Role of the Lectin Pathway Recognition Molecule Ficolin A in Fighting Pneumococcal Infection

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Statement of Originality

This accompanying thesis submitted for the degree of PhD entitled (Role of the lectin pathway recognition molecule ficolin A in fighting pneumococcal infection) is based on work conducted by the author at the University of Leicester, mainly during the period between March 2008 and March 2011.

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: Date:

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Abstract

Complement system is an essential part of innate immune system that plays major role in protection against various pathogens, including *Streptococcus pneumoniae*. Complement is activated via three pathways; the classical pathway, the alternative pathway and the lectin pathway. Lectin pathway of complement activation is mediated via ficolins, MBL and recently described C-type lectin, CL-11, that recognise a wide range of carbohydrates on microbial surfaces and activate the complement system via MASP-2, the effector enzyme of the lectin pathway of complement activation. A significant role of the lectin pathway has been described previously using MASP-2 deficient mice, with a complete deficiency of the lectin activation pathway specific C3 and C5 converting enzymes C4b2a and $C4b2a(3b)_n$ respectively. However, the role of MBL, ficolins and CL-11 in activating lectin pathway against *S. pneumonia*e has not been fully characterised.

In vitro studies demonstrate that ficolin A and CL-11 are the relevant carbohydrate recognition molecules that can activate the lectin pathway of complement on the surface of *S. pneumoniae*. The protective activity of ficolin A was demonstrated *in vivo* using ficolin $A^{-/-}$ mice compared to wild-type controls, the ficolin $A^{-/-}$ mice were highly susceptible to pneumococcal infection with higher mortality and higher bacterial burden in both blood and lungs after intranasal infection with *S. pneumoniae* D39 compared to wild-type controls. These findings imply that the lectin pathway has a significant role in protection against *S. pneumoniae* infection and highlights the importance of non-MBL mediated lectin pathway activation in the innate host defence against *S. pneumoniae*.

The essential role of the lectin pathway in providing protection against *S. pneumoniae* was further described by blocking the lectin pathway by *i.p.* injection of mice with anti-MASP-2 mAb. Mice receiving anti-MASP-2 mAb showed significantly higher mortality after intranasal infection with *S. pneumoniae* when compared to mice receiving control antibody.

Abbreviations

aa	amino acid
AP	Alkaline phosphate
APCs	Antigen presenting cells
BBS	Barbital buffer saline
BgA	Beta-galactosidase A
BHI	Brain/Heart Infusion
bp	Base pair
BSA	Bovine serum albumin
C1-INH	C1-inhibitor
C3aR	C3a receptors
C4bp	C4 binding protein
C5aR	C5a receptors
CbpA	Choline binding protein A
CCP	Complement control protein
cDNA	Complementary deoxy-ribonucleic acid
CFHR1	Factor H related protein 1
CFHR1	Factor H related protein 1
CFP	Complement factor properdin
CFU	Colony forming unit
ChoP	Phosphoryle choline
CL-11	Collectin-11
CL-K1	Collectin kidney 1

CP	Classical Pathway
CPS	Capsular polysaccharides
CR	Complement receptor
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
CUB	C1r/C1s-Uegf-bone morphogenetic protein
DAF	Decay-accelerating factor
dCTP	Deoxycytidine triphosphate
DEPC-H2O	Diethyl pyrocarbonate water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EGTA	Ethylene glycol tetraacetic acid
EDTA	Ethylenediaminetetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
EPS	Extracellular polymeric substance
fB	Factor B
FCS	Foetal calf serum
fD	Factor D
fH	Factor H
FHL-1	Factor H like protein 1
fI	Factor I

GAPDH	Glucose-6-phosphate dehydrogenase others
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
H & E	Haematoxylin and Eosin stain
i.p.	Intraperitoneal
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kb	Kilobase
kDa	Kilodalton
LEA-1	Lectin effector arm 1
LEA-2	Lectin effector arm 2
LP	Lectin Pathway
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAbs	Monoclonal antibodies
MAC	Membrane attack complex
MAp19	19 kDa mannose binding lectin associated protein
MAp44	44 kDa mannose binding lectin associated protein
MASP	Mannose binding lectin-associated serine protease

