sp-D to the innate defence mechanisms during pneumococcal bronchopneumonia and septicaemia. Sp-D knockout and C57BL/6j control mice were infected intranasally with $10^6$ CFU wild-type *S. pneumoniae*. Bacterial growth kinetics in the nasopharynx, trachea, lungs and blood, histology of the development of the inflammation and host immune cells infiltration into the lung were compared in Sp-D -/- and Sp-D +/+ mice.

### 4.2.1 Growth of pneumococci in lungs, trachea, nasopharynx and blood of Sp-D -/- and Sp-D +/+ mice

The growth curve of *S. pneumoniae* in the lungs of Sp-D -/- mice showed differences at time 6h ($P < 0.05$) and 24h ($P < 0.05$) when compared to the wild-type mice (Fig. 4.6). The number of pneumococci in Sp-D -/- lungs remained constant over the 24 h after infection followed by a significant 2 log decrease by 48h post-infection ($P < 0.01$ compared to t 24h). In contrast, numbers of pneumococci in the lungs of Sp-D +/+ mice showed an early decrease at 6h and 24h after infection ($P < 0.05$ compared to time zero) followed by a decrease in numbers of pneumococci recovered from lungs of Sp-D +/+ mice by 48h post-infection (Fig. 4.6).

In the trachea of Sp-D -/- mice, the numbers of pneumococci remained constant over the 48h post-infection whereas bacteria were cleared from the trachea of wild-type mice by 48h after infection ($P < 0.01$, compared to Sp-D -/- mice) (Fig. 4.8).

In the nasopharynx of Sp-D -/- and wild type mice, the numbers of *S. pneumoniae* remained steady over the 48h postinfection (P >0.05 at each time point for both strains of mice) (Fig. 4.9).

In the blood of Sp-D-/- mice, pneumococci were recovered as early as 6h after infection and numbers were further increased by 24h (P <0.05, compared to t 6 h) and decreased by 48h after infection (P <0.05, compared to t 24h). In contrast, pneumococci were detected in blood of Sp-D +/+ mice only at 24h after infection and were present in lower numbers (P <0.05) compared to Sp-D -/- mice. No bacteria were detected in blood of Sp-D +/+ mice by 48h post-infection (Fig. 4.7).

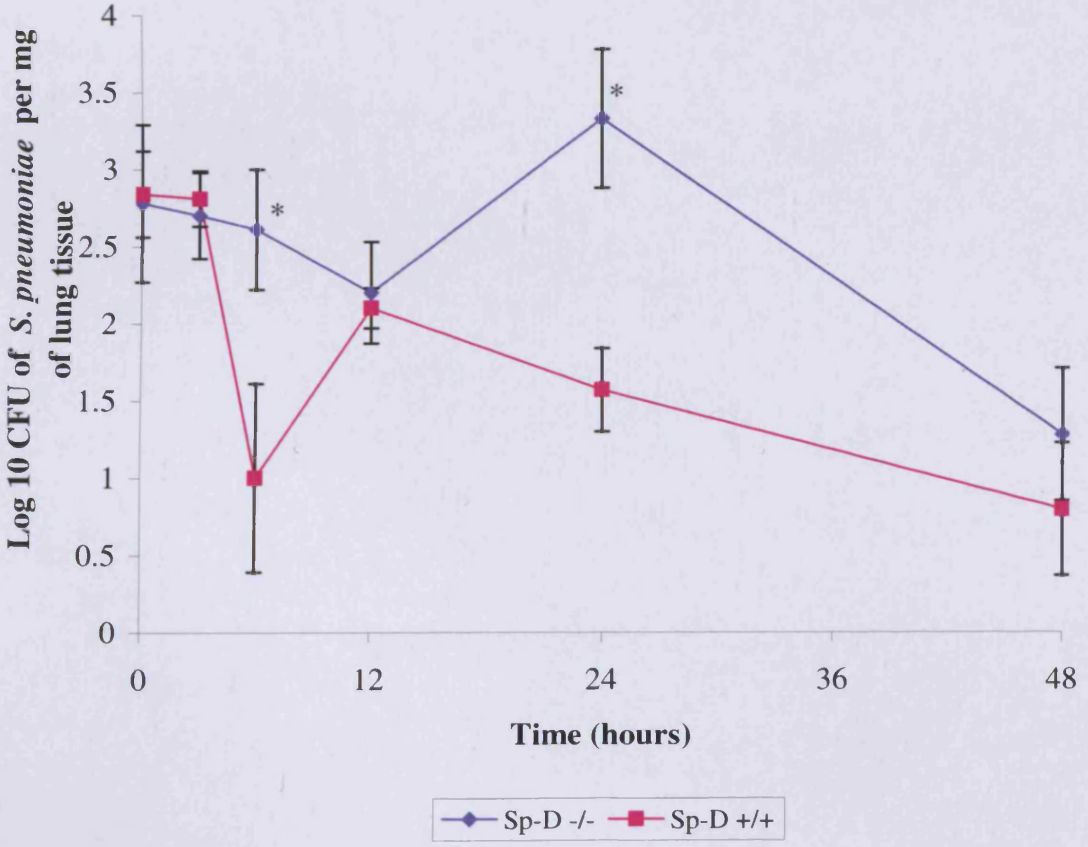



**Fig 4.6.** Time course of the change in numbers of *S. pneumoniae* in the lungs of Sp-D -/- and Sp-D +/+ mice infected intranasally with $10^6$ CFU (n = 5-10 at each time point, error bars indicate SEM). * P< 0.05 for Sp-D -/- when compared to wild-type.

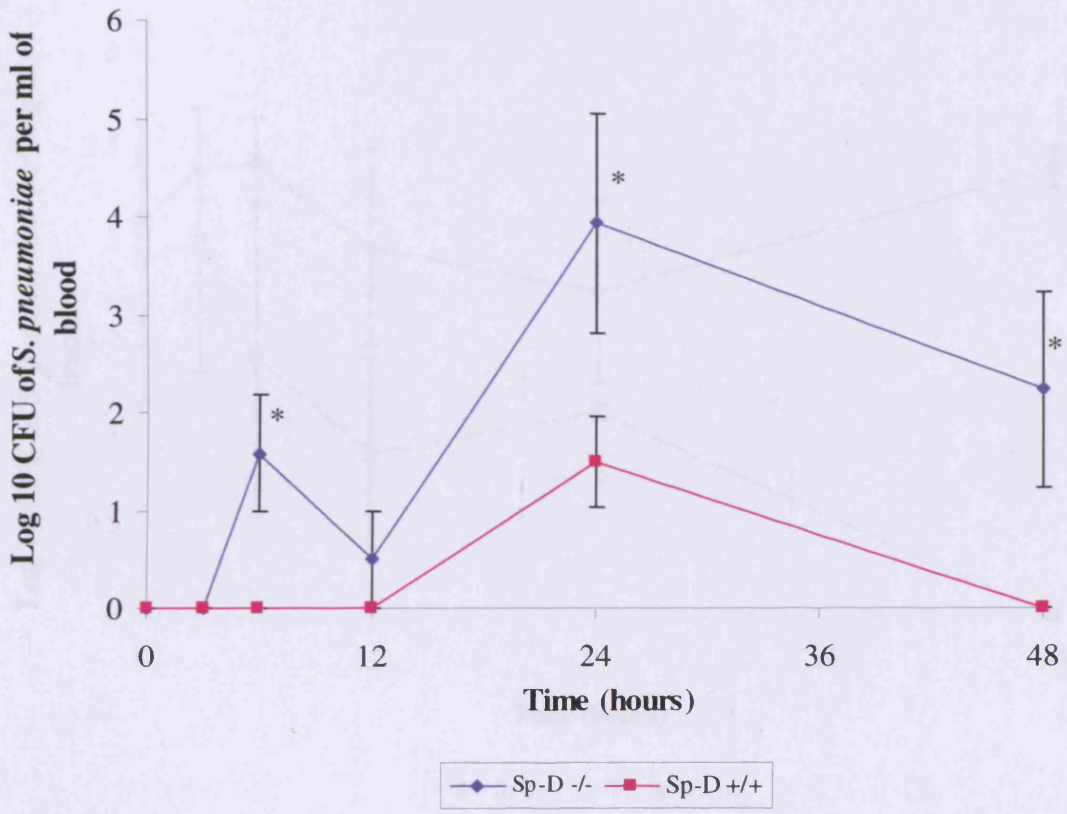



**Fig 4.7.** Time course of the change in numbers of *S. pneumoniae* in the blood of Sp-D -/- and Sp-D +/+ mice infected intranasally with $10^6$ CFU (n = 5-10 at each time point, error bars indicate SEM). * $P < 0.05$ for Sp-D -/- when compared to wild-type.

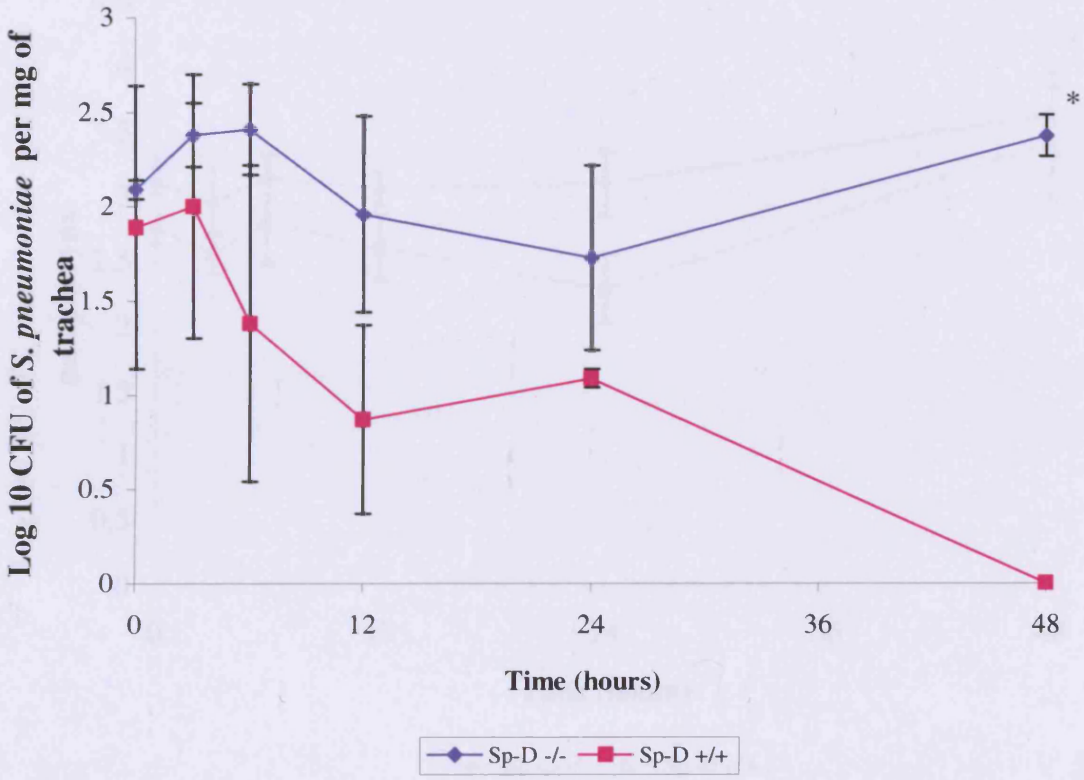



**Fig 4.8.** Time course of the change in numbers of *S. pneumoniae* in the trachea of Sp-D -/- and Sp-D +/+ mice infected intranasally with $10^6$ CFU (n = 5-10 at each time point, error bars indicate SEM). * $P < 0.05$ for Sp-D -/- when compared to wild-type.

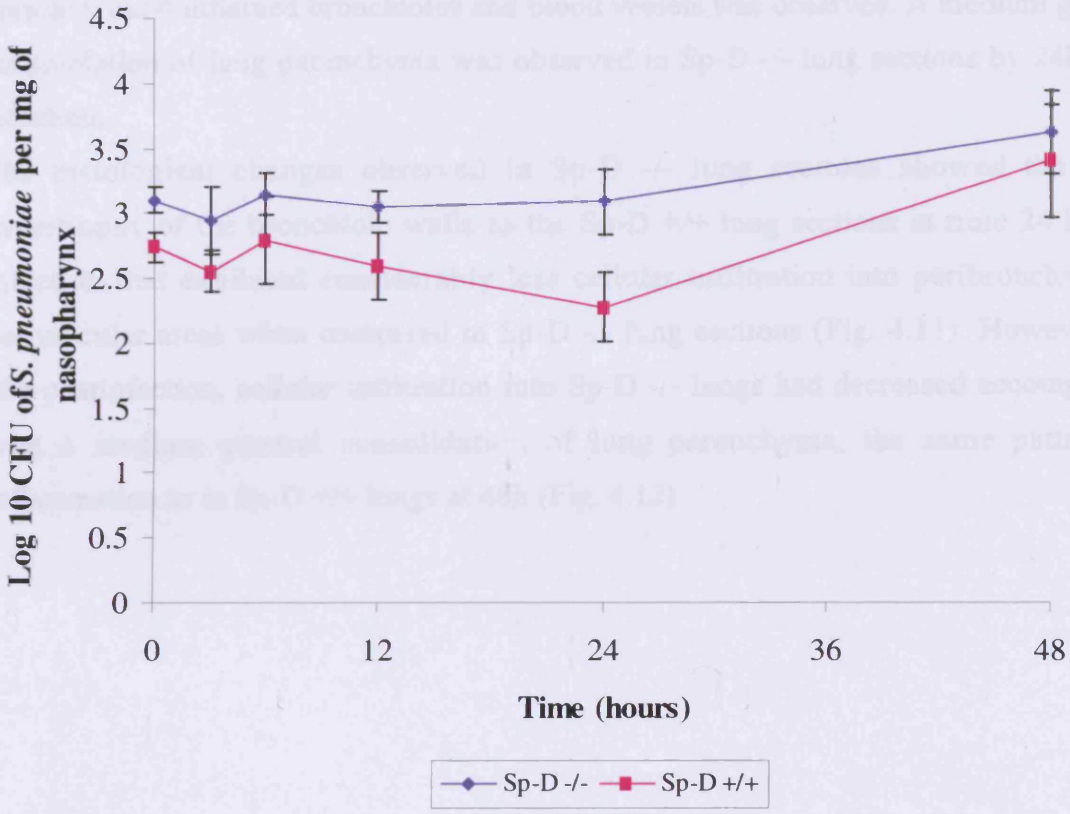



**Fig 4.9.** Time course of the change in numbers of *S. pneumoniae* in the nasopharynx of Sp-D -/- and Sp-D +/+ mice infected intranasally with $10^6$ CFU (n = 5-10 at each time point, error bars indicate SEM).

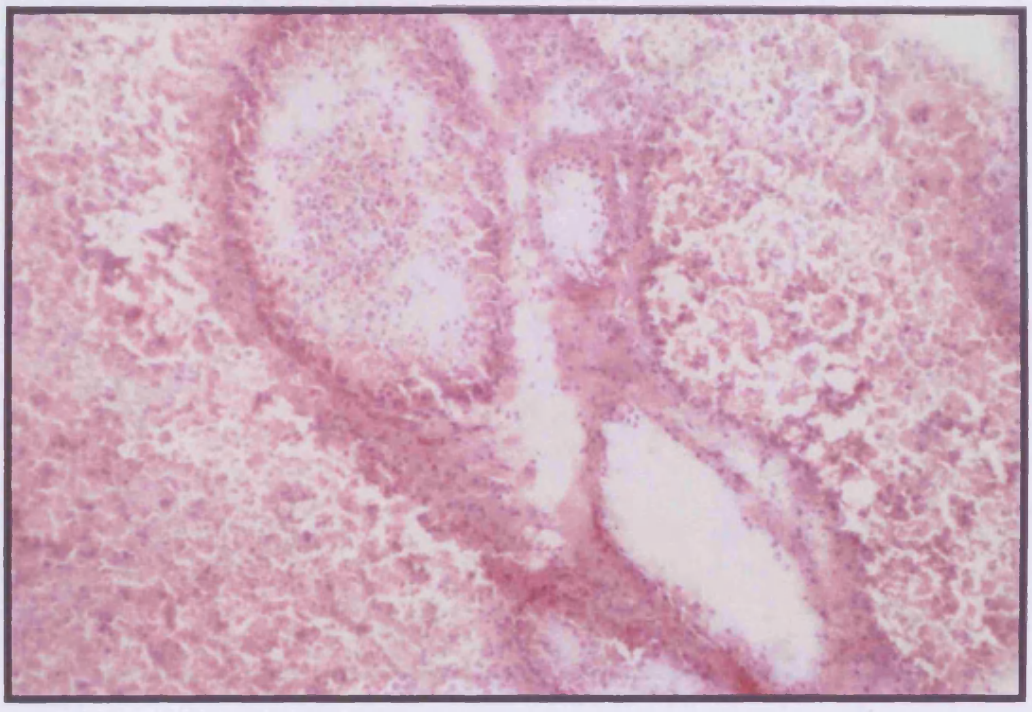
### 4.2.2 Histology of Sp-D -/- and Sp-D +/+ lungs infected with *S. pneumoniae*

Histological examination of lung tissue sections of Sp-D +/+ and Sp-D -/- mice infected with *S. pneumoniae* was done at different time points following the infection.

At 24h after infection, the Sp-D-/- lungs showed a pronounced inflammation shown as medium hypertrophy of the infected bronchiole cell walls and a severe cellular infiltration around infected bronchioles and perivascular areas (Fig. 4.10 and 4.11). Alveolar spaces and bronchioles were filled with exudate. Stretching of the smooth muscle around inflamed bronchioles and blood vessels was observed. A medium general consolidation of lung parenchyma was observed in Sp-D -/- lung sections by 24h after infection.

The histological changes observed in Sp-D -/- lung sections showed the same hypertrophy of the bronchiole walls as the Sp-D +/+ lung sections at time 24 h after infection that exhibited considerably less cellular infiltration into peribronchial and perivascular areas when compared to Sp-D -/- lung sections (Fig. 4.11). However, by 48h postinfection, cellular infiltration into Sp-D -/- lungs had decreased accompanied with a medium general consolidation of lung parenchyma; the same pattern of inflammation as in Sp-D +/+ lungs at 48h (Fig. 4.12).

(A)



(B)

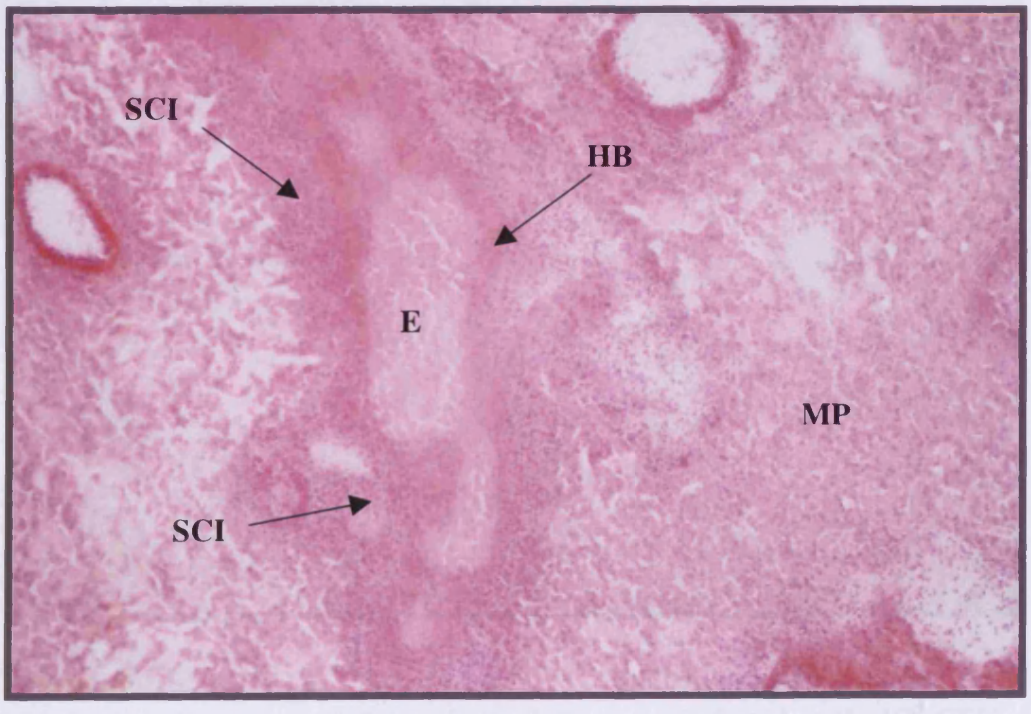
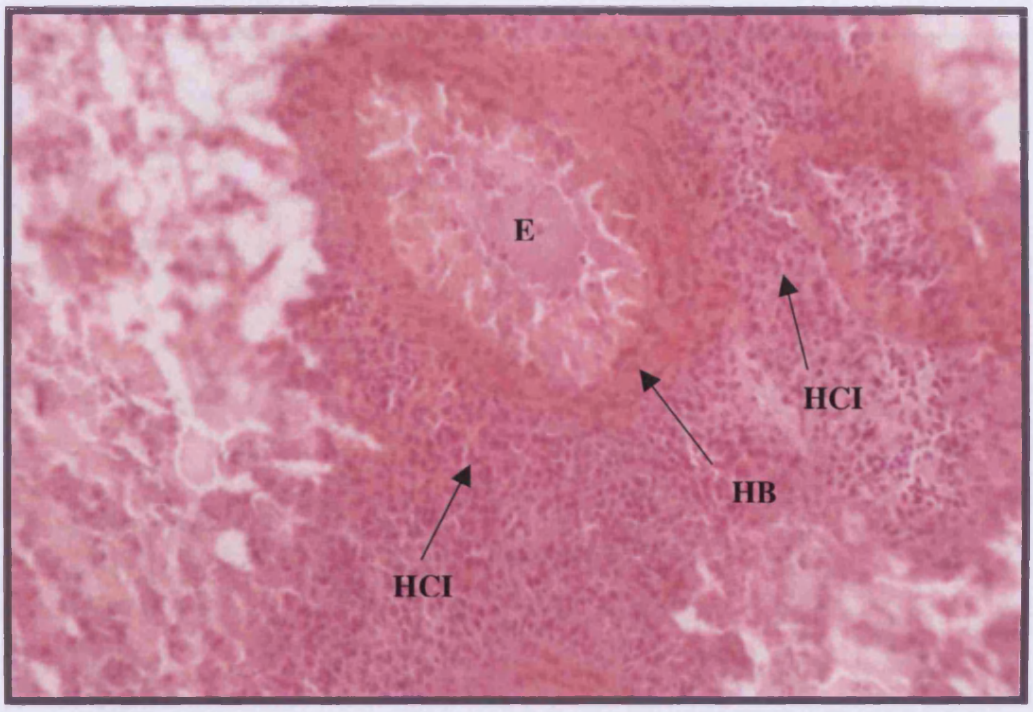



**Fig 4.10** Light microscopy of lung tissue from Sp-D -/- mice infected with $10^6$ CFU of *S. pneumoniae* at (A) 12h and (B) 24h post-infection. Magnification x250. SCI= severe cellular infiltration around inflamed bronchiole, E= exudate in alveolar space, HB= hypertrophy of the bronchiole wall and MP= medium consolidation of lung parenchyma.

(A)




(B)

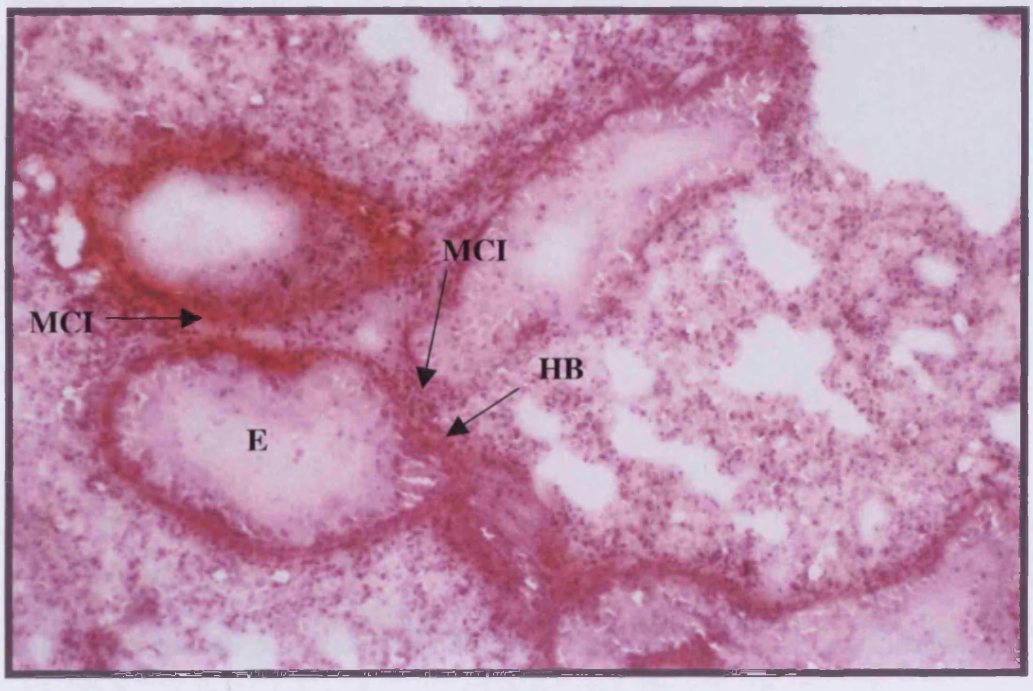
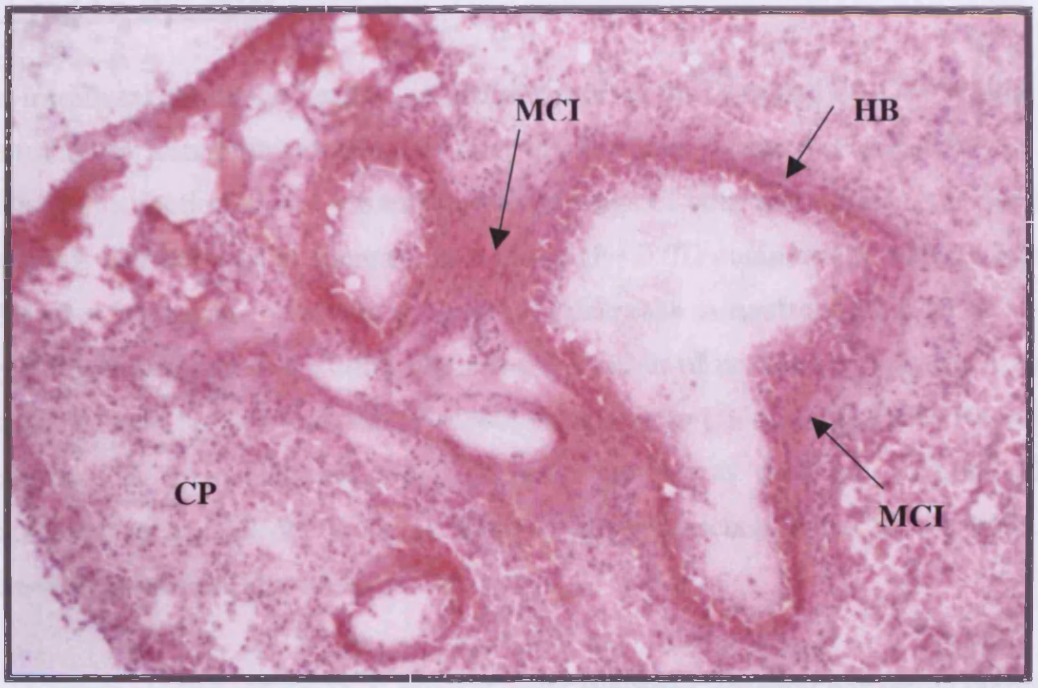



**Fig 4.11** Light microscopy of lung tissue from mice infected with $10^6$ CFU of *S. pneumoniae* (A) Sp-D -/- (B) Sp-D +/+ at 24h post-infection. Magnification (A) x400, (B) x300. HB= hypertrophy of the bronchiole wall, E= exudate in alveolar space, HCI and MCI= heavy and medium cellular infiltration .

(A)




(B)

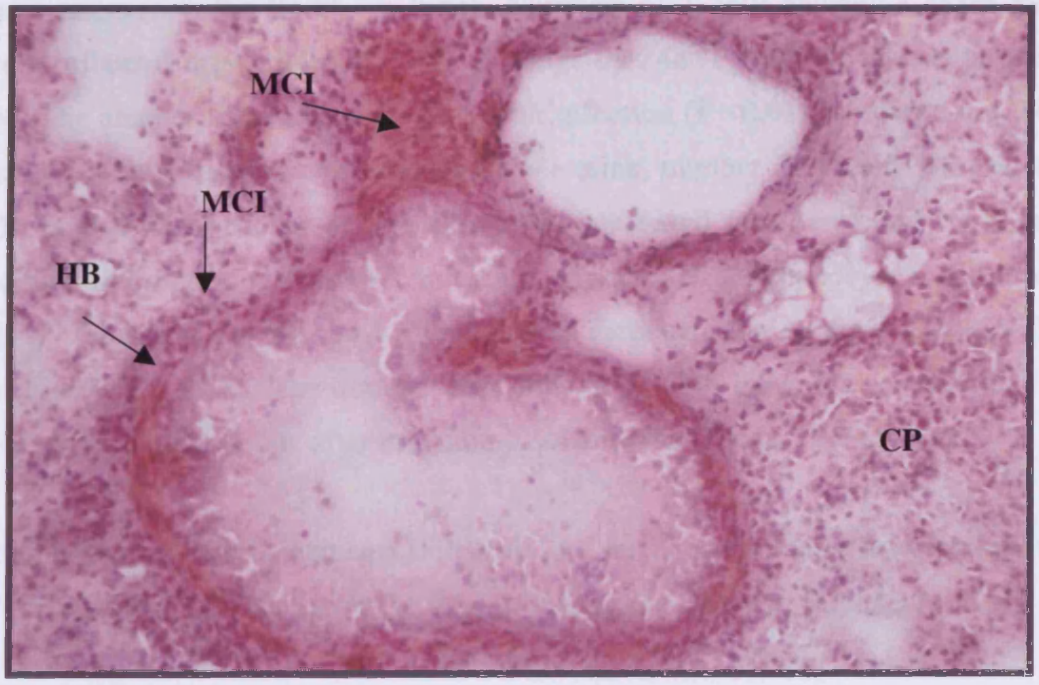



**Fig 4.12** Light microscopy of lung tissue from mice infected with $10^6$ CFU of *S. pneumoniae* (A) Sp-D -/- and (B) Sp-D +/+ at 48h post-infection. Magnification x350. HB= hypertrophy of the bronchiole wall, MCI= medium cellular infiltration and CP= consolidation of lung parenchyma.

### 4.2.3 Immunohistochemical analysis of inflammatory cell infiltrates in lungs of Sp-D +/+ and Sp-D -/- mice

After intranasal challenge, leukocyte infiltration into Sp-D -/- and Sp-D +/+ lung tissue was studied by immunohistochemistry over the 48h after infection (Fig. 4.13).
At 24h after infection, in the inflamed areas of lungs of both mice strains, the number of neutrophils showed a large increase in number ($P < 0.01$, compared to t zero). However at 48h after infection, there was a significant decrease in neutrophils in Sp-D -/- mice lung ($P < 0.05$ compared to t 24h) whereas the number of neutrophils was maintained to the same level in Sp-D +/+ at 48h ($P > 0.05$, compared to t 24 h) (Fig. 4.13). Recruitment of neutrophils into inflamed areas of lung tissue was detected within inflamed bronchioles, in bronchiole walls as well as in perivascular areas in the vicinity of inflamed bronchioles (Fig 4.14).

In lung tissue sections of Sp-D -/- mice, T cells exhibited different pattern of recruitment when compared to the Sp-D +/+ lungs. Numbers of T cells showed a sharp increase around inflamed bronchioles in Sp-D -/- lungs by 24h ($P < 0.01$, when compared to t zero). The number then decreased 48 h after infection ($P < 0.01$, compared to t 24h). In contrast, in lung tissue sections of Sp-D +/+ mice, number of T cells showed only a slight increase in number at 24h ($P > 0.05$, compared to t zero) and their number remained unchanged by 48h after infection. Significant T lymphocyte infiltration into Sp-D -/- lung tissue at 24h post-infection was detected in areas surrounding inflamed bronchioles (Fig 4.15). The number of T cells in Sp-D-/- lungs was greater than Sp-D +/+ lungs at all time points after infection 0, 24 and 48h ($P < 0.01$) (Fig. 4.13)

Macrophages and B cells numbers remained unchanged in the lung tissue sections of Sp-D -/- or Sp-D +/+ ($P > 0.05$) over the time following the infection (Fig. 4.13).

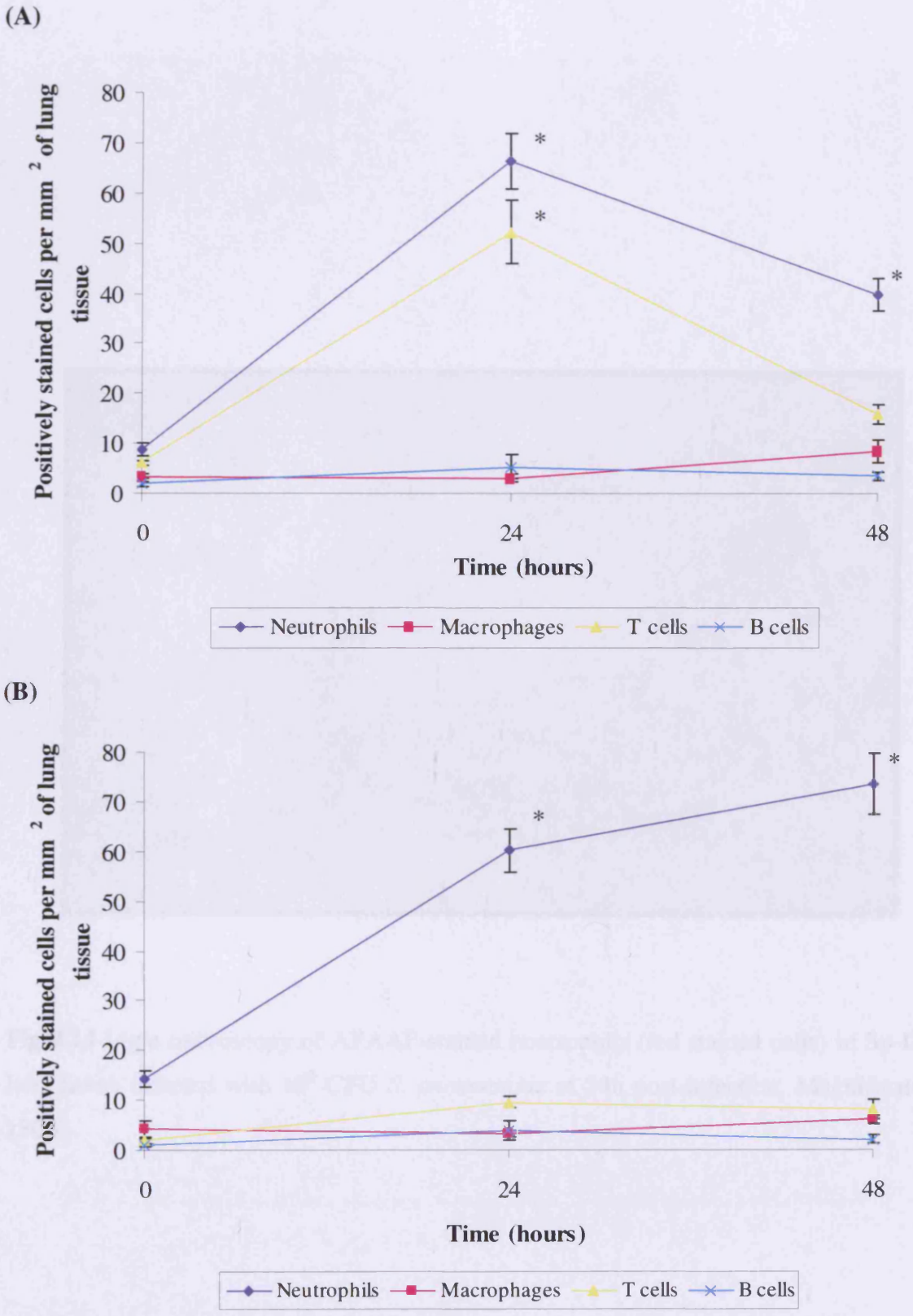



**Fig 4.13.** Numbers of neutrophils, macrophages, T cells and B cells in inflamed tissue sections from lung of Sp-D -/- (A) and Sp-D +/+ (B) mice infected intranasally with $10^6$ CFU of *S. pneumoniae*. (n = 2-3 for each time point, error bars indicate SEM). * P <0.05 when compared to time zero.

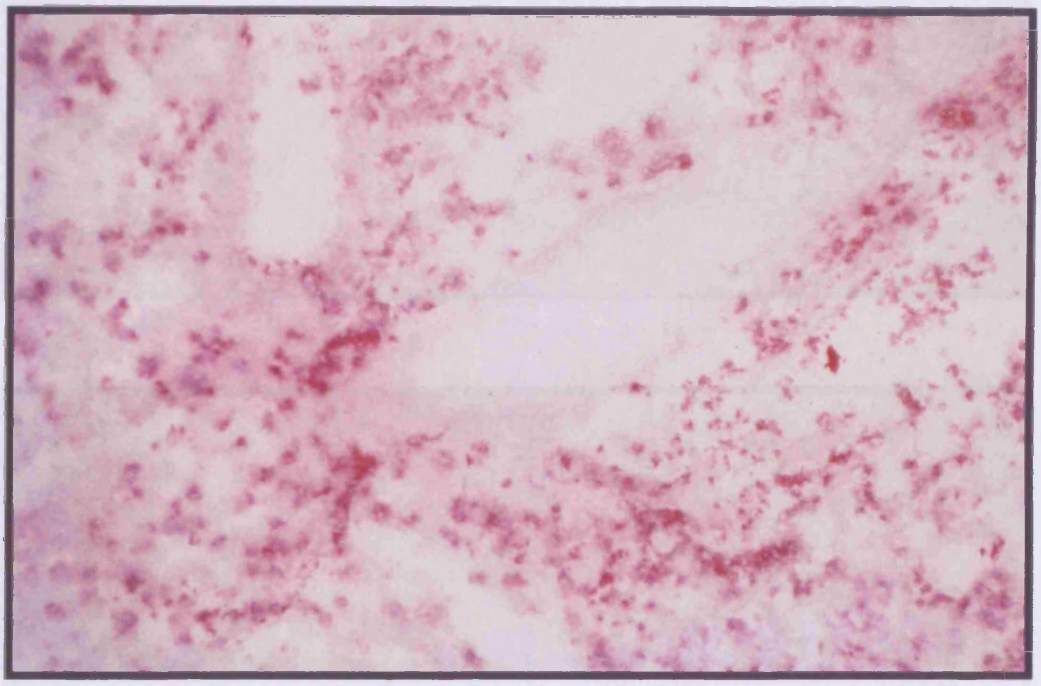
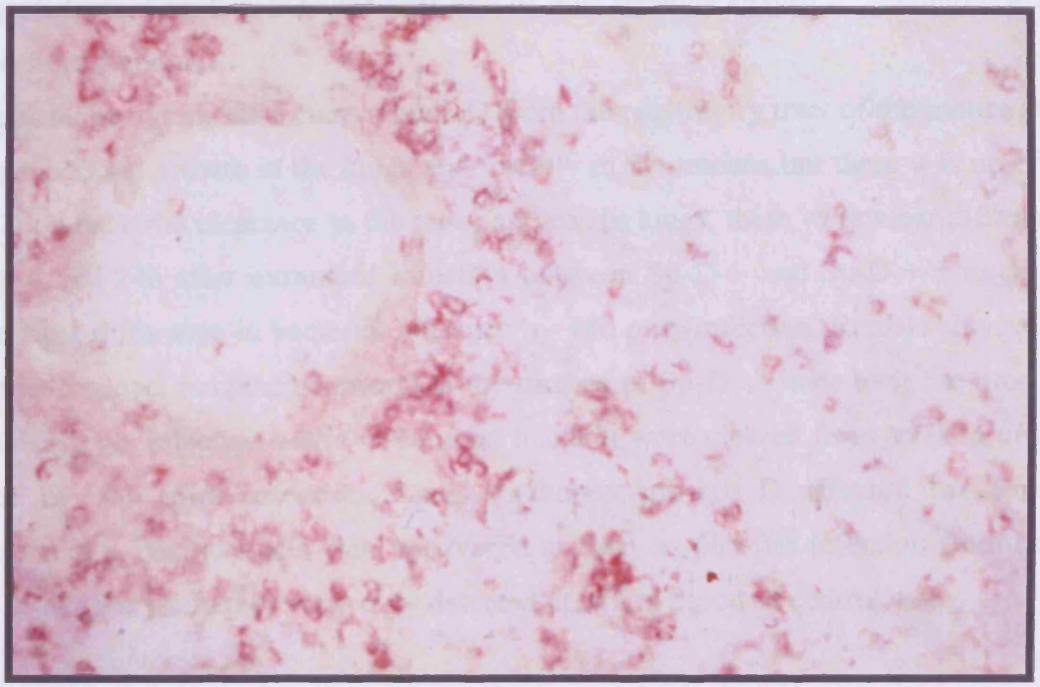


**Fig 4.14** Light microscopy of APAAP-stained neutrophils (red stained cells) in Sp-D-/- lung tissue infected with $10^6$ CFU *S. pneumoniae* at 24h post-infection. Magnification x300.

**(A)**



**(B)**

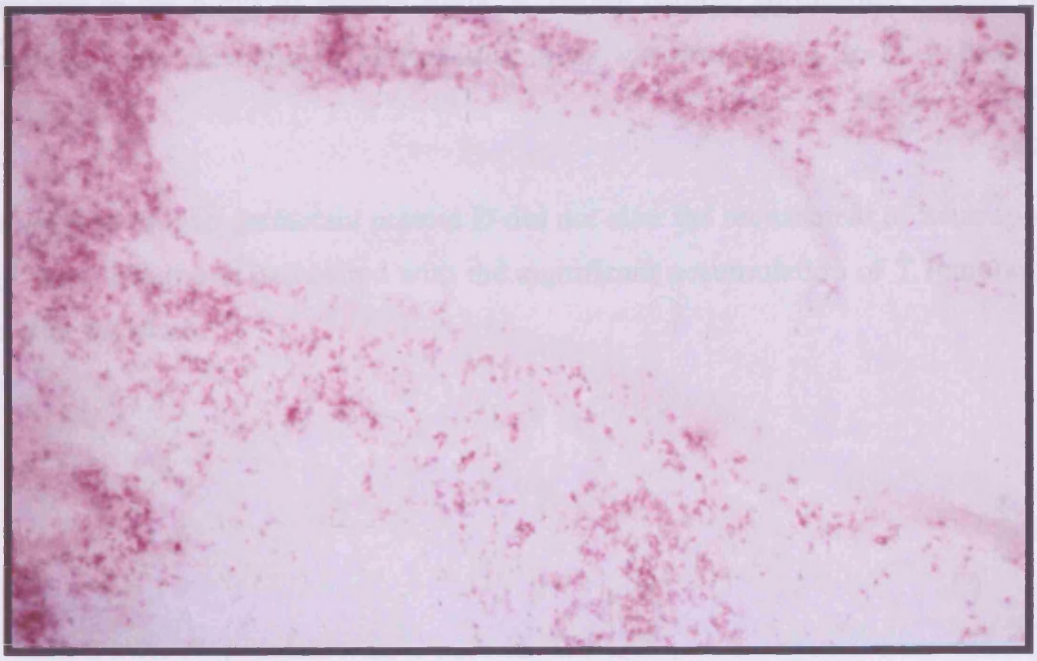


**Fig 4.15** Light microscopy of APAAP-stained T lymphocytes (red stained cells) in Sp-D-/- lung tissue infected with $10^6$ CFU *S. pneumoniae* at 24h post-infection. Magnification x 400. Dramatic infiltration of T cells to the areas surrounding the inflamed bronchioles.

Results presented in this section, indicate the importance of the *in vivo* contribution of lung surfactant protein D to the first line of host defence against *S. pneumoniae* during bronchopneumonia.