MBL	Mannose binding lectin
MIP-2	Macrophage inflammatory protein 2
MLST	Multi-locus sequence typing
MW	Molecular weight
NanA	Neuraminidase A
NanB	Neuraminidase B
NanC	Neuraminidase C
O.D.	Optical density
PAF	Platelet-activating factor
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PfbB	Plasminogen & fibrinogen binding protein
PLY	Pneumolysin
PMNs	Peripheral blood polymorphonuclear cells
pNPP	ρ-nitrophenyle phosphate
PRMs	Pathogen recognition molecule
PRPs	Pathogen recognition patterns
PRRs	Pathogen recognition receptors
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RBCs	Red blood cells
Rnase	Ribonuclease
RT-PCR	Reverse Transcriptase- PCR

SAP	Serum amyloid protein
SERPIN	Serine protease inhibitor
SGI	Syber green I
sIgA	Secretory IgA
sMAP	small MBL-associated protein
SP-A	Surfactant protein A
SP-D	Surfactant protein D
StrH	Beta–N-acetylglucosaminidase
TBS	Tris buffered saline
TCC	Terminal complement complex
TNF	Tumor necrosis factor
URT	Upper respiratory tract
v/v	Volume/volume
w/v	Weight/volume
α–helical	Aplha helical
α–MASP-2	Anti-MASP-2
$\mu^{-/-}$	IgM deficient

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1. Introduction

1.1 Immune system

Humans and other mammals are exposed to a variety of pathogenic microbes, toxic and allergic substances that interfere with normal physiological life functions. Harmful effect of these foreign particles led to the evolution of a complex array of defence mechanisms that compose the host immune system. The immune system includes a large variety of effector proteins, cells, tissues and organs that evolved to protect the hosts from the adverse effects of harmful foreign substances by eliminating or neutralising them. A basic general quality of the immune system is its ability to distinguish between self and non-self (Chaplin, 2010). This enormously complex host defence system is divided into two major categories, the innate and the adaptive immunity that cooperate together to protect the host against infectious agents. The basic difference between both arms of immune system is the receptor types used to identify microorganism. The innate immune system utilises a variety of pathogen recognition patterns (PRPs) encoded by genes in the host's germ line. These PRPs have broad specificities for various patterns on the surfaces of a large variety of microorganisms and provide an essential first line of defence to recognise and eliminate invading microorganisms (Medzhitov *et al.*, 2007). Many groups of pathogens share similar molecular arrays or patterns called pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) (which are common amongst Gram negative bacteria), lipoteichoic acid (LTAs) (which are common amongst Gram positive bacteria), mannan among yeasts, glycolipids among mycobacteria and double-stranded

RNA among viruses (Hoffman *et al*., 1999). PAMPs have some unique features that facilitate innate immune recognition. Common structural motifs of PAMPs help PRPs to bind and recognise a large number of molecules from different microorganisms and are the products of pathways, which are unique to microorganisms that help to distinguish between self and non-self (Medzhitov *et al.,* 2007).

Although innate immunity provides the first line of defence by recognising and eliminating harmful pathogens, its effectiveness is somewhat limited due to the restricted range of specific PAMPs. As pathogens are very diverse and have great variability of antigenic structures they also have the ability to alter their molecular patterns to evade the innate immune response of the host. These challenges led the way to evolution of adaptive immunity to provide a broad and more specific repertoire of recognition of non-self antigens. Unlike the broadly specific and germline encoded PRPs of innate immune system, receptors involved in adaptive immunity use a large number of variable immune receptors that are more specific against a certain antigen. Genes encoding these receptors are somatically rearranged and assemble the receptor molecules with custom-tailored specificity for foreign structures. A signature feature of the adaptive immune system is the immune memory where specific immune cells, called memory cells that are developed during contact with a pathogen in previous episodes of pathogen exposure. These cells can persist dormant in the body until this or similar pathogens cause re-infections and activate these memory cells to mount a cell driven specific immune response. After the initial encounter with pathogens, cells expressing these immune receptors can remain in the host for many years and can respond more rapidly after re-exposure of same antigen (Bonilla & Oettgen, 2010).

Innate and adaptive immunities are generally characterised as separate arms of immune system, but they usually act together. In some cases, PRRs are involved in sending the instructions to lymphocyte in the form of specialised signals and inducing adaptive immune response by activating the antigen specific cells (Chaplin, 2010). The complement system provides a typical example of synergy between innate and adaptive immunity. As a first line of defence, the complement system quickly detects PAMPs on the surface of microbes by PRRs such as C1q, MBLs, ficolins and CL-11 and destroys them by a complex and tightly regulated assembly of proteins. On the other hand, certain byproducts are released during normal complement cascade that induces the adaptive immune response (Ricklin *et al*., 2007).

1.2 Complement system

Complement is a vital component of defence system which plays an integral role in not only the innate immune system but also acts as the effector mechanism of adaptive immunity. Complement system provides a good defence against harmful infectious agents, including bacteria and viruses by a series of physiological processes like clearing the immune complexes and apoptotic cells, and tissue regeneration (Trouw & Daha, 2011). More than 100 years ago, complement was identified as a heat labile component of serum that induced the lysis of bacteria and RBCs by complimenting the effect of specific antibody. Further studies extended its importance in opsonisation and participation in cellular immunity and characterising it as a part of innate immunity. The complement system is composed of a network of more than 30 serum and membrane associated proteins that interact with each other and perform a range of functions from direct cell lysis to the enhancement of B and T cells (Carroll, 2004). Most of these proteins are present as inactive zymogens, which are activated in a

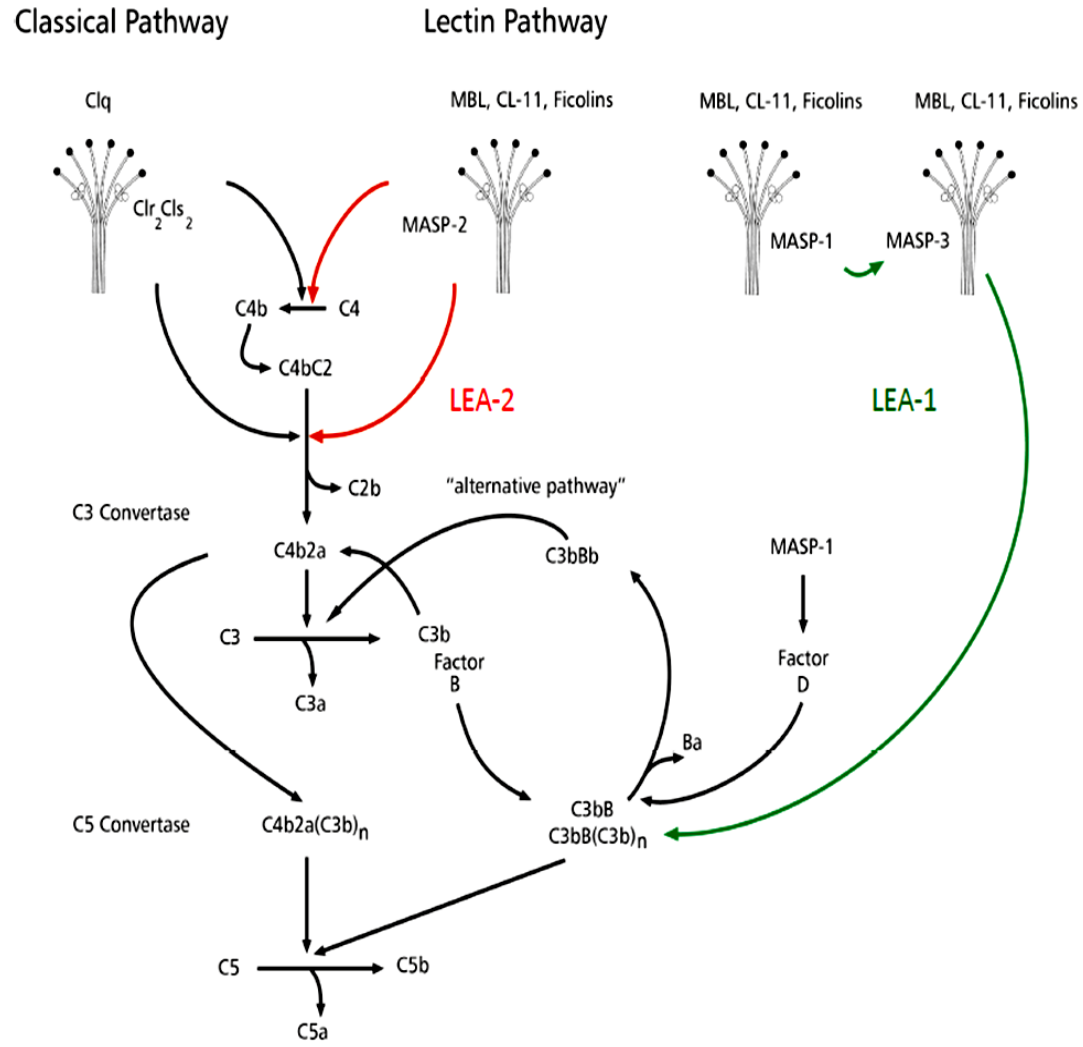
cascade manner mediated after the encounter with pathogen surface structures resulting in a series of modifications in structure, assembly with other components, to be converted into active enzymes that activate their subsequent substrates. After the initiation of the activation cascade, the activation process is amplified and results in powerful effector functions. This whole network is maintained by more than 60 components and activation fragments, including nine central components of cascade (C1-C9), products released after activation of certain components promoting different biological activities, proteases and newly assembled enzymes, regulators and inhibitors, binding proteins, and receptors for effector molecules. Following the identification of more recently discovered regulators and receptors, the number of these components of the complement system is continuously increasing (Zipfel & Skerka, 2009).