The absence of lung surfactant protein D from the respiratory tract of the mouse affected pneumococcal growth in the lungs and notably in the trachea but there was no effect of Sp-D on bacterial clearance in the nasopharynx. In lungs, there were clear differences at time 6 and 24h after intranasal infection between Sp-D-/- and Sp-D +/+ mice but no statistical difference in bacterial numbers by 48h post-infection. Remarkably, numbers of pneumococci remained constant in the trachea of Sp-D -/- mice over the time course following the infection while in contrast bacteria were cleared from trachea of control mice by 48h after infection. Lung surfactant protein D affected the timing of bacteraemia. Pneumococci were recovered as early as 6h after infection from blood of Sp-D -/- mice while they were only detected at 24h in blood of control mice.

Histological changes were more pronounced in Sp-D deficient mice lungs when compared to the lungs of control mice. A severe cellular infiltration around infected bronchioles and surrounding perivascular areas was detected in Sp-D -/- lungs at 24h after infection.

The absence of lung surfactant protein D did not alter the recruitment of neutrophils into lung tissue but it was associated with the significant accumulation of T lymphocytes at 24h after infection.

### 4.3 Interaction of gp-340 and different strains of *S. pneumoniae*

Gp-340, a 340 KDa glycoprotein isolated from bronchoalveolar lavage fluid (BAL), was identified due to its binding to lung surfactant protein D in a calcium dependent manner (Holmskov *et al.,* 1997). Recent studies reported that a salivary agglutinin, identified to be gp-340, was found to bind and aggregate bacteria such as *Streptococcus mutans* and *Helicobacter pylori* (Prakobphol *et al.,* 2000, Ligtenbreg *et al.,* 2001).

Considering the presence of gp-340 in the respiratory tract, its binding to lung surfactant protein D and aggregation effect on microorganisms, in the present study the interaction between human gp-340 and different strains and serotypes of *S. pneumoniae* (those that were already tested with Sp-D) was investigated.

This study was done during a visit to the Department of Microbiology and Immunology, University of Southern Denmark, Denmark.

#### 4.3.1 Binding of gp-340 to *S. pneumoniae*

A solid-phase bacterial ELISA revealed that gp-340 (0.08-5 μg/ml) purified from human BAL, bound to all strains of *S. pneumoniae* used in this study in the presence of calcium (Table 4.4 and 4.5). No significant binding was detected in the presence of 10 mM EDTA emphasizing that it took place in a calcium dependent manner. Results showed that the extent of binding was not different between strains of various capsule or MLSTs ($P > 0.05$) but it was gp-340-concentration dependent (Table 4.4). For instance, strains M393, M110 and M290 of type 3 *S. pneumoniae* were bound to the same extent by gp-340. Also, there was no difference in the extent of binding of gp-340 to pneumococcal strains GM70 (type 19A) and M41 (type 23F) of the same MLST.

In addition, strains of *S. pneumoniae* in which the capsule had been switched in the laboratory were tested for their binding by gp-340 and results showed that there is no difference in the extent of binding between the switched strains. Strain HB565 (type 3), strain Ps3205 (in which the capsule had been switched to type 3 from type 2), strain Ps3221 (in which capsule had been switched to type 3 from 19F) and strain G54 (type 19F parent of PS3221) were bound to the same extent by gp-340 (Table 4.4). Thus, data indicate that the capsule type or the sequence type of the pneumococcal strain did not affect the extent of binding of gp-340.

Whether the presence of the capsule is a pre-requisite for binding of gp-340 to the pneumococcus was tested. Results showed that there was no difference in gp-340 binding ($P > 0.05$) to the encapsulated parent strain D39 (type 2) and the non-

encapsulated isogenic strain RX1 (Table 4.5), thus indicating that gp-340 is binding to the surface of pneumococci even in the absence of the capsule.

The effect of the pneumococcal surface protein C (PspC) on the binding of gp-340 to pneumococci was also assessed using different mutated isogenic strains lacking PspC on the pneumococcal capsule. Also, mutant strains displaying PspC in which the N-terminal of the molecule has been deleted, were used.

As shown in Table 4.5, binding of gp-340 to pneumococcal surface was not affected by the presence or absence of the pneumococcal surface protein C (PspC). For instance, there was no difference in gp-340 binding ($P > 0.05$) to the parent strain type 2 D39 and the three mutant strains, FP30 in which PspC was lacking, FP15 in which the capsule and PspC were not present and FP7 in which the N-terminal of the PspC molecule has been deleted.

| Strain | Serotype | MLST | Gp-340 concentrations | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 µg/ml | 0.08 µg/ml | 1.25 µg/ml | 5 µg/ml | 5 µg/ml+ EDTA |
| M393 | 3 | 260 | 0.034 ± 0.005 | 0.25 ± 0.001 | 1.77 ± 0.006 | 3.32 ± 0.048 | 0.15 ± 0.003 |
| M110 | 3 | 180 | 0.034 ± 0.00 | 0.22 ± 0.002 | 1.16 ± 0.057 | 3.16 ± 0.01 | 0.15 ± 0.004 |
| M290 | 3 | 49 | 0.037 ± 0.00 | 0.30 ± 0.014 | 2.03 ± 0.034 | 3.55 ± 0.03 | 0.16 ± 0.00 |
| M9 | 18C | 102 | 0.032 ± 0.002 | 0.23 ± 0.00 | 1.71 ± 0.045 | 3.17 ± 0.044 | 0.15 ± 0.005 |
| M241 | 18C | 113 | 0.034 ± 0.005 | 0.15 ± 0.00 | 1.23 ± 0.004 | 2.89 ± 0.086 | 0.14 ± 0.009 |
| M208 | 18C | 119 | 0.037 ± 0.00 | 0.23 ± 0.006 | 1.73 ± 0.015 | 3.33 ± 0.05 | 0.16 ± 0.001 |
| GM70 | 19A | 81 | 0.036 ± 0.001 | 0.25 ± 0.005 | 1.74 ± 0.013 | 3.28 ± 0.056 | 0.18 ± 0.011 |
| M41 | 23F | 81 | 0.033 ± 0.005 | 0.19 ± 0.004 | 1.09 ± 0.052 | 3.06 ± 0.09 | 0.14 ± 0.006 |
| M322 | 14A | 156 | 0.033 ± 0.005 | 0.24 ± 0.003 | 1.80 ± 0.017 | 3.35 ± 0.078 | 0.15 ± 0.005 |
| M15 | 9V | 156 | 0.033 ± 0.001 | 0.34 ± 0.00 | 1.52 ± 0.009 | 2.97 ± 0.005 | 0.14 ± 0.004 |
| Ps3221 | 3 | 63 | 0.035 ± 0.00 | 0.23 ± 0.005 | 1.62 ± 0.04 | 3 ± 0.01 | 0.11 ± 0.00 |
| G54 | 19F | 63 | 0.033 ± 0.001 | 0.19 ± 0.001 | 1.40 ± 0.006 | 2.7 ± 0.028 | 0.14 ± 0.005 |
| HB565 | 3 | 378 | 0.033 ± 0.00 | 0.21 ± 0.013 | 1.52 ± 0.046 | 2.9 ± 0.059 | 0.12 ± 0.003 |
| Ps3205 | 3 | ND | 0.036 ± 0.005 | 0.20 ± 0.003 | 1.52 ± 0.04 | 2.83 ± 0.03 | 0.13 ± 0.011 |

**Table 4.4**. Binding of gp-340 to different strains of *S. pneumoniae.*
Binding was assessed by bacterial solid-phase ELISA. Experiments were done in 5mM calcium or EDTA. Values are means ± SEM of optical density at 405 nm. In all cases the OD was significantly increased when compared with control value (in absence of gp-340), $P < 0.05$ except when EDTA was used $P > 0.05$. There is no significant difference $P > 0.05$ in the extent of binding between serotypes.

| | | | | Gp-340 concentrations | | | | |
|---|---|---|---|---|---|---|---|---|
| **Strain** | **Serotype** | **Capsule** | **PspC** | **0 µg/ml** | **0.08 µg/ml** | **1.25 µg/ml** | **5 µg/ml** | **5 µg/ml+ EDTA** |
| D39 | 2 | + | + | 0.038 ± 0.005 | 0.24 ± 0.002 | 1.64 ± 0.015 | 3.14 ± 0.01 | 0.12 ± 0.01 |
| Rx1 | 2 | – | – | 0.037 ± 0.001 | 0.20 ± 0.007 | 1.32 ± 0.16 | 2.53 ± 0.27 | 0.11 ± 0.008 |
| FP30 | 2 | + | – | 0.036 ± 0.001 | 0.20 ± 0.005 | 1.46 ± 0.011 | 2.93 ± 0.049 | 0.12 ± 0.005 |
| FP15 | 2 | – | – | 0.038 ± 0.005 | 0.20 ± 0.003 | 1.43 ± 0.043 | 2.94 ± 0.051 | 0.13 ± 0.003 |
| FP7 | 2 | – | N-terminal deletion | 0.038 ± 0.00 | 0.23 ± 0.005 | 1.13 ± 0.025 | 3.17 ± 0.056 | 0.14 ± 0.002 |
| G54 | 19F | + | + | 0.033 ± 0.001 | 0.19 ± 0.001 | 1.40 ± 0.006 | 2.70 ± 0.028 | 0.15 ± 0.001 |
| FP74 | 19F | + | – | 0.034 ± 0.00 | 0.18 ± 0.002 | 0.98 ± 0.019 | 2.78 ± 0.007 | 0.14 ± 0.009 |
| G374 | 17 | + | + | 0.033 ± 0.001 | 0.26 ± 0.004 | 1.89 ± 0.012 | 3.27 ± 0.008 | 0.18 ± 0.005 |
| FP34 | 17 | + | – | 0.033 ± 0.001 | 0.21 ± 0.002 | 1.58 ± 0.011 | 3.11 ± 0.004 | 0.16 ± 0.002 |
| HB565 | 3 | + | + | 0.033 ± 0.00 | 0.21 ± 0.013 | 1.52 ± 0.046 | 2.90 ± 0.059 | 0.12 ± 0.003 |
| FP8 | 3 | – | N-terminal deletion | 0.037 ± 0.001 | 0.13 ± 0.003 | 0.77 ± 0.04 | 2.62 ± 0.014 | 0.13 ± 0.002 |
| FP20 | 3 | – | – | 0.030 ± 0.001 | 0.18 ± 0.001 | 0.96 ± 0.004 | 2.64 ± 0.006 | 0.11 ± 0.007 |
| FP13 | 3 | + | – | 0.036 ± 0.005 | 0.19 ± 0.003 | 1.09 ± 0.021 | 3.09 ± 0.003 | 0.15 ± 0.002 |

**Table 4.5** Binding of gp-340 to different strains of *S. pneumoniae.* Isogenic mutated strains that lack (-) or have (+) either capsule or pneumococcal surface protein C (PspC) capsule, or have deletion in the N-terminal portion of the PspC molecule were used. Binding was assessed by bacterial solid-phase ELISA. Experiments were done in 5mM calcium or EDTA. Values are means ± SEM of optical density at 405 nm. In all cases the OD was significantly increased when compared with control value, $P < 0.05$ except when EDTA was used ($P > 0.05$). There was no significant difference of binding between different serotypes ($P > 0.05$).

### 4.3.2 Agglutination of *S. pneumoniae* by gp-340

To further investigate the physical effect resulting from gp-340 binding to *S. pneumoniae,* different strains were incubated with human gp-340 (10 μg/ml). Agglutination was measured as sedimentation by following the change in optical density of 500 nm at 37 °C at 30 min intervals for 3 hours. There was no agglutination in the presence of 10 mM EDTA emphasizing that it took place in a calcium dependent manner. Encapsulated serotype 2 strain D39 and non-encapsulated strain RX1 *S. pneumoniae* were agglutinated to the same extent in the presence of gp-340 suggesting no role for capsule (Fig 4.16). In addition, agglutination by gp-340 was also tested using pneumococcal strains in which the capsule had been switched in the laboratory. As shown in Fig 4.17, the capsule also did not appear to have a role in these experiments. Strain HB565, serotype 3 was strongly agglutinated by gp-340, whereas strain Ps3205, in which the capsule had been switched to type 3 from type 2 was not agglutinated by gp-340. Furthermore, strain Ps3221 in which the capsule had been switched to type 3 from 19F showed agglutination by gp-340, while strain G54, type 19F parent of Ps 3221 did not show any agglutination (Fig 4.18).
Interestingly, pneumococcal strains type 2 D39 and RX1, HB565 and Ps3221 were agglutinated by gp-340 as well as by lung surfactant protein D as reported previously, in contrast strains G54 and Ps3205 were not agglutinated either with Sp-D or gp-340.

Results presented in this section showed that human gp-340 isolated from BAL, bound in a calcium dependent manner to different strains and serotypes of *S. pneumoniae.* The extent of binding was not affected by the presence or absence of the capsule, the pneumococcal surface protein C (PspC), the capsule or the sequence type of the pneumococcal strain. Gp-340 induced agglutination of several strains of *S. pneumoniae* in the presence of calcium. Although binding of gp-340 to all pneumococcal strains was detected, aggregation of bacteria by gp-340 was not detected for all *S. pneumoniae* strains such as G54 and Ps3205. Furthermore; data of agglutination experiments suggested that the capsule seemed to have no effect on the process of agglutination of bacteria by gp-340.

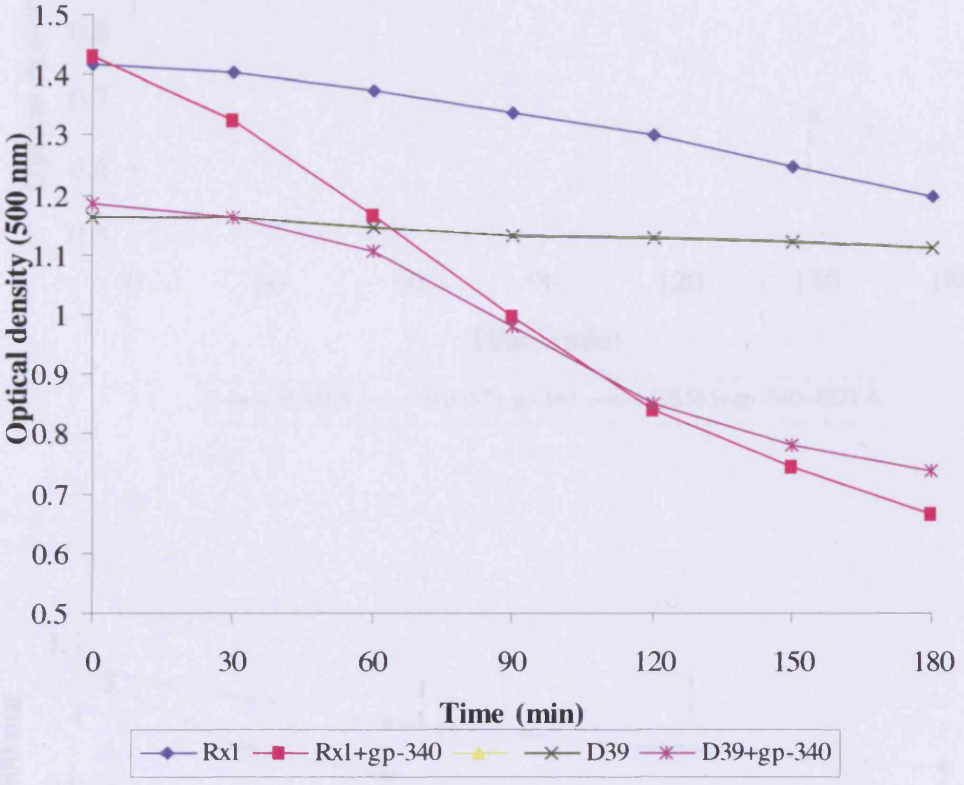
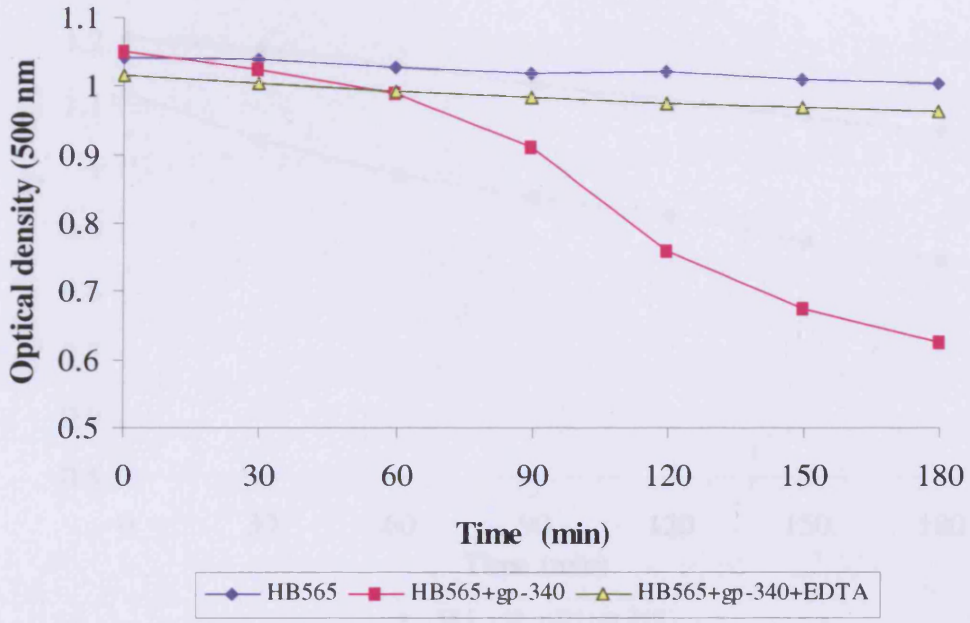



**Fig 4.16.** Agglutination by gp-340 of type 2 *S. pneumoniae* encapsulated strain (D39) and non-encapsulted strain RX1. Bacteria were incubated with gp-340 in the presence of calcium or EDTA. Agglutination was measured as sedimentation at an optical density of 500 nm at 37 °C at 30 min intervals for 3 hours.

(A)




(B)

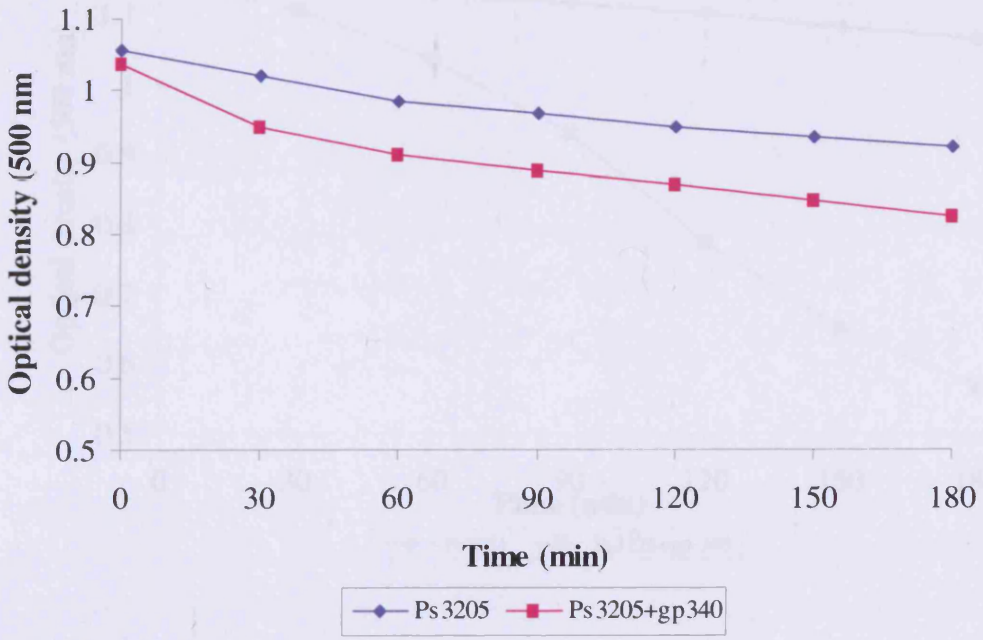
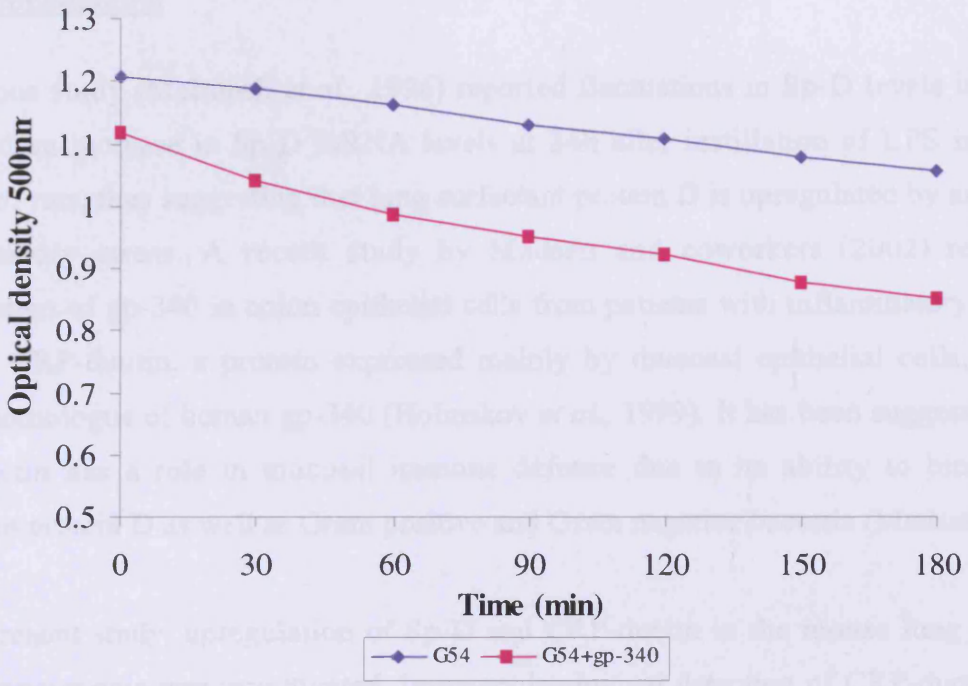



**Fig 4.17.** Agglutination by gp-340 of *S. pneumoniae* strains (A) HB565 and (B) Ps3205. Bacteria were incubated with gp-340 in the presence of calcium or EDTA. Agglutination was measured as sedimentation at an optical density of 500 nm at 37 °C at 30 min intervals for 3 hours.