Complement activation takes place sequentially and can be divided into four parts. The first part is the initiation of complement activation (or the activation pathway) that can either be induced by spontaneous activation on biological surfaces that readily promote spontaneous activation or through recognising antibody bound to a target antigen or through binding of carbohydrate recognition complexes to carbohydrates on microbial surfaces. Complement is activated via three pathways: the classical, the lectin and the alternative pathway (Fig 1.1 a). Classical pathway is activated by antigen-antibody complexes. Lectin pathway is activated after carbohydrate recognition on the surface of microbes by carbohydrate recognition subcomponents including mannose binding lectins (MBLs), ficolins and recently described C type lectin CL-11. Activation of the alternative pathway does not involve any recognition molecule and is initiated by covalent binding of small amounts of C3 to hydroxyl or amine groups on cell surface

molecules of microorganisms (Fujita *et al.,* 2004; Hansen *et al.,* 2010; Schwaeble *et al.,* 2011). These pathways will be subsequently described in more details in Chapter 1.2.

The second step is the formation of the C3 convertase (C4b2a) forming after the cleavage of C2 and C4. The C3 convertase cleaves C3 in C3a and C3b. C3a is involved in recruitment and activation of innate immune effector cells. Third step is the formation of C5 convertase that cleaves C5 into C5a and C5b. C5a is a powerful anaphylactic peptide that induces inflammation whereas C5b can initiate the terminal pathway by assembling the terminal pathway components C5, C6, C7, C8 and C9 ultimately leading to the formation of membrane attack complex (Zipfel & Skerka, 2009: Walport, 2001).

A.



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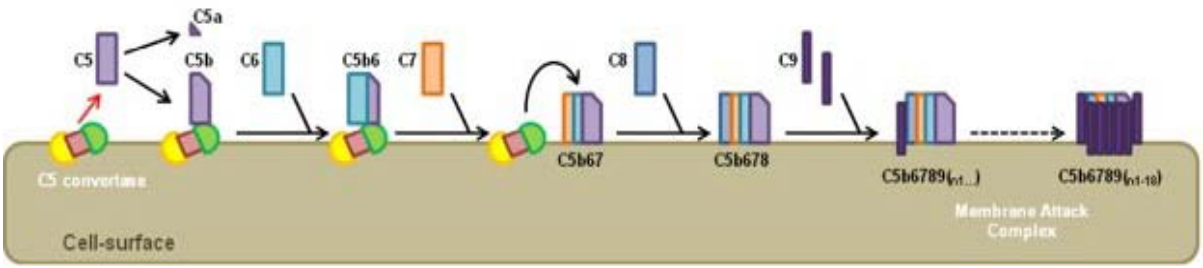


Figure 1.1: A schematic diagram of complement activation. (a) Activation of the classical, the lectin and the alternative pathway. LEA-1 represents lectin effector arm-1, LEA-2 represents lectin effector arm-2 (figure kindly provided by Professor Wilhelm Schwaeble). (b) formation of MAC (figure modified after Carroll & Sim, 2011).