(A)




(B)

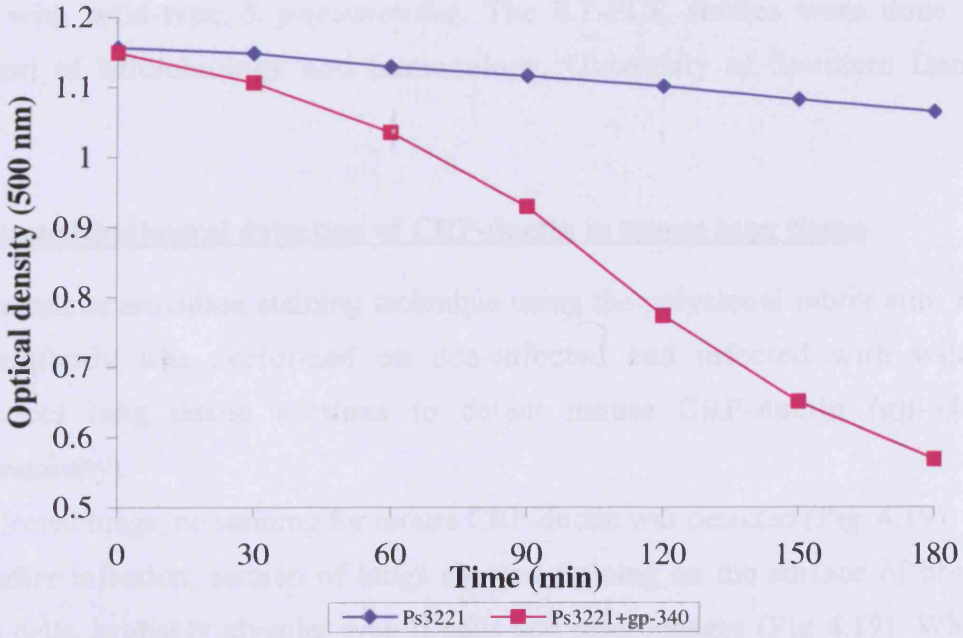



**Fig 4.18.** Agglutination by gp-340 of *S. pneumoniae* strains (A) G54 and (B) Ps3221. Bacteria were incubated with gp-340 in the presence of calcium or EDTA. Agglutination was measured as sedimentation at an optical density of 500 nm at 37 °C at 30 min intervals for 3 hours.

## 4.4 Upregulation of lung surfactant protein D and CRP-ductin in mice lungs during bronchopneumonia

A previous study (McIntosh *et al.*, 1996) reported fluctuations in Sp-D levels in BAL fluid and an increase in Sp-D mRNA levels at 24h after instillation of LPS into the trachea of rats, thus suggesting that lung surfactant protein D is upregulated by an acute inflammatory stress. A recent study by Madsen and coworkers (2002) reported upregulation of gp-340 in colon epithelial cells from patients with inflammatory bowel disease. CRP-ductin, a protein expressed mainly by mucosal epithelial cells, is the mouse homologue of human gp-340 (Holmskov *et al.*, 1999). It has been suggested that CRP-ductin has a role in mucosal immune defence due to its ability to bind lung surfactant protein D as well as Gram positive and Gram negative bacteria (Madsen *et al.*, 2001).

In the present study, upregulation of Sp-D and CRP-ductin in the mouse lung during bronchopneumonia was investigated. Immunohistological detection of CRP-ductin and RT-PCR studies were performed on lung tissues, where the mice were intranasally infected with wild-type *S. pneumoniae*. The RT-PCR studies were done at the Department of Microbiology and Immunology, University of Southern Denmark, Denmark.

### 4.4.1 Immunohistological detection of CRP-ductin in mouse lung tissue

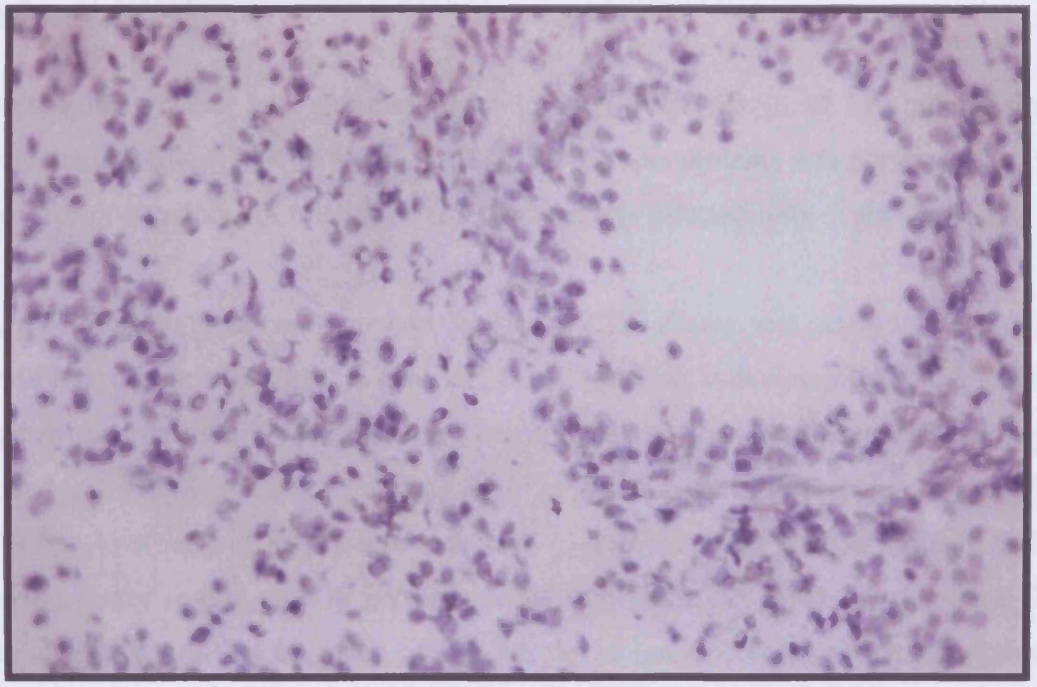
Indirect immunoperoxidase staining technique using the polyclonal rabbit anti- human gp-340 antibody was performed on non-infected and infected with wild-type pneumococci lung tissue sections to detect mouse CRP-ductin (gp-340-like immunoreactivity).

In non-infected lungs, no staining for mouse CRP-ductin was detected (Fig. 4.19).

At 48 h after infection, section of lungs showed staining on the surface of bronchial epithelial cells, probably alveolar type II cells and macrophages (Fig 4.19). When the anti-gp-340 was omitted, no staining was seen. However, using frozen lung sections, the morphology of the tissue after immunostaining was not clear thus leading to the use of RT-PCR as a sensitive method to study upregulation of CRP-ductin in mouse lung after intranasal infection.

No suitable monoclonal antibody directed against gp-340 was available.

(A)



(B)

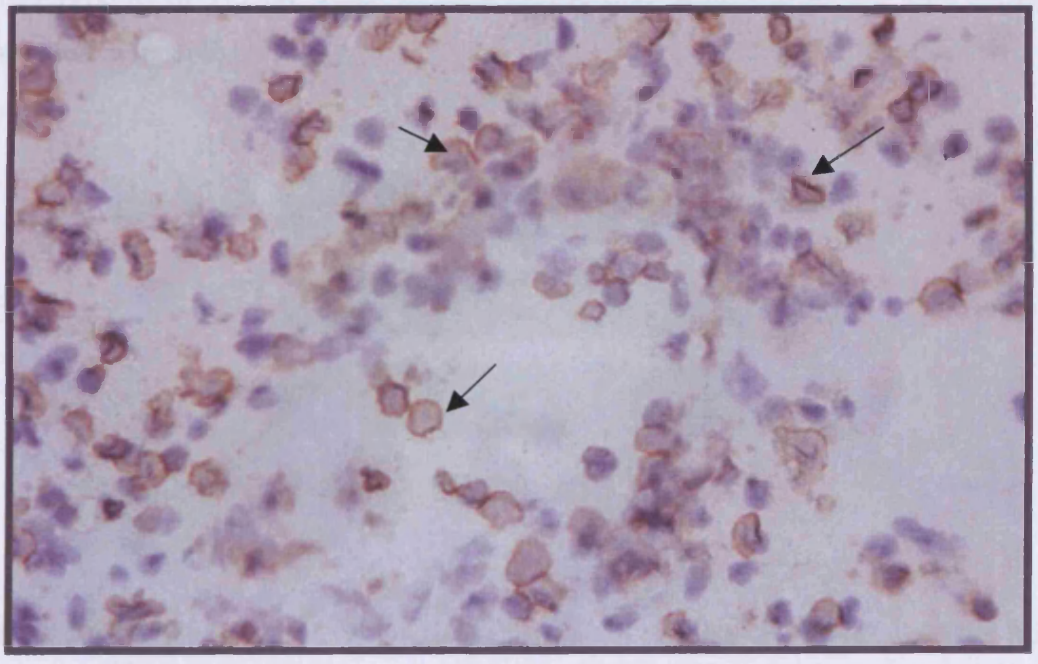


**Fig 4.19.** Light microscopy of Biotin-Streptavidin peroxidase stained CRP-ductin (gp-340-like immunoreactivity) in lung tissue (A) non-infected (B) infected with$10^6$ CFU of wild-type *S. pneumoniae* 48h post-infection. Magnification (A) x 300 and (B) x 400.

### 4.4.2 RT-PCR analysis of Sp-D and CRP-ductin expression in mice lungs during bronchopneumonia

To investigate whether lung surfactant protein D and CRP-ductin are upregulated during bronchopneumonia, RT-PCR for the mRNA for the two proteins was performed on lung tissues from two strains of mice that were intranasally infected with *S. pneumoniae*.

First, the tissue expression of mouse Sp-D and CRP-ductin was analysed in a pair of infected lungs from MF1 mice at time 0, 24 and 48h after infection (Fig. 4.20). RT-PCR was performed on total RNA extracted from lung tissues. A pair of primers spanning the neck and CRD coding region of mouse Sp-D gene was used and gave rise to a fragment of 459 bp. Another pair of primers was used to amplify the $8^{th}$ SRCR domain and the following linker region rich in proline, serine and threonine residues of mouse CRP-ductin and gave rise to a fragment of 381 bp. As an internal standard for the amount of RNA used in PCR, a primer pair of the mouse transferrin receptor was used and gave rise to a product of 371 bp. Thus, all lung tissues have been normalized with respect to the transferrin receptor.

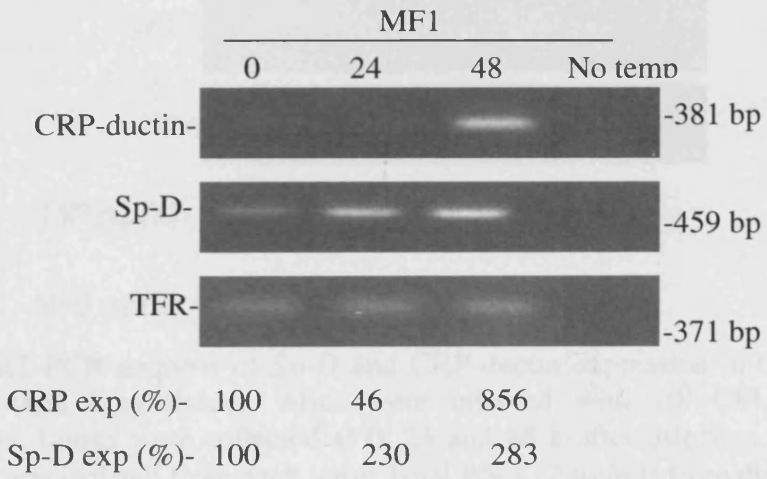



**Fig 4.20.** RT-PCR analysis of Sp-D and CRP-ductin expression in MF1 infected mouse lung tissues. Mice were infected with $10^6$ CFU of wild-type *S. pneumoniae*. Lungs were collected at 0, 24 and 48 h after infection, homogenised and total RNA was isolated from each lung. Total RNA (2 µg/ml) from different lung tissues were used for first strand synthesis and Sp-D, CRP-ductin or the transferrin receptor transcripts were amplified by PCR using gene-specific primers. The products were separated by 1.2 % (w/v) agarose gel electrophoresis.

The densities of the PCR products were measured and ratios Sp-D/TFR and CRP-ductin were calculated. The ratio was set for 0h as 100%.

Based on the signals and the calculated percentage for the expression of the two proteins, expression of Sp-D mRNA was more pronounced in the lungs of MF1 mice by 24 and 48h after infection compared to time zero (Fig. 4.20). Expression of CRP-ductin mRNA in the lungs of MF1 mice was decreased by 24h followed by a significant increase by 48h after infection.

It is worth mentioning that when genomic DNA was used as a template in the PCR, no bands were seen indicating that the amplified fragment did not originate from contaminating genomic DNA in the RNA samples.

To fortify these findings, RT-PCR for the mRNA for CRP-ductin and Sp-D was performed on another pair of MF1 and a pair of C57BL/6 mice that were infected with *S. pneumoniae* (Fig. 4.21). This work was done by Dr. Jens Madsen (Department of Microbiology and Immunology, University of Southern Denmark, Denmark).

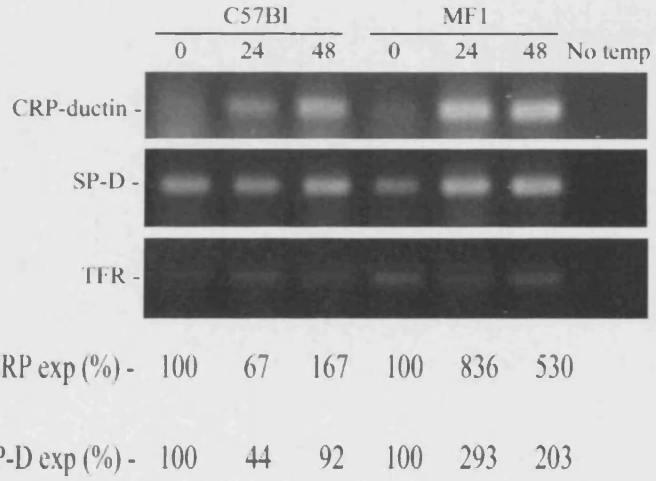



**Fig 4.21.** RT-PCR analysis of Sp-D and CRP-ductin expression in C57BL/6 and MF1 infected mouse lung tissues. Mice were infected with $10^6$ CFU of wild-type *S. pneumoniae*. Lungs were collected at 0, 24 and 48 h after infection, homogenised and total RNA was isolated from each lung. Total RNA (2 μg/ml) from different lung tissues were used for first strand synthesis and Sp-D, CRP-ductin or the transferrin receptor transcripts were amplified by PCR using gene-specific primers. The products were separated by 1.2 % (w/v) agarose gel electrophoresis.

Based on the signals and the calculated percentage, expression for both Sp-D and CRP-ductin, in C57BL/6 lung tissues, decrease by 24h after infection followed by an increase to normal level for Sp-D and for a greater level for CRP-ductin by 48h after infection. For the MF1 strain, the expression of both proteins rises by 24 and 48h after infection.

Assuming that translation efficiency is similar for all these proteins, these RT-PCR results strongly suggest that CRP-ductin and Sp-D are up-regulated in the lung of MF1 mice during bronchopneumonia and only CRP-ductin is up-regulated in the lung of C57BL/6 mice.

# Chapter 5: Discussion

The host innate immune system has a crucial role in the early control of *S. pneumoniae* infection and affects the later establishment of pneumococcal disease. The objective of the study was to investigate the contribution of complement and lung surfactant protein D in the innate resistance to *S. pneumoniae.*

Complement is known to be an important innate factor in host resistance to the pneumococcus. The overall contribution of the classical complement pathway activation and the effects of the ability of pneumolysin to activate complement were investigated in a murine model of bronchopneumonia.

In the last decade, lung surfactant protein D has been reported to be an important component in the innate host defence of the lung. *In vitro* interaction between Sp-D and the pneumococcus and an investigation of the *in vivo* contribution of Sp-D to innate host defence during bronchopneumonia are reported in this thesis. Additionally, the interaction between Sp-D receptor (gp-340) and the pneumococcus and upregulation of Sp-D and CRP-ductin (homologue of gp-340 in the mouse) during bronchopneumonia were investigated.

## 5.1 Pneumococcal behaviour and host responses during bronchopneumonia are affected differently by the cytolytic and complement activating activities of pneumolysin

The multifunctional toxin pneumolysin, produced by all clinical isolates of *S. pneumoniae*, is strongly implicated in the pathogenesis of pneumococcal bronchopneumonia and septicaemia (Canvin *et al.,* 1995, Kadioglu *et al.,* 2000). Site-directed mutagenesis studies demonstrated that distinct parts of the toxin molecule are involved in its cytolytic and complement activation properties (Boulnois *et al.,* 1991). Subsequent work lead to the production of pneumococcal strains producing pneumolysin lacking one or other of its wild-type activities. These isogenic mutant strains were used in bronchopneumonia and lobar pneumonia models and showed that both anti-cellular and complement activation activities of pneumolysin contributed to virulence of the pneumococcus (Rubins *et al.,* 1996, Alexander *et al.,* 1998). Pneumolysin's complement activating activity was associated with bacterial growth in lung tissue and blood at 24h after endotracheal instillation, while the cytolytic activity correlated with impairment of the alveolar capillary barrier and increase in bacterial numbers during the first 6h of

infection (Rubins *et al.,* 1996). Similarly, after intranasal challenge both of these activities were required for full virulence of *S. pneumoniae* (Alexander *et al.,* 1998).

The studies reported in this thesis extended the previous observations by analysing in more detail how the growth of the pneumococcus and the pulmonary host response are affected by the individual complement activation property of pneumolysin.
A similar study was conducted by Dr. Aras Kadioglu (Dept. of Microbiology and Immunology, University of Leicester), investigating the effect of pneumolysin's cytolytic property using the strain H2-/C+. My work will be discussed along with his data to give a full picture on the individual effect of each of the pneumolysin's properties on the *in vivo* events during bronchopneumonia.

The wild-type parent type 2 strain D39 and two isogenic mutant strains were used in these studies: strain H+/C- carrying one point mutation (Asp385>Asn) which produces pneumolysin lacking complement activity but with complete cytolytic activity (Mitchell *et al.,* 1991) and strain H2-/C+ carrying one point mutation (His367>Arg) which produces pneumolysin with only 0.02% cytolyic activity but has 100% complement activation activity (Alexander *et al.,* 1998). The effect of deletion of cytotoxic activity or complement activation activity of pneumolysin on bacterial growth in lungs and blood, histological changes and the pattern of inflammatory cell recruitment into infected lung tissue were examined.

The deletion of either cytotoxic or complement activating activity affected the early growth of the pneumococcus in the lung. Surprisingly, removal of one or other of pneumolysin wild-type activities enhanced pneumococcal survival. Absence of complement activation improved pneumococcal survival in the first twelve hours after infection whereas a reduction in cytolytic activity was associated with better pneumococcal survival in the first six hours (Fig 5.1). However, the same numbers of mutants and wild-type pneumococci were present in the lungs by 48h after intranasal challenge. This indicates that pneumolysin's properties make distinct contributions to pneumococcal growth in the lung and that these mutations do not affect the outcome for the bacteria in lungs. It has been shown previously that the mutations Asp385>Asn and His367>Arg in the pneumolysin gene increased the survival time of mice after intranasal challenge (Alexander *et al.,* 1998). Conversely, from the present findings no correlation

could be established between the survival time and the number of pneumococci in the lungs.

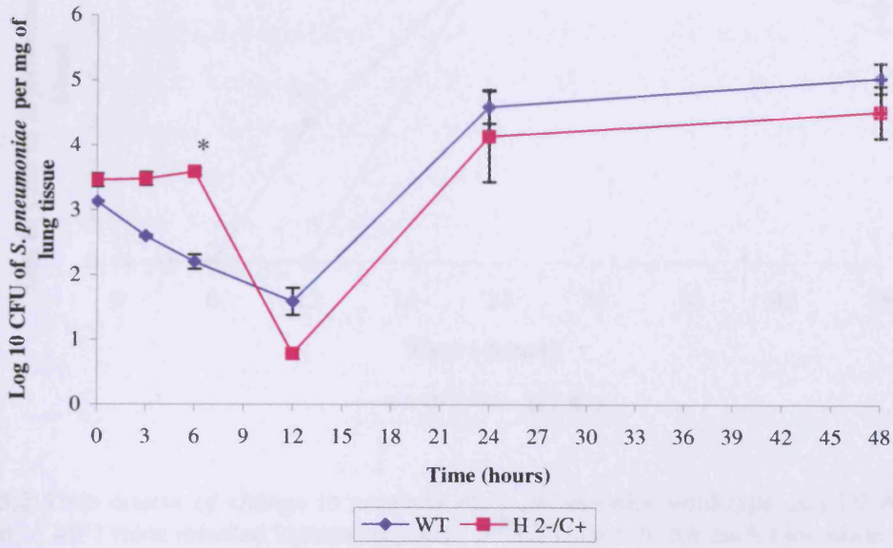



**Figure 5.1** Time course of change in numbers of *S. pneumoniae* wild-type and H2-/C+ in the lungs of MF1 mice infected intranasally with $10^6$ CFU (n = 10 for each time point, error bars indicate SEM). * P <0.05 for H2-/C+ at 6h compared to wild-type.

The deletion of either cytotoxic or complement activating activity influenced differently the timing of bacteraemia. The mutant H+/C- was detected as early as 6h in the blood suggesting that complement activation by pneumolysin has a crucial role in delaying bacteraemia. In contrast, the mutant H2-/C+ showed a later appearance in the blood at 24h (Fig 5.2) that could be associated with an increase in survival time as stated previously (Kadioglu *et al.*, 2000). It is noteworthy that the mutants H+/C-, H2-/C+ and the wild-type pneumococci increased at the same rate in the blood. These results are in agreement with a previous study reporting that after intravenous challenge, isogenic mutant strains having reduced complement activation activity or cytotoxic activity did not individually alter the growth of pneumococci in blood (Benton *et al.*, 1997).

Furthermore, Kadioglu *et al.* (2000) reported that production of pneumolysin conferred to the bacterium resistance against anti-microbial mechanisms that limit their numbers in the blood. From the result presented here, it can be concluded that presence of either cytolytic or complement activation activities is sufficient for the bacterium resistance and thus enhancing its virulence.