1.2.1 The Classical Pathway

The Classical pathway was the first complement activation pathway described and is often referred to as the antibody-dependent pathway as it is initiated after the recognition of Fc portion of IgM and IgG (IgG1, IgG2 and IgG3, not IgG4) clusters binding to the cell surface. This pathway may also be activated independently in the absence of specific antibodies by C-reactive protein (CRP) binding to a specific antigen. Proteins involved in this pathway were designated as C1 through C9. However, these numbers do not represent the order of reaction of protein activation (Pettgrew *et al.,* 2009; Janeway, 2005; Walport, 2001). Classical pathway is triggered by C1, a 790 kDa multimolecular protease formed by association of pattern recognition molecule, C1q and two proteases, C1r and C1s (Fig. 1.2). C1r is responsible for internal activation of C1 whereas C1s is responsible to transfer the signal mediating the cleavage of C4 and C2. Hence, C1 has combined abilities to recognise distinct structures on microbial surfaces and convert the recognition signal into highly specific proteolytic activity (Gaboriaud *et al.,* 2004; Arlaud *et al.,* 2002). C1r and C1s are attached to each other as zymogens forming a 340 kDa C1rs tetramer (C1s-C1r-C1r-C1s). C1q recognises immune complexes or directly binds to microbial surface inducing the autocleavage of C1r. Activated C1r then cleaves and activates C1s. C4 is the first substrate of C1s, which cleaves it into C4a and C4b. A previously hidden thioester is exposed after cleavage of C4, which allows deposition of C4b on nearby surfaces close to the activation sites. C4b also binds to C2 which upon binding of C4b can be cleaved by C1s into C2a and C2b. The C2a fragments remains attached to C4b to form C3 convertase (C4b2a) (Wallis *et al.,* 2010).

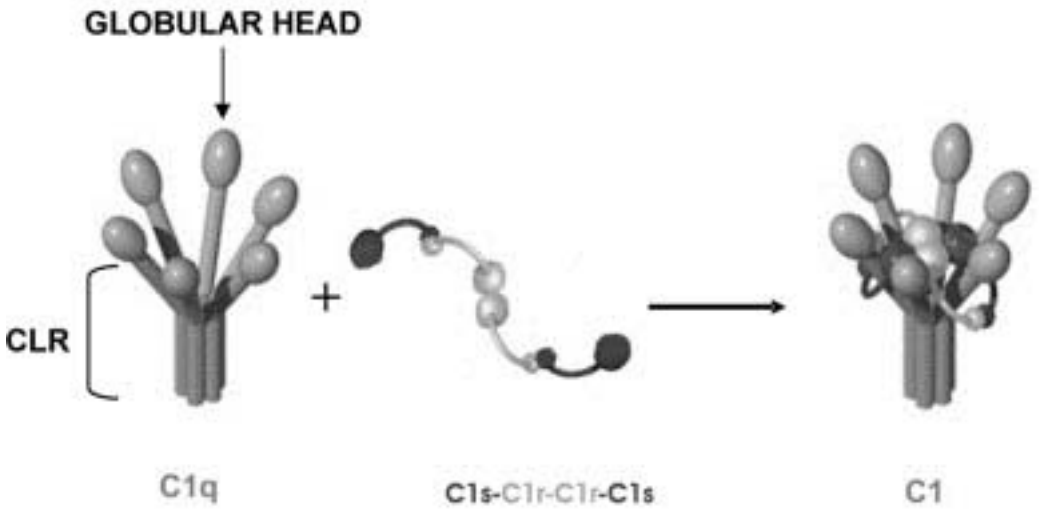


Figure 1.2: Association of C1q with C1r/C1s tetramer to form C1 complex (Figure modified after Pflieger *et al.*, 2010).