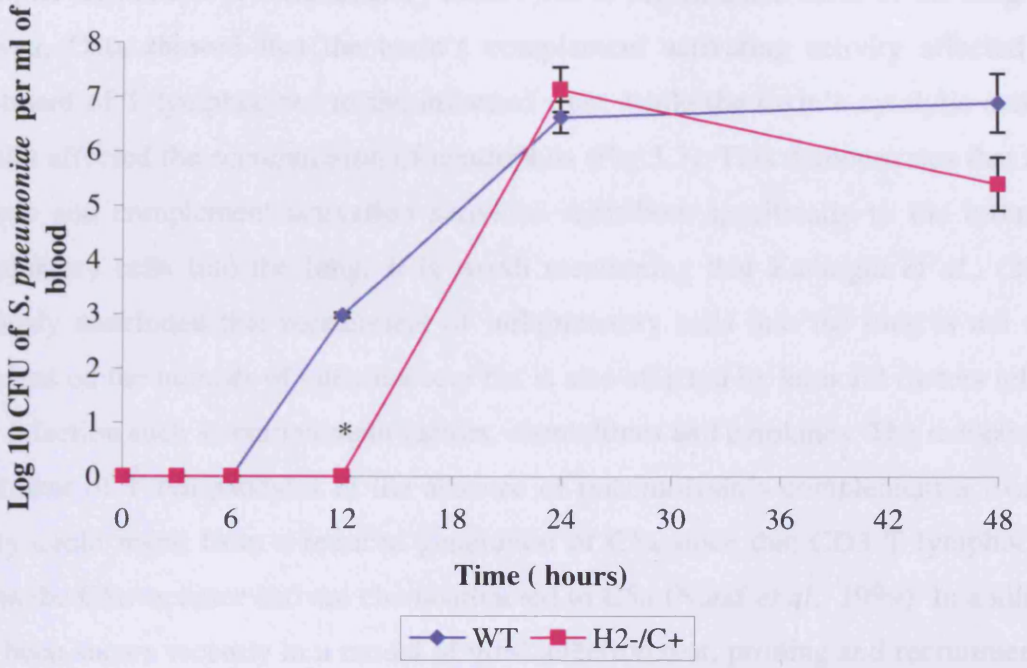



**Fig 5.2** Time course of change in numbers of *S. pneumoniae* wild-type and H2-/C+ in blood of MF1 mice infected intranasally with $10^6$ CFU (n = 10 for each time point, error bars indicate SEM). * P <0.05 for H2-/C+ at 12h compared to wild-type.

In opposition to these observations, it has been shown in a model of pneumococcal bacteraemia that the absence of complement activating activity resulted in greater pneumococcal clearance from blood (Alcantra *et al.,* 1999). However, another study demonstrated that pneumolysin's cytolytic activity but not its complement activation activity contributed to virulence during systemic infection in mice (Berry *et al.,* 1995).

Histological studies showed that both properties of pneumolysin are involved in the induction of inflammation. A less severe histological picture was observed in lungs infected with mutant strains H+/C- and H2-/C+ early after infection. At 24h after intranasal challenge, no correlation could be seen between inflammation and number of pneumococci in the lungs. However, by 48h the extent of inflammation was similar in lungs of mice infected with wild-type and the mutant strains. These results contrast the situation after infection with pneumococci unable to produce pneumolysin. Absence of pneumolyin was associated with delay and lower severity in pulmonary inflammation (Kadioglu *et al.,* 2000). Remarkably, in tissue infected with H+/C- inflammatory cells were less tightly packed around the bronchioles. This indicates that complement activation determines local localisation of cells.

Sequential infiltration of inflammatory leukocytes to the inflamed areas of the lung was observed. Data showed that the toxin's complement activating activity affected the recruitment of T lymphocytes to the inflamed sites, while the toxin's cytolytic activity crucially affected the accumulation of neutrophils (Fig 5.3). This demonstrates that both cytolytic and complement activation activities contribute specifically to the influx of inflammatory cells into the lung. It is worth mentioning that Kadioglu *et al.*, (2000) previously concluded that recruitment of inflammatory cells into the lung is not only dependent on the number of pneumococci but is also affected by humoral factors related to the infection such as complement factors, chemokines and cytokines. The reduction in recruitment of T lymphocytes in the absence of pneumolysin's complement activating activity could result from a reduced generation of C5a since that CD3 T lymphocytes express the C5a receptor and are chemoattracted to C5a (Nataf *et al.*, 1999). In addition, it has been shown recently in a model of viral infection that, priming and recruitment of T lymphocytes into the lung are severely impaired in C3-deficient mice (Kopf *et al.*, 2002). In accord with previous work (Kadioglu *et al.*, 2000), the current findings indicate that T cells play a role in the inflammatory host immune response to the pneumococcal toxin pneumolysin.

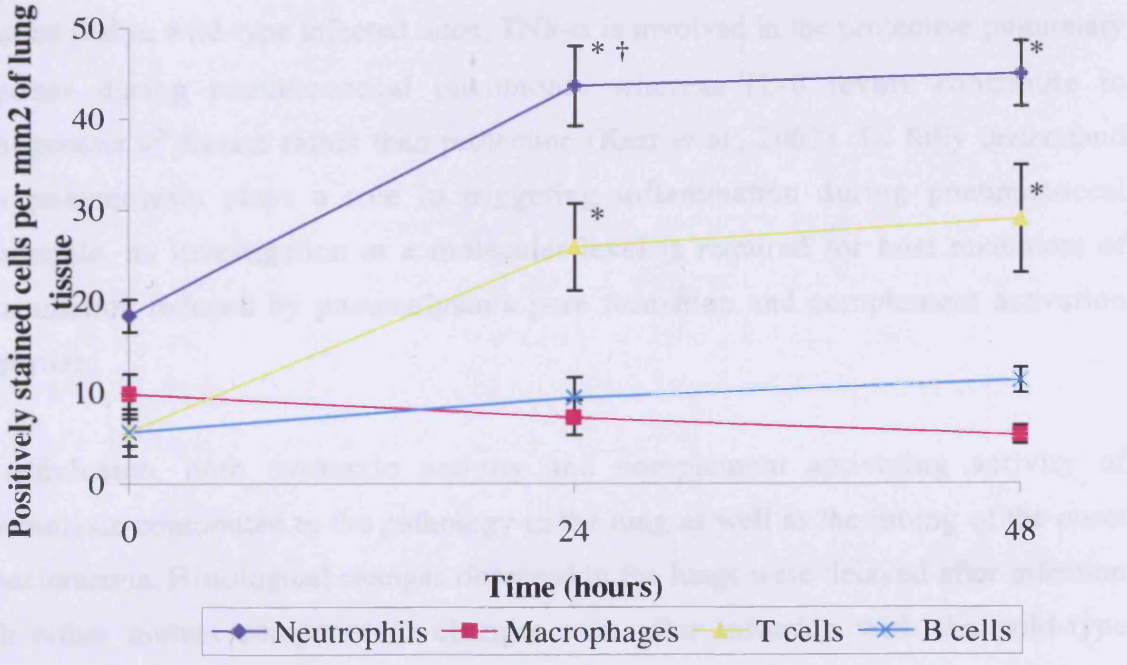



**Figure 5.3** Numbers of neutrophils, macrophages, T cells and B cells in tissue sections from lungs of MF1 mice infected intranasally with $10^6$ CFU of H2-/C+ *S. pneumoniae* (n = 4 for each time point, error bars indicate SEM). * $P < 0.05$ significantly different when compared to time zero. * $P < 0.05$ compared to time zero and † $P < 0.01$ compared to wild-type at 24h.

Several studies suggested the presence of a third unknown activity of pneumolysin that also contributes to virulence (Benton *et al.*, 1997, Alexander *et al.*, 1998, Berry *et al.*, 1999, Baba *et al.*, 2002). A remarkable recent study reported that mutant mice lacking functional Toll-like receptor (TLR)4 are significantly more susceptible to invasive disease and death after exposure to pneumolysin-producing pneumococci when compared to control mice (Malley *et al.*, 2003). Thus, investigators concluded that TLR4 mediates innate immune response to pneumococci through its interaction with pneumolysin (Malley *et al.*, 2003). Interestingly, a mutant of pneumolysin with undetectable cytotoxicity and no complement-activating properties is able to stimulate macrophages in a TLR4-dependent manner (Malley *et al.*, 2003). Thus, this study reported an additional property of pneumolysin, independent of its cytotoxic or complement-activating properties, that contributes to its inflammatory activity.

According to a recent study (Baba *et al.*, 2002), the mutant H2-/C+ is capable of inducing interferon-γ and consequently more nitric oxide (NO) than the wild-type or the H+/C- mutant. However, the observation of a reduced inflammation and decreased cell recruitment with the mutant H2-/C+ does not indicate that interferon-γ or nitric oxide are the principal mediators induced by pneumolysin during pneumonia. A recent study reported that in wild-type infected mice, TNF-α is involved in the protective pulmonary response during pneumococcal pneumonia whereas IL-6 levels contribute to pathogenesis of disease rather than protection (Kerr *et al.*, 2002). To fully understand how pneumolysin plays a role in triggering inflammation during pneumococcal pneumonia, an investigation at a molecular level is required for host mediators of inflammation induced by pneumolysin's pore formation and complement activation properties.

In conclusion, both cytotoxic activity and complement activating activity of pneumolysin contributed to the pathology in the lung as well as the timing of the onset of bacteraemia. Histological changes observed in the lungs were delayed after infection with either mutant compared to changes seen after infection with the wild-type pneumococcus. The complement activating activity affected the accumulation of T cells whereas the toxin's cytolytic activity influenced the neutrophil recruitment into lung tissue.

## 5.2 Pneumolysin's activation of complement C3 in lungs of mice during bronchopneumonia

Previous studies showed that the toxin pneumolysin has a vital role in pneumococcal disease. It has been reported that during bronchopneumonia, pneumococci not expressing pneumolysin have reduced virulence, slower growth in the lung associated with a delayed bacteraemia, a reduced inflammation and altered host cellular response compared to the wild-type organism (Canvin *et al.*, 1995, Kadioglu *et al.*, 2000). Interestingly, *in vitro* studies demonstrated that low levels of pneumolysin are able to activate the classical complement pathway in human serum, in the absence of anti-toxin antibodies, due at least in part to non-specific binding of the toxin to the Fc region of human immunoglobulin G IgG (Paton *et al,* 1984, Mitchell *et al.,* 1991). However, the direct ability of pneumolysin to activate local complement C3 *in vivo* was not examined until now. The hypothesis that production of pneumolysin by the bacterium significantly activates complement C3 in lungs of mice during bronchopneumonia was investigated.

The influence of the complement activation property of pneumolysin on the local activation of complement C3 was revealed clearly by comparison of the amounts of C3-activation products in lung tissue following intranasal infection with wild-type pneumococci and the isogenic mutant strain unable to produce pneumolysin (PLN-A). The amounts of C3 –activation products were substantially higher in lungs infected with wild-type *S. pneumoniae* than lungs infected with PLN-A by 24 and 48h after infection. These results are in agreement with the hypothesis and exhibit the important effect of pneumolysin on activating the complement pool of the lung during bronchopneumonia. The overall effect of the ability of pneumolysin to activate complement on the innate immunity to *S. pneumoniae* was investigated by using a strain of mice with genetic deficiency in C1q affecting the classical complement pathway.

## 5.3 Critical contribution of the classical complement pathway to the innate immunity during pneumococccal bronchopneumonia

Several studies culminated in giving strong evidence that the complement system is critical for innate immunity to *S. pneumoniae* (Alper *et al.,* 1970, Winkelstein 1981, Sampson *et al.,* 1982, Mold *et al.,* 2002). An increase in the susceptibility to pneumococcal disease has been demonstrated in human patients and in several animal

models, due to deprived levels of complement. Pnemococcal bacteraemia is life threatening in patients with low serum levels of C3 and/or C4 (Dee *et al.*, 1977).
Injection of cobra venom factor into mice and guinea pigs depleted complement levels and resulted in a decreased pneumococcal blood clearance and higher mortality rates compared to the control animals with an intact complement system (Brown *et al.*, 1983, Winkelstein *et al.*, 1975). Some studies investigated the effect of complement on the clearance of *S. pneumoniae* from the lung. Decomplemented mice that were intratracheally infected with virulent *S. pneumoniae* were unable to clear the bacteria efficiently from their lungs and developed a severe pneumonia (Gross *et al.*, 1978, Coonrod *et al.*, 1981, Coonrod and Yoneda 1982).

Several pieces of evidence showed that the classical complement pathway, in the absence of specific immunity, plays a central role in host defence against Group B streptococci (Wessels *et al.*, 1995), the intracellular parasite *Salmonella enterica* Serovar Typhimirium (Warren *et al.*, 2002) and in polymicrobial peritonitis (Celik *et al.*, 2001). However, at the time this investigation began, nothing had been reported on the involvement of the classical complement pathway in the innate resistance to pneumococcal infection.

In the present study, the overall contribution of the classical complement pathway in the innate immunity to *S. pneumoniae* on the *in vivo* events during bronchopneumonia was revealed by comparison of events following intranasal infection of C1q deficient mice (C1q -/-) and control mice (C1q +/+) having a complete complement system. It is worth mentioning that these mice had no antibodies directed against type 2 *S. pneumoniae.*
The hypothesis that the classical complement pathway is essential in the innate immunity to *S. pneumoniae* and that its absence results in increased susceptibility to pneumococcal pneumonia associated with severe septicaemia and alteration in the host immune response, was studied. An investigation on how the pattern of pneumococcal growth in lungs and blood, host tissue response in terms of inflammation and infiltration of inflammatory leukocytes into the lung and local activation of lung complement C3 are affected by the removal of the classical complement pathway was done. Analysis of observations showed clear differences in the outcome of bacterial growth in lungs and blood, severity of inflammation and the extent of activation of local complement C3 in the lungs between C1q-deficient mice and control mice.

Removal of the classical complement pathway increased the susceptibility to pneumococcal infection in mice and reduced the clearance of pneumococci from the lungs and blood. The growth kinetics of *S. pneumoniae* organisms in lungs showed an early and sharp decline in numbers of pneumococci in control mice significantly more intense than the clearance observed in C1q-deficient mice during the first 6h after infection. Additionally, control mice virtually cleared all the pneumococci from their lungs by 48h after infection whereas a sharp increase in pneumococcal number was observed in lungs of C1q-deficient mice. It has been reported that complement components are present and available at the alveolar epithelium in mice and a functional classical pathway has been detected in broncho-alveolar lavage fluid (Coonrod and Yoneda 1981, Watford *et al.,* 2000) thus combined with the presented data indicate an important role for the classical complement pathway in clearance of the pathogen from the lung. The results support the hypothesis that the classical complement pathway contributes greatly in the clearance of *S. pneumoniae* from the lungs.
The lack in C1q influenced critically the levels but not the timing of bacteraemia. However, control mice (C1q +/+) successfully cleared *S. pneumoniae* from their blood by 48h after infection whereas a rapid increase in the numbers of pneumococci was detected in blood of C1q-deficient mice. This underscores that the classical complement pathway activation has a remarkable influence on limiting bacteraemia and progression of pneumococcal disease.

To further investigate how the host mechanisms are affected by the absence of the classical complement pathway, the inflammation and the influx of inflammatory cells were analysed during bronchopneumonia. As determined by a histological study, deficiency in C1q was associated with lower severity in pulmonary inflammation early after infection, thus indicating that activation of complement through the classical pathway significantly contributes to the induction of inflammation in lung tissue. Activation of complement results in the release of high levels of chemotactic and anaphylatoxins factors (C3a) and (C5a). These anaphylatoxins could be responsible for inducing the inflammation seen in lungs of control mice (C1q +/+) since a high level of complement C3 is activated in these mice when compared with C1q-deficient mice (C1q -/-) that showed less severe inflammation in the lung.

Surprisingly, the infiltration of neutrophils into the inflamed sites of the lung was not affected by the absence of the classical pathway activation. This could be related to the data presented in section 5.1 for the mutant H2-/C+ and a recent study using purified pneumolysin mutants (Rijneveld *et al.*, 2002), both reported that the cytolytic activity of pneumolysin rather than its complement activation activity is responsible of neutrophils recruitment to the lung. However, a weak accumulation of macrophages and T cells was observed at 48h after infection in lungs of C1q-deficient mice (C1q -/-). High numbers of pneumococci are present in the lungs of (C1q -/-) mice by 48h that could enhance the observed accumulation of inflammatory leukocytes thus leading to a subsequent establishment of a primary adaptive immunity.
These results support the hypothesis that the lack of the classical complement pathway alters host inflammatory response during bronchopneumonia.

To elucidate more directly the effect of the classical complement pathway absence on the total activation of complement pool in the lung during bronchopneumonia, activation products of local complement C3 were quantified. Lung homogenates of control mice (C1q +/+) had significantly greater activation products of complement C3 at 24 and 48h after infection, in comparison to lung homogenate of C1q-deficient mice (C1q -/-). This demonstrates that the activation of complement C3 through the classical pathway is dominant (estimation of the contribution of the classical pathway is 70%) and significantly influences the levels of consumption of lung complement C3 during bronchopneumonia.

Lung complement C3 was slightly activated in C1q-deficient lung homogenates that could be as a result of complement activation through the alternative and lectin pathways. However, previous studies reported that MBL levels are very low in the lung of the mouse (Reading *et al.*, 1997) and that MBL binds poorly to the pneumococcus (Neth *et al.*, 2000). In addition, it has been shown that the teichoic acid of the pneumococcal cell wall is responsible for alternative pathway activation (Winkelstein *et al.*, 1978). Thus, this activation of complement C3 in C1q-deficient mice is probably accounted for the alternative pathway activation rather than the lectin pathway. It is worth mentioning that activation of complement through the alternative pathway will result in the deposition of C3 activation products on the pneumococcal cell wall, thus the polysaccharide capsule of the bacterium is considered to act as a mechanical barrier to

the recognition of fixed complement by phagocytes (Winkelstein 1984). Therefore, the activation of the classical complement pathway is essential for opsonisation of pneumococci and deposition of complement C3b on the capsule (Brown *et al.,* 1983).

Previous studies showed that opsonization of *S. pneumoniae* by complement is a critical determinant for their destruction by phagocytic cells (Winkelstein 1981, Brown *et al.,* 1981, 1985, Gordon *et al.,* 1988, 2000). In the non-immune host, C3 is the primary opsonin for the pneumococcus. Activation of complement will result in a covalent deposition of C3b on the surface of the pneumococcus. Subsequently C3b will be cleaved by serum proteases to smaller fragments such as iC3b and C3d (Hostetter 1986). Interestingly, the C3b cleavage pattern differs among pneumococcal serotypes and is considered as serotype-specific (Hostetter 1986). Data showed that the C3 fragment of serotype 2 strain D39 is iC3b. This latter easily serves as ligand for complement receptor type 3 on the neutrophils (Hostetter 1999), thereby enhancing phagocytosis and clearance of pneumococci.
Although the infiltration of neutrophils was observed in infected lungs of C1q-deficient and control mice, efficient clearance was only seen in control mice in which lungs contained high levels of opsonic fragment iC3b. This indicates that opsonisation of pneumococci with iC3b is a prerequisite for efficient bacterial clearance from lungs and blood of mice during bronchopneumonia. In addition, there is evidence that C1q can trigger the generation of toxic oxygen radicals by neutrophils (Hamada *et al.,* 1988, Tenner and Cooper 1982). This also could contribute to the decreased pneumococcal clearance in C1q-deficient mice.

In the non-immune host, *S. pneumoniae* could activate the classical complement pathway through several mechanisms. The first candidate able to activate the classical complement pathway is pneumolysin. *In vitro* studies demonstrated that low levels of pneumolysin are able to activate the classical complement pathway, in the absence of anti-toxin antibodies, due at least in part to non-specific binding of the toxin to Fc region of human immunoglobulin G IgG (Paton *et al.,* 1984, Mitchell *et al.,* 1991). Similarities in amino-acid sequences were detected between pneumolysin and the human acute-phase reactant C-reactive protein (CRP) that is able to bind to *S. pneumoniae* and also to activate the classical complement pathway in the absence of antibody by binding C1q (Mitchell *et al.,* 1991, Volanakis and Kaplan 1974). Furthermore, immunization of mice

with human CRP provided protection against *S. pneumoniae* infection (Horowitz *et al.*, 1987, Szalai *et al.*, 1996, Mold *et al.*, 2002). Thus, it appears that CRP is an important innate component through the activation of the classical complement pathway but it is unlikely to have a role in the mouse lung during bronchopneumonia due to the low levels of CRP expression. In addition, the major acute-phase reactant in mouse, serum amyloid P (SAP) component fails to protect mice from pneumococcal infection when bound to bacterial pathogens (Yother *et al.*, 1982, Noursadeghi *et al.*, 2000). Another mechanism by which the classical complement pathway could be activated in the non-immune host is through naturally occurring anti-phosphocholine (PC) antibodies. It has been reported that these natural IgM antibodies bind to PC (teichoic acid) on the cell wall C-polysaccharide and then activate complement (Briles *et al.*, 1981, Mold *et al.*, 2002), but these workers also suggested only a minor role for these natural antibodies in the protection from *S. pneumoniae* infection (Mold *et al.*, 2002).

During the writing-up of this thesis and consistent with the presented findings, Brown and co-workers (2002) published data demonstrating that the classical complement pathway provide a critical defence line in the innate immune response to *S. pneumoniae* infection. It was reported after using C1q-deficient mice and secretory IgM-deficient mice, that the activation of the classical complement pathway in the innate immunity requires natural IgM (Brown *et al.*, 2002).
Brown and colleagues reported that the median survival time is 48h for C1q deficient mice after intranasal infection. This thesis reports that 50% of the C1q deficient mice showed signs of illness by 48h after infection. In the study of Brown *et al.* (2002), the recovery of *S. pneumoniae* from lungs and blood of mice was only monitored at 24h after infection and was significantly different between C1q deficient and control mice. Data presented in this thesis report a detailed time course of change in bacterial numbers in lungs and blood of C1q deficient and control mice. The results showed significant differences in CFU between lungs of C1q deficient mice and wild-type control mice at 6 and 48h after infection but in contrast to Brown *et al.* 2002, there was no difference in bacterial numbers at 24h after infection.

In comparison with the work presented in this thesis, the study of Brown and colleagues did not report any histology or immunohistology work on infected lungs. By using flow cytometry, they showed that the percentages of activated macrophages and activated

CD4 lymphocytes in the lungs of C1q deficient mice at 24h were reduced compared with wild-type mice, but Brown *et al.* (2002) did not report any data on the recruitment of neutrophils.

Some of the presented data in the study of Brown and colleagues indicates that the classical activation of complement occurs through several mechanisms and not only through the natural antibodies. For instance, after intranasal challenge with *S. pneumoniae* the mortality rate was high in C1q-deficient mice while it was moderate in IgM-deficient mice. Additionally, after intraperitoneal challenge the numbers of pneumococci in blood and spleen were lower in IgM-deficient mice compared with C1q-deficient mice. However, Brown *et al.* (2002) did not take into account the contribution of pneumolysin in the activation of complement, even a number of studies highlighted the important effects of the complement-activating activity of pneumolysin on the *in vivo* events during pneumococcal pneumonia. The observations described in this thesis, combined with the work of Brown and co-workers (2002), suggest that the classical complement pathway is dominant during the innate immunity to *S. pneumoniae* and its activation is dependent on the toxin pneumolysin and natural antibodies IgM. However, as discussed in 5.2, due to the high levels of C3 activation associated with the presence of pneumolysin, the toxin is strongly implicated in activation of the classical pathway during bronchopneumonia.

In conclusion, activation of the classical complement pathway is critical in the innate immunity of the non-immune host. The deletion of the classical complement pathway resulted in increased susceptibility to pneumococcal infection, deficiency in bacterial clearance from lungs and blood, a less severe inflammation, an altered host inflammatory response and considerable reduction in the activation of complement C3 of the lung. To fully explore the effects of the ability of pneumolysin to activate the classical complement pathway on the innate immunity during bronchopneumonia, it will be ideal to infect C1q-deficient mice with *S. pneumoniae* isogenic strain (PLN-A) unable to produce pneumolysin.

Previous studies reported that mouse strains differ in their susceptibility to the pneumococcus (Briles *et al.*, 1980, Gingles *et al.*, 2001). Phenotypical differences between susceptible (outbred MF1) and resistant (C57BL/6) mice strains are observed

clearly in data presented in this thesis. Bacterial growth kinetics revealed that MF1 mice showed proliferation of *S. pneumoniae* in lungs and blood leading to a moribund status at 48h postinfection, whereas C57BL/6 mice were able to clear pneumococci from their lungs and blood. A number of investigators have indicated that the genetic background of mice provides their susceptibility to pneumococcal infection. To date, the factors that confer susceptibility to MF1 mice and resistance to C57BL/6 mice are not reported.

Gingles and coworkers (2001) identified murine strains resistant (BALB/c) and susceptible (CBA/Ca) to *S. pneumoniae* infection and suggested the association of the susceptibility with recruitment of neutrophils and/ or function of neutrophils. They found a greater inflammatory response and recruitment of neutrophils in BALB/c mice compared to CBA/Ca. Therefore the mechanism of resistance or susceptibility in these mice are probably different from in MF1 and C57BL/6. In these mice, after pneumococcal infection, the histology study showed that inflammatory lesions were more severe in MF1 mice than in C57BL/6 mice and in the lungs, the number of neutrophils at 24h after infection was similar in both strains of mice. However, the infiltration of T lymphocytes into the lung tissue was only observed in MF1 mice.

Studies are in progress in order to identify the host genetic factors that confer resistance or susceptibility to pneumococcal disease in BALB/c and CBA/Ca mice and have localised a major susceptibility locus to a 8 cM region of chromosome 7. However, no obvious candidate genes have been identified yet within this region (P. W. Andrew and P. Denny, unpublished).

## 5.4 The capsule-type is not the individual determinant of agglutination of *S. pneumoniae* by lung surfactant protein D

In the early stage after infection, natural pulmonary defence mechanisms are required for efficient resistance and clearance of the respiratory pathogen *S. pneumoniae*. Recent studies have drawn attention to the important role of lung surfactant protein D expressed in the respiratory tract and other mucosal surfaces (Madsen *et al.*, 2000), in the first-line of innate host immunity and maybe later on to the modulation of the adaptive immunity (Wright 1997, Holmskov 1999, Lawson and Reid 2000, Crouch and Wright 2001). A great deal of *in vitro* studies demonstrated the ability of Sp-D to agglutinate a number of pulmonary pathogens such as *Mycobacterium tuberculosis*, *Klebsiella pneumonaie*,

*Aspergillus fumigatus* and Influenza A virus (Madan *et al.,* 1997, Hartshorn *et al.,* 1994, 1996, Ferguson *et al.,* 2001, Ofek *et al.,* 2001). All these studies agreed that Sp-D interacts with microorganisms via binding of the CRD to carbohydrate structures on the surface of the pathogens leading to different effector mechanisms, such as aggregation or modulation of the microbial uptake by immune cells, thus probably affecting the outcome of microbial invasion (Lim *et al.,* 1994, Kishore *et al.,* 1996, Hakansson *et al.,* 1999).

A previous study reported that highly multimerized Sp-D molecules bound to serotype 4, 19 and 23 *S. pneumoniae*, inducing their agglutination and enhancing their uptake by neutrophils (Hartshorn *et al.,* 1998). Assuming that agglutination of *S. pneumoniae* will have an important role in collecting and limiting the spread of the pathogen through the lung tissue, the interaction between lung surfactant protein D and different strains and serotypes of *S. pneumoniae* was studied. Although carbohydrate was implicated in the binding of Sp-D by other bacteria, no examination of its role in pneumococcal binding has been reported. This thesis reports an investigation of whether the capsule type is a determinant of *S. pneumoniae* aggregation by lung surfactant protein D. To this end, different strains of pneumococci having the same capsule-type or the same multilocus sequence-type were assessed for their binding and agglutination by Sp-D. Moreover, encapsulated and non-encapsulated strains of *S. pneumoniae* were used to investigate whether Sp-D enhanced uptake of different strains of pneumococci by neutrophils.

Data presented in this study show that the recombinant Sp-D (rSp-D), composed of the head/neck of the molecule and three carbohydrate recognition domains, binds to different strains and serotypes of *S. pneumoniae* in the presence of calcium. From these assays, it can be concluded that the extent of binding is not significantly affected by the capsule- or the sequence-type. Notably, the rSp-D expressed in yeast was not effective in binding to *S. pneumoniae* as the rSp-D expressed in *E. coli.* This is probably due to differences in their collagen repeats, amino acid compositions and the degree of multimerization of the head/neck part of the molecule that is higher in the recombinant expressed in the bacteria. Previous studies highlighted the importance of the neck region as a trimerizing agent in bringing together three CRDs and suggested that its multivalency is important, affecting the solid binding of Sp-D to carbohydrate targets

(Kishore *et al.,* 1996). Presented data indicate that the recombinant of the Sp-D molecule is sufficient for binding to the surface of the pneumococcus.

Agglutination of microorganisms is considered to be an important innate host defence mechanism. Results show that in the presence of calcium, full length Sp-D molecule causes agglutination of *S. pneumoniae.* This is consistent with a previous study (Hartshorn *et al.,* 1998). However, the different strains and serotypes were not agglutinated to the same extent by Sp-D. In addition, as have been recently reported for *K. pneumoniae* (Ofek *et al.,* 2001), the pneumococcal agglutination was effectively inhibited by maltose as a competitive sugar. Consistent with previous studies (Kuan *et al.,* 1992, Madan *et al.,* 1997), agglutination of *S. pneumoniae* by Sp-D was inhibited in the presence of EDTA. These results indicate that Sp-D molecule, via its calcium-dependent C-type lectin domains binds to carbohydrate structures present on the pneumococcal surface. Recombinant Sp-D, lacking the collagen domain, does not agglutinate *S. pneumoniae.* This is may not be surprising giving that previous studies suggested that the long, rigid structure of the Sp-D collagen domain is essential and allows better linking and interactions between the agglutinated bacteria (Lu *et al.,* 1993, Crouch *et al.,* 1994).

Interestingly, although binding of rSp-D was detected for all pneumococcal strains studied not all of them were agglutinated by full length Sp-D. It is tempting to speculate that the first step of binding occurs but does not necessarily lead to the agglutination of bacteria. Furthermore, in binding assays, dead bacteria were used, while in the agglutination studies whole alive bacteria in suspension were utilized. It is possible to suggest that Sp-D has access to carbohydrate groups on the pneumococcal surface that are not available for binding and/or agglutination when the pneumococci are in suspension. For instance, human CRP binds more avidly to killed than to live *S. pneumoniae* (de Beaufort *et al.,* 1997).

Studies on several strains and switched serotypes of *S. pneumoniae* reported in this thesis, show no correlation between the aggregating effect of Sp-D and the capsule or the multilocus sequence-type of the pneumococcus. It is possible to conclude that the capsule-type and/or the multilocus sequence-type of the pneumococcus are not the sole determinants of agglutination of *S. pneumoniae* by lung Sp-D. Probably, it is a combination of factors specific for each pneumococcal strain.

Using a solid phase-based binding assay, van de Wetering and colleagues (2001) showed that two major cell wall components of Gram-positive bacteria, lipoteichoic acid (LTA) of *Bacillus subtilis* and peptidoglycan (PepG) of *Staphylococcus aureus* were bound by Sp-D in a calcium-dependent and carbohydrate-inhibitable manner. Thus, it is tempting to suggest that binding of Sp-D to the pneumococcal surface could occur on the capsule consisting of repeating oligosaccharide as well as on the cell wall consisting of LTA and PepG. For instance, encapsulated serotype 2 and non-encapsulated mutant Rx1 were agglutinated by Sp-D.

Additionally, the data show that the interaction between Sp-D or rSp-D with capsulated and non-encapsulated *S. pneumoniae* did not enhance killing of pneumococcus by neutrophils. It can be concluded that Sp-D is not an opsonin for *S. pneumoniae* with neutrophils. This may not be surprising given that to date no receptors for Sp-D on neutrophils have been described. However, it was previously reported using flow cytometry that Sp-D enhances uptake of *S. pneumoniae* by neutrophils (Hartshorn *et al.*, 1998). Several publications studied the interaction between Sp-D and neutrophils. Crouch and colleagues (1995) reported that Sp-D binds and activates a chemotactic response in neutrophils and other groups demonstrated that Sp-D enhances the neutrophil uptake of various pathogens. For example, Influenza A virus (Hartshorn *et al.*, 1994, 1997), *E. coli*, and *Staphylococcus aureus* (Hartshorn *et al.*, 1998). Sp-D also has been reported to promote the oxidative burst of neutrophils in response to *Aspergillus fumigatus* conidia (Madan *et al.*, 1997). In addition, Sp-D enhances phagocytosis of *P. aeruginosa* and *K. pneumoniae* by alveolar macrophages (Restrepo *et al.*, 1999, Ofek *et al.*, 2001) and inhibits phagocytosis of *P. carini* (Vuk-Pavlovic *et al.*, 1998), *C. albicans* (van Rozendaal *et al.*, 2000) and *M. tuberculosis* by alveolar macrophages (Ferguson *et al.*, 2002). Thus, Ferguson and colleagues (2002) suggested that Sp-D is not simply a non-specific host defence protein since it can inhibit or enhance interaction between microorganisms and host cells.

The concentration of Sp-D in broncho-alveolar lavage fluid isolated from healthy volunteers is 1 μg/ml (Honda *et al.*, 1995) and is expected to be extremely diluted compared to the concentration found in the airways. Furthermore, Sp-D concentration showed an increase in several lung diseases indicating that Sp-D could be used as a disease marker. For instance, an increase of 20% in the concentration of Sp-D was

detected in serum of pneumonia patients (Honda *et al.,* 1995). Thus it is possible that Sp-D binding and aggregation of *S. pneumoniae* were detected *in vitro* at concentrations comparable to their physiological concentration in the host and specially in case of infection.

*In vitro* studies presented in this thesis implicated a role for lung surfactant D in the innate immunity against the pneumococcus. The physical mechanism of agglutination of the pneumococcus may have an important role *in vivo* in limiting the spread of the bacteria through the lung tissue and enhancing mucociliary clearance and binding of pathogens to phagocytes resident within the lung. Furthermore, recent studies demonstrated that Sp-D has a greater ability than MBL or conglutinin in binding to *S. pneumoniae.* A chimeric collectin consisting of the Sp-D molecule in which the neck and CRD were replaced with those of MBL, bound to live *S. pneumoniae* type 23 more intensely than native MBL (Hartshorn *et al.,* 2002). It is likely that the formation of large pneumococcal aggregates by Sp-D may immobilize the pathogen in the lung and then alveolar macrophages and marginating neutrophils can detect the aggregates and migrate to the site of infection. Gp-340 molecule existing in a soluble form and in a form associated with alveolar macrophages, binds to Sp-D in a calcium-dependent manner and has been suggested as a putative receptor for Sp-D (Holmskov *et al.,* 1997).

In summary, *in vitro* findings show that lung surfactant protein D interacts with *S. pneumoniae*, inducing agglutination of several strains and serotypes of the pneumococcus in the presence of calcium. Inhibition of the interaction was observed in the presence of a competitive saccharide thus indicating that Sp-D binds to carbohydrate structure on the pneumococcal surface. The physical effect of aggregation of *S. pneumoniae* by lung surfactant protein D is not only dependent on the capsule-type or the multilocus sequence-type but most likely is due to a combination of factors specific to each pneumococcal strain. Sp-D does not enhance killing of *S. pneumoniae* by neutrophils.

Finally, these *in vitro* observations strongly implicate lung surfactant protein D as playing an important role in the host innate immunity during pneumococcal infections.

## 5.5 Increased susceptibility of Sp-D deficient mice to *S. pneumoniae*

The generation of Sp-D knockout mice allowed an interesting investigation of the potential role of Sp-D in host defence along with the assessment of the physiological importance of its activities. Thus far, Sp-D deficient mice have been infected with *H. influenzae*, group B streptococcus and Influenza A virus (IAV). After intratracheal instillation of bacteria, Sp-D deficient mice showed no decrease in bacterial killing in lung tissue but increased inflammation, cellular recruitment, oxidant production and decreased macrophage phagocytosis (LeVine *et al.*, 2000). However, after intranasal infection with IAV, Sp-D deficient mice showed decreased viral clearance and uptake by alveolar macrophages and increased production of inflammatory cytokines in response to viral challenge (LeVine *et al.*, 2001). Although there is *in vitro* evidence that Sp-D interacts with *S. pneumoniae*, its role in the innate host defence mechanisms during pneumococcal infection had not been investigated. To this end, Sp-D deficient mice were infected intranasally with *S. pneumoniae*. Pneumococcal growth kinetics in nasopharynx, trachea, lungs and blood, histology of the development of the inflammation and host immune cells infiltration into the lung were compared in Sp-D -/- and Sp-D +/+ mice during bronchopneumonia.

Results presented in this thesis support a role for Sp-D in the innate host defence to *S. pneumoniae* and are the first to show an increased susceptibility to bacterial infection in Sp-D deficient mice. Removal of Sp-D affected bacterial clearance from the respiratory tract. Early after infection (6 and 24h), a reduction in pulmonary clearance of intranasally administered *S. pneumoniae* was observed in Sp-D deficient mice. Furthermore, an interesting finding is that the absence of Sp-D was associated with a defect in the ability of mice to clear pneumococci from the trachea. In contrast to Sp-D-/- mice, wild-type mice cleared efficiently *S. pneumoniae* from their trachea by 48h after infection. However, neither Sp-D -/- or Sp-D +/+ mice were able to clear pneumococci from their nasopharynx. These results indicate that lung surfactant protein D has a crucial role in the early clearance of *S. pneumoniae* from the lungs and from the trachea of mice during bronchopneumonia.

The effect of Sp-D on the bacterial clearance from the trachea may not be surprising given that Sp-D is synthesised and secreted not only by pulmonary epithelial cells but also by epithelial cells and submucosal glands of the trachea of the adult mouse (Wong *et al.*, 1996). Sp-D was also detected at low concentration (56 ng/ml) in nasopharyngeal

washings of normal mice (Reading *et al.*, 1997), but results show that Sp-D does not have a role at this site during pneumococcal infection.

The absence of Sp-D affected the onset and the levels of bacteraemia during bronchopneumonia. Mice lacking Sp-D showed an early timing of bacteraemia, at 6h after intranasal infection, whereas the pneumococci appeared only at 24h after infection in blood of wild-type mice. In addition, the numbers of pneumococci were significantly high in blood of Sp-D -/- mice compared to Sp-D +/+ mice, which cleared the bacteria by 48h after infection. It is worth mentioning that the numbers of pneumococci in the blood reflected their levels in the lung. These results indicate that lung surfactant protein D plays a crucial role in delaying the appearance of pneumococci in the blood and in limiting their numbers in the bloodstream.

The local production of Sp-D in the respiratory tract, which is the natural portal of entry of *S. pneumoniae*, implicates this collectin as having an important role in the first-line of host defence. As discussed in section 5.5, Sp-D binds and agglutinates *S. pneumoniae* in the presence of calcium and thus may enhance mucociliary and phagocytic clearance. In addition, binding of Sp-D to lipoteichoic acid of Gram-positive bacteria (van de Wetering *et al.*, 2001), an important mediator of adherence of epithelial cells, suggests a role for Sp-D in preventing bacterial colonisation of the alveolar epithelium thus enhancing host defence independent of phagocytosis. All these suggestions could be reasons for the decreased pneumococcal clearance from lungs, trachea and blood and the early onset of pneumococcal septicaemia observed in Sp-D deficient mice.

As determined by the histology study, absence of Sp-D was associated with greater inflammation in the lungs. After pneumococcal infection, the inflammation was greatly increased by 24h in the lungs of Sp-D deficient mice, in comparison with wild-type control in which less cellular infiltration into the lung was observed. This result is consistent with previous studies that also showed an increased inflammation in Sp-D -/- mice after viral or bacterial challenge, as indicated by increased total cell counts and pro-inflammatory cytokines IL-6 and TNF in the lung (LeVine *et al.*, 2000, 2001). The increase in cytokine production may be the reason for increased perivascular and peribronchial cellular infiltration observed in lungs of Sp-D-/- mice. Thus, it appears that Sp-D plays an anti-inflammatory role *in vivo*.

As reported for other strains of mice, pneumococcal infection was coupled with an influx of inflammatory immune cells into the lung tissue of Sp-D -/- mice. Recruitment of neutrophils into infected lung tissue was not affected by the absence of Sp-D. Although Sp-D has been reported as chemotactic for neutrophils *in vitro* (Crouch *et al.*, 1995), data demonstrate that Sp-D is not a critical determinant for neutrophil chemotaxis *in vivo*. Although a similar accumulation of neutrophils was observed in the lungs of both strains of mice by 24h after infection, pneumococcal killing was decreased only in Sp-D -/- mice. This could be the result because of the deficiency in binding of the pneumococcus by Sp-D as well as to other reasons related to the neutrophil's activity.

Consistent with the presented data, LeVine and colleagues (2000, 2001) also showed that neutrophil accumulation was similar in the lungs of Sp-D -/- and wild-type mice after bacterial infection and even greater in Sp-D -/- mice after viral infection. For instance, workers suggested that the impairment of viral clearance in Sp-D -/- mice is attributed to the decrease in neutrophil myeloperoxidase (MPO) activity (LeVine *et al.*, 2001).

In addition, Sp-D deficient mice, despite their healthy appearance, develop progressive alveolar proteinosis and emphysema and have increased numbers of foamy alveolar macrophages (Korfhagen *et al.*, 1998, Botas *et al.*, 1998). Thus, it is possible to suggest that the lipid excess in Sp-D -/- lungs may inhibit the neutrophil respiratory burst, as demonstrated *in vitro* (Ahuja *et al.*, 1996).

Furthermore, previous studies reported that Sp-D inhibits T lymphocyte proliferation and local response *in vitro* and demonstrated accumulation of peribronchial and perivascular CD4 and CD8 T lymphocytes in lungs of Sp-D -/- mice in the absence of an infection (Borron *et al.*, 1998, Fisher *et al.*, 2002). The presented data show that the levels of T lymphocytes were significantly higher in Sp-D deficient mice before and after the infection in comparison with wild-type control mice. Interestingly, in contrast to wild-type control mice, lungs deficient in surfactant protein D showed heavy CD3 T lymphocyte infiltration into the inflamed areas at 24h after pneumococcal infection. This finding indicates that Sp-D affected the accumulation of T cells in response to pneumococcal infection. Thus, an immuno-regulatory role for Sp-D could be suggested.

The physiological concentration of surfactant protein D in the lavage fluid of C57BL/6 aged 6-8 weeks old is about 552 ng/ml (Reading *et al.,* 1997), which is greatly more concentrated in the alveolar spaces and most likely will increase rapidly after the intranasal infection. Several studies showed that surfactant protein D levels increase in many diseases thus implicating this protein as a useful biomarker in various lung diseases in humans and in rats (Honda *et al.,* 1995, Pan *et al.,* 2002).

Interesting recent studies showed that Sp-D deficient mice have 5- to 10- fold increase in the number of apoptotic and necrotic alveolar macrophages compared to the wild-type mice thus workers suggest a contributory role for Sp-D in immune homeostasis by recognising and promoting removal of apoptotic cells *in vivo* (Vandivier *et al.,* 2002, Clark *et al.,* 2002). It would be interesting to assess the clearance of infected apoptotic neutrophils during pneumococcal infection in Sp-D deficient and sufficient mice.

The potential of truncated recombinant forms of surfactant protein D as a novel therapy for infectious and inflammatory diseases has been reported in several studies. Treatment by intranasal administration of Sp-D and a 60-KDa recombinant of Sp-D (rSp-D) has a good protective effect in a murine model of fungal infection and allergy caused by an opportunistic pathogen, *Aspergillus fumigatus* (Madan *et al.,* 2001). The survival rate of mice increased to 60 and 80 % after treatment with Sp-D and rSp-D respectively (Madan *et al.,* 2001). In addition, intrapulmonary administration of rSp-D reduces the number of apoptotic and necrotic alveolar macrophages and partially corrects lipid accumulation of Sp-D -/- mice (Clark *et al.,* 2002). Thus, it would be of a great interest to investigate whether the co-administration of Sp-D or rSp-D with *S. pneumoniae* would correct the defects observed in Sp-D deficient mice during pneumococcal bronchopneumonia. Also, administration of Sp-D after infection has begun, will indicate at what times in the disease process this protein is most heavily involved.

In summary, the absence of lung surfactant protein D increased the susceptibility to pneumococcal infection and was associated with decreased pneumococcal clearance from lungs, trachea and blood, early onset and increased levels of bacteraemia. In addition, the inflammation in the lungs was more severe in Sp-D -/- mice, suggesting a role for Sp-D in modulating the inflammation during bronchopneumonia. Accumulation of neutrophils was unchanged in the absence of Sp-D, however, T cell recruitment was

detected in lungs of Sp-D-/- mice suggesting that Sp-D alters T cells response during pneumococcal infection. Finally, lung surfactant protein D plays an immediate role in the host innate defence mechanisms to inhaled *S. pneumoniae*.

## 5.6 Ability of gp-340, a putative receptor of lung surfactant protein D, to bind and agglutinate *S. pneumoniae*

Accumulating data suggested a direct involvement of gp-340 in innate immunity. Glycoprotein-340, found in soluble form in human lung washings and associated with the membranes of alveolar macrophages, binds specifically to lung surfactant protein D in a protein-protein interaction (Holmskov *et al.*, 1997, 1999). Thus, gp-340 is suggested to be a putative opsonin receptor for Sp-D (Holmskov *et al.*, 1999). More recent studies demonstrated that a human salivary agglutinin isolated from human parotid saliva is identical to gp-340. This agglutinin is able to interact with Sp-D and to bind and aggregate bacteria such as *Helicobacter pylori* and *Streptococcus mutans* (Prakobphol *et al.*, 2000, Ligtenberg *et al.*, 2001). However, the interaction between gp-340 and *S. pneumoniae* has not been reported.
Due to the presence of gp-340 in the respiratory tract and its aggregating effect on microorganisms, it was interesting to study its interaction with the respiratory pathogen *S. pneumoniae*, independently of Sp-D.

Data presented in this thesis show that human gp-340, isolated from the broncho-alveolar lavage fluid, is able to bind and agglutinate several strains of *S. pneumoniae* in the presence of calcium. The extent of binding was not different between the different strains having different capsule type or multi-locus sequence type. This indicates that the capsule or the sequence type of the pneumococcal strain by themselves do not affect the binding of gp-340 to the pneumococcus. Furthermore, pneumococcal surface protein C (PspC), a surface protein of *S. pneumoniae* that is able to bind secretory IgA, C3 and complement factor H (Hammerschmidt *et al.*, 1997, Cheng *et al.*, 2000, Janulczyk *et al.*, 2000), was considered as a ligand for gp-340. Strains possessing and others lacking PspC were used in this study. Data show that the extent of binding of gp-340 to the surface of the pneumococcus was not affected by the absence of PspC. Thus, it can be concluded that binding of gp-340 to *S. pneumoniae* is not dependent on PspC presence.

However, although gp-340 was able to bind to *S. pneumoniae*, the protein did not aggregate all the pneumococcal strains but it was able to aggregate encapsulated and non-encapsulated strains of *S. pneumoniae*. These results indicate that the presence of the capsule is not a pre-requisite for the binding and aggregating effect of gp-340 on the pneumococcus. Observations with the switched serotypes indicated that the capsule-type or sequence-type is not a determinant for aggregation of *S. pneumoniae* by gp-340. Thus, the surface properties of the pneumococcus that determine its interaction with gp-340 remain to be discovered.

There is no extensive data showing which structure of the gp-340 molecule is binding to the surface of bacteria. It has been reported that the salivary agglutinin similar to gp-340 binds to the surface protein antigen (PAc) of *S. mutans* (Oho *et al.,* 1998). Another recent study demonstrated that only one of the fourteen Scavenger receptor cysteine rich domains of the agglutinin, is able to bind and remarkably to agglutinate *S. mutans* (Bikker *et al.,* 2002).

The interaction between *S. pneumoniae* and gp-340, found in the lungs, trachea and salivary glands (Holmkov *et al.,* 1999), suggests a role for this protein in limiting the spread of the bacteria through lung tissue and in clearing this respiratory pathogen. It is worth mentioning that gp-340 stimulates random migration of alveolar macrophages (Tino and Wright 1999). Thus, gp-340 could enhance the interaction between the pneumococcus and the host immune cells.

Furthermore, without the presence of Sp-D, gp-340 is able to interact with *S. pneumoniae* thus indicating that this molecule may have a role in pneumococcal clearance alone or in combination with Sp-D, either through the mucosal defence mechanisms (ciliated epithelial cells) or by increased presentation to phagocytic cells. A cooperative action between Sp-D and soluble form of gp-340 in the defence against microorganisms has been shown recently in a viral model of aggregation and neutralization (K. Hartshorn, University of Boston, Personal communication).

Investigators also demonstrated that gp-340, like Sp-D, is located at different mucosal surfaces, for example, human salivary glands, small and large intestine (J Madsen, University of Southern Denmark, Personal communication), suggesting a wide-ranging role for these proteins. A recent study demonstrated the presence of gp-340 in epithelial

cell lines and supposed the involvement of this molecule not only in the innate immunity but also in epithelial growth (Kang *et al.*, 2002).

In conclusion, in the presence of calcium and independently of lung surfactant protein D, human gp-340 is able to bind to the pneumococcus and induces agglutination of several strains of *S. pneumoniae.* The presence of the capsule and/or the pneumococcal surface protein C is not a pre-requisite for gp-340 binding to the surface of the pneumococcus. In addition, the capsule or sequence-type of the pneumococcal strain is not a determinant for its aggregation by gp-340. By its interaction with the pneumococcus, gp-340 is predicted to have a role in the innate immunity to this pathogen. A further understanding of the interaction between gp-340 and *S. pneumoniae* at the structural level is required. Also, it will be interesting in future experiments to investigate the effect of this interaction on *in vivo* events during pneumococal infection.

## 5.7 Upregulation of Sp-D and CRP-ductin in the mouse lung during bronchopneumonia

Several studies have implicated Sp-D as a disease marker and as a useful indicator of lung injury in humans and rats (Honda *et al.*, 1995, Pan *et al.*, 2002). Investigators also demonstrated that the levels of expression of Sp-D were increased in murine models of allergic pulmonary inflammation, Influenza A virus infection and *Helicobacter pylori*-associated gastritis (Haley *et al.*, 2002, LeVine *et al.*, 2001, Murray *et al.*, 2002). Furthermore, immunohistochemistry studies showed that the expression of gp-340 was clearly enhanced in inflamed colon epithelial cells of patients with inflammatory bowel disease as compared with normal colon tissue (J. Madsen, University of Southern Denmark, personal communication).

CRP-ductin is the mouse homologue of gp-340, not only at the protein structure level but also at the genomic level (Cheng *et al.*, 1996, Holmskov *et al.*, 1999, Mollenhauer *et al.*, 1999). Mainly synthesised in the pancreas, CRP-ductin is also produced by epithelial cells at different body locations (Cheng *et al.*, 1996). Furthermore, recent work demonstrated that mouse CRP-ductin, like human gp-340, is able to bind to lung surfactant protein D and to agglutinate different Gram-positive (*S. aureus, S. mutans*) and Gram-negative (*H. influenzae, K. oxytoca*) bacteria (J. Madsen, personal communication). Thus, a role for CRP-ductin in the innate immunity of the mouse has

been suggested. Considering the *in vitro* results of the interaction between gp-340 and the pneumococcus and the presence of Sp-D and CRP-ductin in the mouse lung, it was of great interest to investigate whether the expression of these proteins increases in the lung tissue during pneumococcal bronchopneumonia.

RT-PCR analysis of lung RNA from MF1 mice infected intranasally with *S. pneumoniae* gave a strong signal for CRP-ductin and Sp-D mRNA in the lung by 24 and 48h after infection, compared to time zero. This indicates that the expression of the genes for these two proteins increases in the lung of MF1 mice after pneumococcal infection and suggests that protein levels also will be increased.

However, data also indicated that only the expression of CRP-ductin and not Sp-D increases in the lungs from pneumococci infected C57Bl/6 mice. This may reflect the situation that MF1 mice had a high numbers of bacteria in lungs and blood and were lethargic by 48h after infection whereas C57BL/6 mice virtually cleared the bacteria by 48h after infection.

In conclusion, lung surfactant protein D and CRP-ductin genes are upregulated in the lung during bronchopneumonia thus strongly supporting a role for these proteins in the innate immunity to the pneumococcus in mice. Further investigations are required to characterize the individual role of CRP-ductin and its cooperation with lung surfactant protein D in the innate immune defence mechanisms to *S. pneumoniae*.

## 5.8 Summary

Briefly, the results of this thesis reveal the essential contribution of complement and lung surfactant protein D to innate immunity during *S. pneumoniae* infection.

The effects of the ability of pneumolysin to activate the classical complement pathway on *in vivo* events were studied. The use of the isogenic mutant strain H+/C- showed that lack of complement activation activity of pneumolysin affected pneumococcal behaviour and host response during bronchopneumonia. The absence of the ability of pneumolysin to activate complement was associated with increased bacterial growth in the lung in the first twelve hours after infection, early bacteraemia, less severe inflammation in lung tissue by 24h and absence of T lymphocyte recruitment into the inflamed sites of the lung. In addition, complement C3 was substantially activated in the lung after intranasal infection with wild-type *S. pneumoniae*, in contrast with the situation after infection with

the isogenic mutant strain (PLN-A) unable to produce pneumolysin. C1q-deficient mice, lacking the classical complement pathway, showed increased susceptibility to pneumococcal infection. Defects in pneumococcal clearance from lungs and blood associated with pneumonia and severe septicaemia were observed in C1q deficient mice.

*In vitro* and *in vivo* studies reported in this project strongly implicated lung surfactant protein D as having a role in the host innate immunity to the pneumococcus.
Lung surfactant protein D and its receptor gp-340 are able to bind and agglutinate several strains of *S. pneumoniae*. The capsule- or the multilocus sequence-type is not a determinant for aggregation of the pneumococcal strain by Sp-D or gp-340. Furthermore, the presence of the capsule is not a pre-requisite for these interactions.
Sp-D deficient mice showed increased susceptibility to pneumococcal infection associated with reduced bacterial clearance from the lungs, trachea and blood, early onset of bacteraemia, increased inflammation and dramatic infiltration of T lymphocytes into the lung by 24h after infection. Furthermore, Sp-D and gp-340 were found to be upregulated in the lung during pneumococcal bronchopneumonia.
Thus these observations indicate an immediate role for lung Sp-D in the clearance of inhaled *S. pneumoniae* and control of the lung inflammation.

Further study investigating whether wild-type Sp-D or recombinant Sp-D has therapeutic potential in *S. pneumoniae* infection, is of great interest. This novel therapy may be important particularly in the neonatal period, in non-immune hosts and in states of impaired immune functions.

Evidence arising from this project demonstrates that the complement system and lung surfactant protein D are important components of the pulmonary innate immunity to pneumococcal challenge. It would be interesting to investigate the contribution of other innate immune factors, such as surfactant protein A and MBL, to host resistance during pneumococcal infection.

# Chapter 6: References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# Pneumococcal Behavior and Host Responses during Bronchopneumonia Are Affected Differently by the Cytolytic and Complement-Activating Activities of Pneumolysin

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**Pneumolysin, a multifunctional toxin produced by all clinical isolates of *Streptococcus pneumoniae*, is strongly implicated in the pathogenesis of pneumococcal bronchopneumonia and septicemia. Using isogenic mutant strains, we examined the effect of deletion of the cytotoxic activity or complement-activating activity of pneumolysin on bacterial growth in lungs and blood, histological changes in infected lung tissue, and the pattern of inflammatory cell recruitment. Both of the activities of pneumolysin contributed to the pathology in the lungs, as well as the timing of the onset of bacteremia. Histological changes in the lungs were delayed after infection with either mutant compared to the changes seen after infection with the wild-type pneumococcus. The complement-activating activity of pneumolysin affected the accumulation of T cells, whereas the toxin's cytolytic activity influenced neutrophil recruitment into lung tissue.**

Previous studies in our laboratory showed that the pneumococcal toxin pneumolysin has a crucial role in the pathogenesis of pneumococcal bronchopneumonia and septicemia (7, 12). Other studies showed that pneumolysin alone can reproduce the symptoms of pneumococcal disease in the lungs (8), and inactivation of the pneumolysin gene of *Streptococcus pneumoniae* resulted in an avirulent mutant (5). Subsequent studies showed that mutant pneumococci unable to produce pneumolysin generated much less inflammation, delayed bacteremia, limited multiplication of bacteria within the lungs (7, 12, 18), and a different pattern of host inflammatory cell infiltration into the lung tissue (12) compared with wild-type pneumococci.

Several pieces of evidence showed that pneumolysin is cytolytic to all eukaryotic cells tested, probably as a result of pore formation in target membranes, and at sublytic concentrations it alters normal functioning of immune cells (9, 17) (for example, by inhibiting human neutrophil and monocyte respiratory burst and chemotaxis [15]). In addition, low levels of the toxin can activate the complement pathway in human serum in the absence of antipneumolysin antibody (13, 16), as well as stimulate production of other host inflammatory molecules (10).

Site-directed mutagenesis showed that distinct parts of the toxin molecule are responsible for pore formation and the accompanying cytolytic and anticellular activities and for complement activation (6, 14). The site-directed mutagenesis results allowed production of pneumococcal strains that make pneumolysin which lacks one of its wild-type activities. By using these strains, it was shown previously that both anticellular (pore formation) and complement-activating activities of pneumolysin contributed to virulence during pulmonary infection following intratracheal challenge in mice (2, 19). Complement-activating activity was associated with bacterial growth in lung tissue and blood at 24 h after pulmonary infection, while the cytotoxic activity correlated with impairment of the alveolar capillary barrier and an increase in the number of bacteria during the first 6 h of infection (19). A model of pneumococcal bronchopneumonia following intranasal challenge in which complement-activating activity and anticellular activity of pneumolysin have discrete roles has also been described (2). The previous studies also suggested that a third activity contributes to virulence (2), an observation that seemed to be confirmed by recent studies (3) which showed that a fragment of pneumolysin that lacked cytolytic and complement-activating regions was able to induce gamma interferon release.

In this study we extended a previous study (2) by investigating the mechanisms by which the activities of pneumolysin individually influence in vivo events. Below we describe the histological changes and the host cellular immune response to pneumococci producing modified pneumolysin in a murine model of bronchopneumonia and septicemia. The following two isogenic mutant strains producing pneumolysins with specific amino acid substitutions that modify the toxin activity were used: strain H+/C−, carrying a point mutation (Asp385 → Asn) which results in a pneumolysin that lacks complement-activating activity but has complete anticellular activity; and strain H2−/C+, carrying a point mutation (His367 → Arg) which results in pneumolysin that has only 0.02% of the anticellular activity associated with pore formation but has 100% of the complement-activating activity (2).

## MATERIALS AND METHODS

**Pneumococcal strains.** Three isogenic strains of *S. pneumoniae* were used. The wild-type strain was serotype 2 strain D39 (= NCTC 7466 [National Collection

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of Type Cultures, London, United Kingdom]). The mutant strains were H+/C− and H2−/C+, which had single point mutations (Asp385 → Asn and His367 → Arg, respectively) in the chromosomal pneumolysin gene (2). The pneumococci were grown on blood agar base containing 5% (vol/vol) horse blood or in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) containing 20% (vol/vol) fetal bovine serum (Gibco, Paisley, United Kingdom) supplemented with 1 mg of erythromycin (Sigma, Poole, United Kingdom) per ml for the mutant strains.

Before use in vivo, *S. pneumoniae* was passaged through the peritoneal cavities of mice and then stored at −70°C (5). Pneumococci can be stored for at least 3 months at −70°C with no significant loss of viability. When needed, an aliquot was thawed at room temperature, and bacteria were harvested by centrifugation before resuspension in sterile phosphate-buffered saline (PBS) (7).

The Gram reaction, Quellung reaction, and optochin sensitivity of all strains were confirmed prior to use.

**Intranasal challenge of mice.** Female MF1 outbred mice weighing 30 to 35 g were obtained from Harlan Olac, Bicester, United Kingdom. At the start of the experiment the mice had no detectable levels of antitype antibodies. Mice were each infected intranasally with $1 \times 10^6$ *S. pneumoniae* CFU, as described previously (12). At predetermined times following infection, groups of mice were deeply anesthetized with 5% (vol/vol) fluothane (Astra-Zeneca, Macclesfield, United Kingdom), and blood was collected by cardiac puncture. Subsequently, the mice were killed by cervical dislocation, and the lungs were removed, placed into 10 ml of sterile distilled water, weighed, and then homogenized with a Stomacher-Lab blender (Seward Medical, London, United Kingdom). Viable counts in homogenates and blood were determined by serial dilution in sterile nanopure water and plating onto blood agar plates (Oxoid) supplemented with 5% (vol/vol) horse blood.

**Histology.** At the predetermined times following infection as described above, whole-lung samples were excised, embedded in Tissue-Tec OCT (Sakura), and frozen in liquid nitrogen with an isopentane heat buffer to prevent snap freezing and tissue damage. The samples were stored at −70°C. One day before sectioning, the lungs were transferred to storage at −20°C. Sections (thickness, 10 to 20 μm) were cut at −18 to −25°C with a Bright cryostat and then allowed to dry at room temperature. Once dried, the sections were stained with hematoxylin and eosin and subsequently fixed with DPX mountant (BDH) for permanent storage.

**Immunohistochemistry.** Leukocyte recruitment into lung tissue was analyzed by an alkaline phosphatase anti-alkaline phosphatase staining method (11). Sections were fixed with acetone for 10 min at 4°C, air dried, and washed in PBS for 5 min. Normal rabbit serum, diluted 1:5 in PBS, was overlaid onto each section. The excess serum was tapped off, and 50-μl portions of rat anti-mouse monoclonal antibodies to T cells (pan-T-cell marker CD3), B cells (pan-B-cell marker CD19), macrophages (F/480), or neutrophils (7/4) (Serotec, Oxford, United Kingdom), all previously diluted 1:50 in PBS, were layered onto the sections. Four whole lungs collected at predetermined times were sectioned completely, and 20 sections from each lung were used for each antibody tested. After incubation for 30 min, the sections were washed for 5 min in PBS and incubated with 50 μl of rabbit anti-rat antibody (1:25; Dako, Glostrup, Denmark) for 30 min at room temperature. A 5-min wash was followed by addition of rat alkaline phosphatase anti-alkaline phosphatase monoclonal antibody diluted 1:50 in PBS. Each preparation was washed again with PBS before addition of the substrate, fast red, and levamisole to inhibit endogenous phosphatase activity. After 20 min of incubation with the substrate, the sections were washed, counterstained briefly with hematoxylin, and finally washed in tap water. The sections were mounted in aqueous mounting medium (DPX mountant). Once stained, the positively stained cells in the vicinity of inflamed bronchioles were enumerated. Sections from throughout the lung were taken, and at least 10 sections per lung were analyzed.

**Statistical analysis.** Data were analyzed by analysis of variance, followed by the Bonferroni test. Statistical significance was defined as a $P$ value of <0.05.

## RESULTS

**Growth of wild-type and mutant pneumococci in lung tissue and blood.** There were clear differences in the growth curves of the wild-type and mutant strains (H+/C− and H2−/C+) in the lungs (Fig. 1) and in the blood (Fig. 2). The number of H+/C− pneumococci in the lungs remained constant for 24 h postinfection, in contrast to the number of wild-type pneumococci, which declined sharply during the first 12 h postinfection ($P <$ 0.01, compared to the zero-time value) (Fig. 1). After this, the

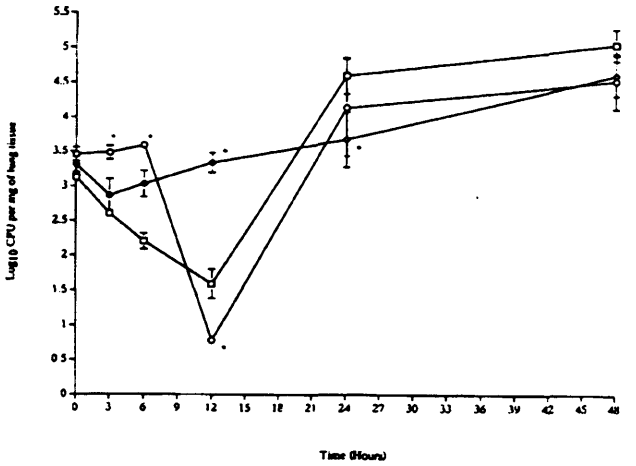


FIG. 1. Time courses for changes in the numbers of the *S. pneumoniae* wild-type (□), H+/C− (◇), and H2−/C+ (○) strains in the lungs of MF1 mice infected intranasally with $10^6$ CFU ($n$ = 10 for each time point). The error bars indicate standard errors of the means. An asterisk indicates that the $P$ value is <0.05 for a comparison of the H+/C− or H2−/C+ mutant with the wild-type strain.

wild-type levels increased, and by 48 h postinfection the numbers of the wild-type and H+/C− pneumococci were the same. The ability of pneumolysin to activate complement influenced the timing of bacteremia after intranasal infection. The wild-type pneumococci were detected in the blood at 12 h after infection, while the H+/C− pneumococci were detected earlier, at 6 h after infection, but after 24 and 48 h there were no differences ($P > 0.05$) in the numbers of the wild-type and H+/C− bacteria in the blood (Fig. 2).

The growth of H2−/C+ pneumococci in lungs and blood also was assessed. In the lungs, the growth curve for H2−/C+

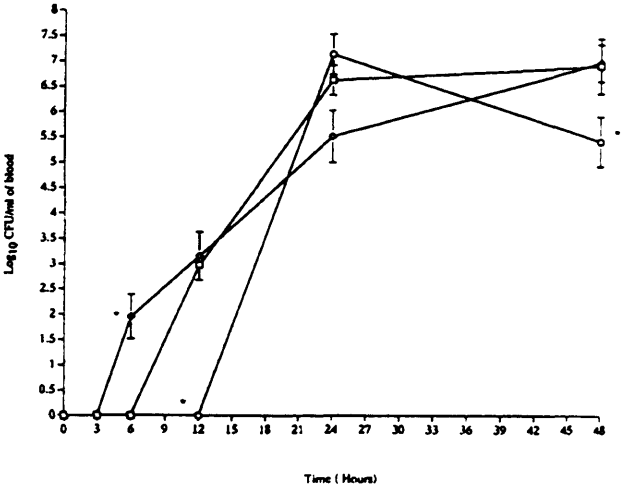


FIG. 2. Time courses for changes in the numbers of the *S. pneumoniae* wild-type (□), H+/C− (◇), and H2−/C+ (○) strains in the blood of MF1 mice infected intranasally with $10^6$ CFU ($n$ = 10 for each time point). The error bars indicate standard errors of the means. An asterisk indicates that the $P$ value is <0.05 for a comparison of the H+/C− or H2−/C+ mutant with the wild-type strain.

showed a sharp decline between 6 and 12 h postinfection ($P < 0.001$, compared to the zero-time value), and this was followed by notable bacterial multiplication during the following 12 h ($P < 0.05$ for 24-h data versus 12-h data) (Fig. 1). Compared with growth of the wild-type pneumococcus, the lack of the anticellular activity of pneumolysin influenced bacterial growth in the lungs during the very early hours after infection. Analysis of the wild-type and H2−/C+ mutant growth levels in lung tissue showed that there were significant differences at 3, 6, and 12 h postinfection ($P < 0.01$) but no significant differences after 12 h ($P > 0.05$). Elimination of the anticellular activity of pneumolysin also affected bacteremia. H2−/C+ pneumococci were not detected in the blood before 24 h postinfection (Fig. 2), and the number of H2−/C+ pneumococci in the blood after 48 h was significantly less than the number of wild-type pneumococci ($P < 0.01$) at the same time.

**Histology.** At intervals following infection, a histological examination of lung tissue sections from mice infected with the wild-type and the mutants (H+/C− and H2−/C+) was performed. The data obtained for the wild type were identical to data obtained previously (12), and the new data are not shown.

At 24 h after infection, the H+/C− pneumococcus-infected lung sections showed a heavy cellular infiltrate within infected bronchioles (Fig. 3A), and the H2−/C+ pneumococcus-infected lung sections showed slight cellular infiltration of infected bronchioles (Fig. 3B). In lungs from mice infected with either mutant, the general lung parenchyma was not involved in inflammation (Fig. 3A and B), and interstitial alveolitis (thickening of alveolar walls, along with the presence of fibrosis) was not detected at 24 h after infection (data not shown). With either mutant, the hypertrophy of bronchiole walls (enlargement of bronchial epithelial cells in the bronchiole wall) was less than that in the wild-type pneumococcus-infected tissue sections at 24 h. The main difference between H+/C− and H2−/C+ pneumococcus-infected lungs at 24 h was the difference in the extent of cellular infiltrate (Fig. 3A and B).

At 48 h postinfection, wild-type pneumococcus-, H+/C− pneumococcus-, and H2−/C+ pneumococcus-infected sections showed hypertrophy of the inflamed bronchiole walls, severe multifocal peribronchial infiltration of inflammatory cells, and extensive infiltration of the lung parenchyma (Fig. 3C and D). The bronchioles and lung alveoli appeared to be filled with exudate. Overall, at 48 h after infection, the majority of the lung surface in the mice infected with each strain showed consolidation of bronchiolar spaces and associated parenchyma (due to heavy infiltration of inflammatory cells and the presence of exudate) and hypertrophy of infected bronchiole cell walls.

**Immunohistochemical analysis of inflammatory cell infiltrates.** An immunohistochemistry analysis was performed to identify leukocytes and to analyze the change in the number of leukocytes in lung tissue over time after intranasal infection with the wild-type and mutant pneumococci.

In the inflamed areas of H+/C− pneumococcus-infected lung tissue, neutrophils showed the same pattern of recruitment that was observed in the wild-type pneumococcus-infected lung tissue. Infiltration of neutrophils was detected within inflamed bronchioles, in bronchiole walls, and in perivascular areas near inflamed bronchioles, as well as in the alveolar spaces. The number of neutrophils increased progressively over 24 h and was significantly greater ($P < 0.05$) at 12 and 24 h postinfection than at zero time (Fig. 4A and B). The number of neutrophils then decreased significantly ($P < 0.05$) by 48 h postinfection compared to the 24-h levels. Similar results were obtained for lungs infected with wild-type pneumococci (there were no significant differences between the tissues infected with the wild-type and H+/C− pneumococci at 12, 24, and 48 h).

In mice infected with H2−/C+ pneumococci, the neutrophils showed a pattern of recruitment into lung tissue that was different than the pattern observed in wild-type pneumococcus-infected tissue (Fig. 4C). At 12 h postinfection the numbers of neutrophils were the same as they were in the wild-type pneumococcus-infected tissue, but after this the numbers did not change until 48 h postinfection and were significantly lower than the numbers in the mice infected with the wild-type and H+/C− pneumococci ($P < 0.01$ for comparisons with wild-type and H+/C− data).

In lung tissue sections from mice infected with H2−/C+ or wild-type pneumococci, the numbers of T cells were significantly greater at 24 and 48 h postinfection than at zero time ($P < 0.05$ for H2−/C+ and $P < 0.001$ for the wild type) (Fig. 4A and C). There were not significant differences in the numbers of T cells between lungs infected with the wild type and lungs infected with the H2−/C+ mutant at these times. In contrast, the numbers of T cells in lung tissue sections remained constant and did not increase during the 48 h following infection with H+/C− pneumococci (Fig. 4B).

The numbers of macrophages and B cells remained unchanged ($P > 0.05$) over the course of infection with either mutant pneumococcus (H+/C− or H2−/C+).

## DISCUSSION

The influence of cytotoxic (hemolytic pore formation) and complement-activating properties of pneumolysin on the in vivo events in a model of bronchopneumonia was revealed by comparing events following intranasal infection with two mutant pneumococci, H+/C− and H2−/C+, and the wild-type parent strain. It was shown previously that both of these activities are required for full virulence of the pneumococcus in bronchopneumonia (2) and lobar pneumonia (19). In the studies that we describe here we extended these observations by analyzing the pulmonary growth of pneumococci in more detail and by analyzing the effects of individual activities of the toxin on the host immune response during bronchopneumonia. Our results showed that pore formation and complement activation by pneumolysin make distinct contributions to pneumococcal growth in the lungs and to bacteremia. Also, both activities are involved in the inflammation and cellular influx seen in pneumonia. Significantly, however, they contribute differently to the level of the host immune response. To our knowledge, this is the first detailed description of the pulmonary immune responses to individual pneumolysin activities.

Removal of either cytotoxic or complement-activating activity had an affect on the early growth of the pneumococci in the lungs. This may not be surprising given that these mutations significantly decrease virulence and the absence of pneumolysin has previously been shown to change the pattern of pneumococcal growth in the lungs (2, 12). However, as shown here,

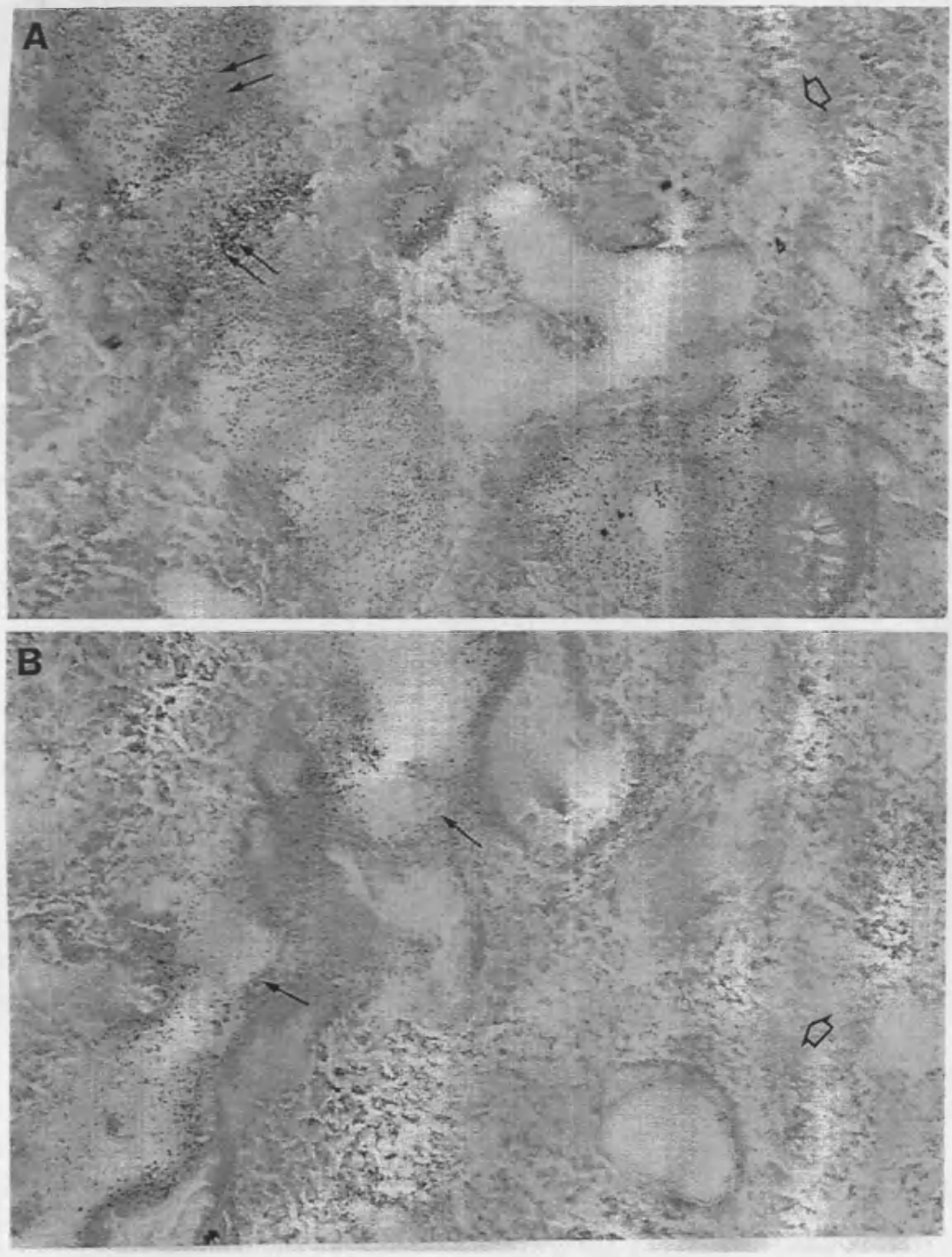


FIG. 3. Light microscopy of lung tissue from a mouse infected with $10^6$ CFU of *S. pneumoniae* H+/C− at 24 h postinfection (A), from a mouse infected with $10^6$ CFU of *S. pneumoniae* H2−/C+ at 24 h postinfection (B), from a mouse infected with $10^6$ CFU of *S. pneumoniae* H+/C− at 48 h postinfection (C), and from a mouse infected with $10^6$ CFU of *S. pneumoniae* H2−/C+ at 48 h postinfection (D). In panel A the double arrows indicate heavy cellular infiltrate in infected bronchioles. In panel B the single arrows indicate slight cellular infiltration of infected bronchioles. In panels A and B the open arrows indicate general lung parenchyma that was not involved in inflammation. In panels C and D the thin arrows indicate hypertrophy of the inflamed bronchiole walls, the arrowheads indicate severe multifocal peribronchial infiltration of inflammatory cells, and the thick arrows indicate extensive infiltration of lung parenchyma. Magnification, ×250.

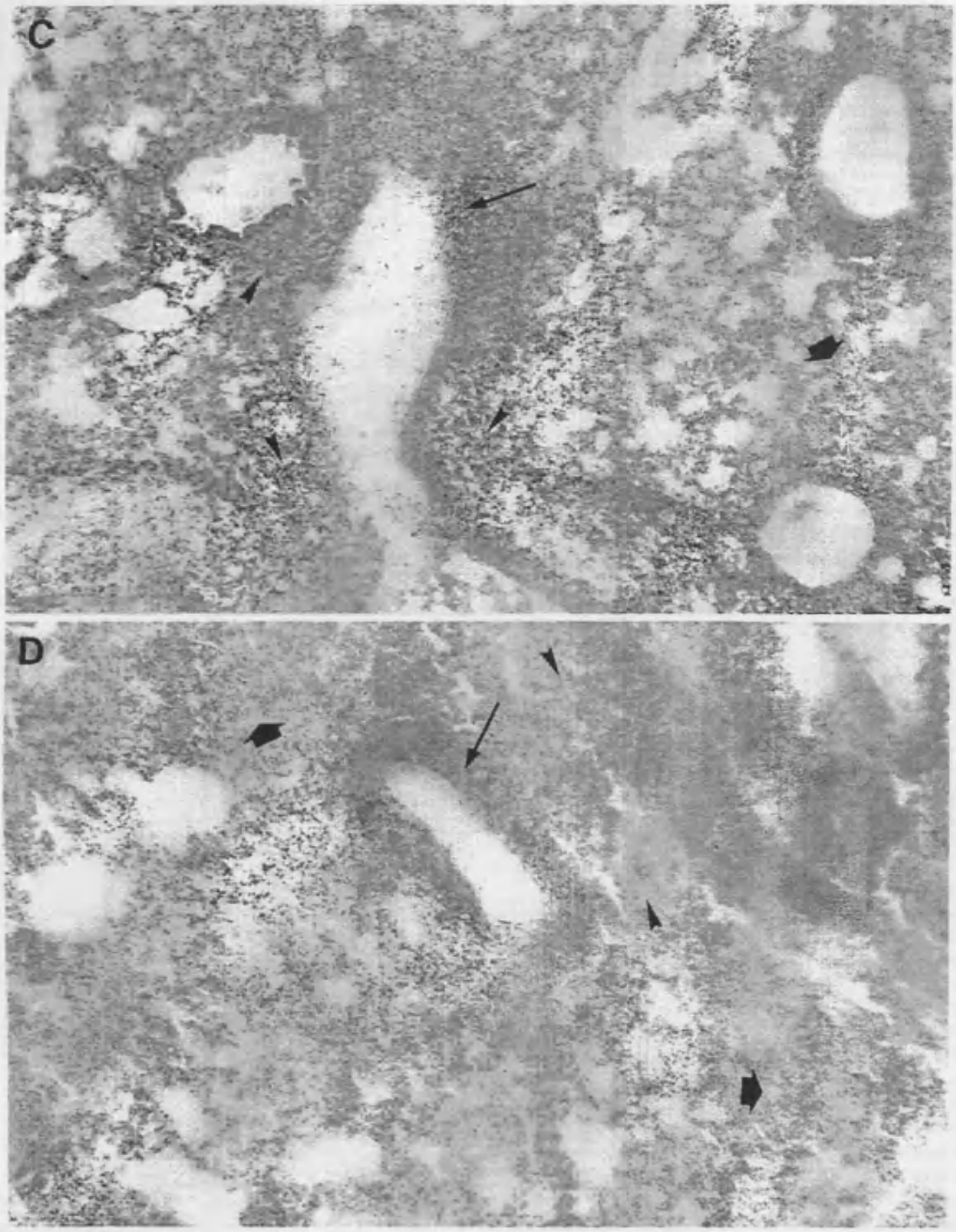


FIG. 3—*Continued.*

how the growth was affected was curious. We observed that at certain times after infection, the absence of one of the pneumolysin activities improved pneumococcal survival. Reduced cytotoxic activity was accompanied by better pneumococcal survival in the first 6 h, whereas the absence of complement activity was accompanied by significantly better pneumococcal survival at around 12 h postinfection. Therefore, for these experiments, conclusions about whether the mutations in the *ply* gene affect virulence depend on the definition of virulence. Judged by the outcome for the bacteria in the lungs and blood,

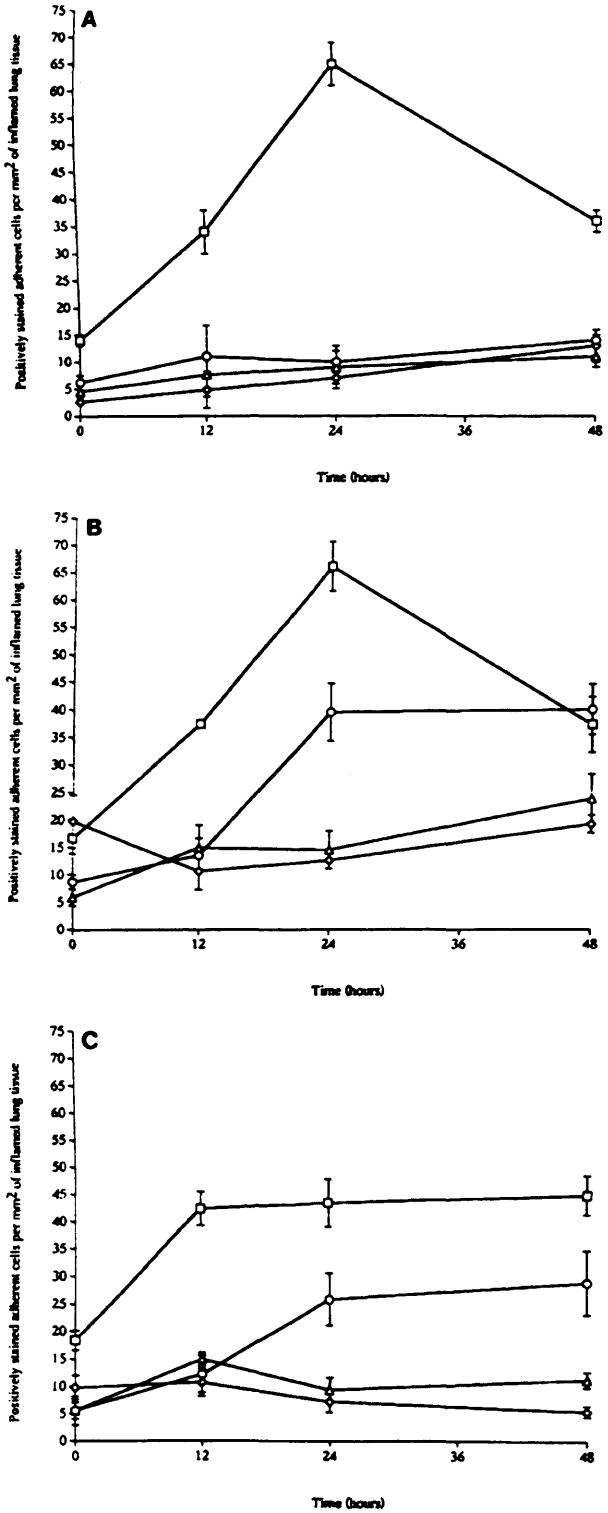


FIG. 4. Numbers of neutrophils (□), macrophages (◇), T cells (○), and B cells (△) in tissue sections from lungs of MF1 mice infected intranasally with $10^6$ CFU of the *S. pneumoniae* wild-type *strain* (A), with $10^6$ CFU of *S. pneumoniae* H+/C− (B), and with $10^6$ CFU of *S. pneumoniae* H2−/C+ (C) ($n$ = 4 for each time point) The error bars indicate standard errors of the means.

the mutations have no effect by 48 h, but judged by the effect on the host, the absence of either activity is an important determinant of the outcome. Why does the pneumococcus retain a system that leads to damage of the host but does not apparently benefit the bacterium directly?

It has been shown previously (2) that the presence of the Asp385 → Asn and His367 → Arg mutations in the pneumococcal pneumolysin extend the survival time of mice after intranasal infection. In the study reported here we observed a correlation between survival time and timing or extent of cellular inflammation, but we did not observe a correlation between survival or inflammation and the numbers of pneumococci in the lungs. It is noteworthy that by 48 h postinfection the same numbers of mutant and wild-type pneumococci were present in the lungs. Also, there was no correlation between the timing of the onset or the extent of bacteremia and host survival time. The later appearance of H2−/C+ in the blood is consistent with the previously stated view (12) that a delay in bacteremia is associated with a delay in the time of death. However, the timing of bacteremia due to H+/C− is not consistent with this hypothesis. The early detection of H+/C− in the blood suggests that complement activation by pneumolysin plays a pivotal role in delaying bacteremia. Benton and colleagues (4) reported that point mutations in the *ply* gene that reduced complement activation or cytotoxicity did not individually alter the growth kinetics of pneumococci injected directly into the blood. In our study the numbers of the mutants and the wild type also increased at the same rate in the blood.

Previously, it was reported that when pneumolysin was present, pneumococci were more resistant to antimicrobial mechanisms that limit their numbers in the blood (12). The data in this paper show that the presence of either the pore formation or complement-activating activity of pneumolysin is sufficient for this resistance. In contrast to these observations, Alcantra et al. (1) suggested that an absence of complement-activating activity results in greater pneumococcal clearance after intravenous infection of rats.

It has been reported previously that an absence of pneumolysin was associated with a significant delay in and a lower severity of pulmonary inflammation (12). Also, an absence of pneumolysin resulted in significantly less intense accumulation of neutrophils and T cells at inflamed sites in the lungs compared to the responses observed with wild-type pneumococci (12). Based on the histological analysis described here, it seems that both of the pneumolysin activities that were investigated contribute to the induction of inflammation. Thus, a less severe histological result was seen with both mutant strains soon after infection. However, by 48 h the extent of inflammation was as severe as the extent of inflammation after wild-type infection. This contrasts with the situation after infection with the pneumolysin-negative mutant (12). What was also noteworthy was the observation that after infection with H+/C− pneumococci, the responding inflammatory cells were less tightly packed around the bronchioles. This implicates complement activation in mediating local localization of cells.

Pneumococcal infection is associated with a sequential influx of inflammatory cells into the lungs. We concluded that accumulation of host cells in lesions was not simply a consequence of pneumococcal numbers in the lungs but was related to the signals related to the infection, such as chemokines, cytokines,

and complement factors. The data obtained in this study support this conclusion. Two other conclusions can be drawn from the data: both the cytolytic and complement-activating activities contribute to the influx of cells, and there is specificity in the consequences of each activity. Crucially, the toxin's complement-activating activity seems to be more important for the recruitment of T cells to inflammatory lesions. To our knowledge, the previous work in our laboratory was the first work to demonstrate the important involvement of T cells during pneumococcal bronchopneumonia (12). The findings presented in this paper significantly advance our understanding of the role that T cells play as part of the inflammatory host immune response to the pneumococcal toxin pneumolysin.

Conversely, the toxin's cytolytic activity is the predominant influence on neutrophil accumulation. Extrapolating from the observations of Baba et al. (3), it is likely that the H2−/C+ toxin stimulated more gamma interferon and consequently more nitric oxide than the wild-type or H+/C− toxin stimulated. Therefore, the reduced inflammation and decreased cell recruitment observed with the H2−/C+ mutant do not support the hypothesis that gamma interferon and nitric oxide are major mediators of the effects of the toxin in pneumonia. It has previously been shown that in wild-type pneumococcus-infected mice, tumor necrosis factor alpha is involved in the protective pulmonary response during pneumococcal pneumonia and that interleukin-6 levels contribute to pathogenesis of the disease rather than protection (13). Analysis at a molecular level of induction of host mediators of inflammation by pneumolysin's anticellular and complement properties is required to obtain a fuller understanding of how this toxin acts as a principal trigger of inflammation during pneumococcal pneumonia.

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