1.2.2 The Alternative pathway

Unlike the lectin and the classical pathway of complement that require specific recognition molecules for activation, the initiation of the alternative pathway of complement system requires spontaneous covalent binding of C3 to hydroxyl or amine groups on the surface of microorganism and is further activated by low grade cleavage of C3 (Fujita *et al.*, 2004; Walport 2001). The alternative pathway activation can be divided into two phases. The initiation phase and amplification/regulation phase. Initiations phase involves spontaneous conformational changes in structure of C3. C3, a 187kDa protein is the central component of the alternative pathway. It is quite inert in its native form displaying only few ligands. Due to spontaneous conformational changes in its structure, when the C3 molecule is hydrolysed into $C3_{H2O.}$, this results in the exposure of new binding sites including a highly reactive thioester bond that promotes the attachment to other molecules. $C3_{H2O}$ binds to the Factor B (fB) zymogen which upon binding to C3b or $C3_{H2O}$, can be cleaved by factor D (fD) into Ba and Bb.

The Ba fragment is released whereas Bb remains attached to $C3_{H2O}$ (or C3b) and forms an alternative pathway C3 convertase ($C3_{H2O}$ Bb or C3bBb). This C3 convertase can further activate the complement by cleaving C3 into active fragments C3a and C3b, further exposing thioester bonds which allow C3b to covalently bind to any surface structure containing amine groups and initiating the second phase, the amplification/regulation phase. Amplification or regulation depends on the surface where the reaction takes place. On foreign surfaces, reaction is amplified by continuous generation of C3b and binding with fB. This reaction is regulated on human cells by fluid phase regulators such as Factor H, FHL-I and some membrane-bound regulators such as decay accelerating factor (CD55) and protectin (CD59). The interaction of these fluid phase inhibitors with surface structures of cells allows differentiation between self and non-self since, for example factor H has higher affinity to bind to C3b and fB bound C3b on the surface of host cells and decays C3b on the surface of host cells and C3b from complexes with fB and serves as a cofactor in the factor I mediated conversion of C3b to iC3b and further to C3dg (Janssen *et al.*, 2005; Zipfel *et al.*, 2007; Pangburn *et al.*, 2008).

Recent studies provided new evidence that the alternative pathway can be initiated in PRM-based pattern recognition complexes of the lectin pathway. Some recent papers claim that properdin may recognise and bind pathogen associated molecular patterns or apoptotic cells and initiates the complement by providing a platform for assembly of C3 convertases (Spitzer *et al.*, 2007). This is also good evidence showing that lectin pathway components like the serine protease MASP-1 and MASP-3 are involved in the maturation and possibly activation of the alternative pathway. MASP-1 appears to be required to control zymogen factor D into its enzymatically active form (Takahashi *et*

al., 2008 & 2010) and MASP-3 (activated by MASP-1) may catalyse the cleavage C3b bound fB on the pathogen surface (Iwaki *et al.,* 2011) (Fig 1.1 a).

1.2.3 The Lectin pathway

The lectin activation pathway of complement involves the recognition of an array of pathogen associated molecular patterns (PAMPs) by pathogen recognition molecules (PRMs) of the lectin pathway. This activation pathway can neutralise pathogens by complement lysis and/or opsonisation in the absence of antibodies (Schwaeble *et al.,* 2002; Walport 2001, Wallis *et al.,* 2007). PRMs of the lectin pathway either belong to the collectin family or the ficolin family of proteins. Rodents have four lectin pathway recognition molecules that can activate the lectin pathway, including two MBLs, MBL-A and MBL-C along with ficolin A and recently described C-type lectin, collectin-11 (CL-11). The human lectin pathway recognition molecules include MBL-2 (Mannan binding lectin-2), CL-11 and three ficolins namely L-ficolin, H- ficolin and M-Ficolin (Hansen *et al.,* 2010; Schwaeble *et al.,* 2011; Endo *et al.,* 2007). All the PRMs of the lectin pathway described so far can activate the complement except mouse ficolin B, the murine orthologue of human M-ficolin (Endo *et al.,* 2005). Recent studies described that rat ficolin B can drive lectin pathway by activating MBL-associated serine protease MASP-2 (Girija *et al.,* 2011). All pathogen recognition components of the lectin pathway can recognise an array of carbohydrate structures on the surface of bacteria viruses and parasites. MBLs binds to sugars with 3- and 4-OH groups through carbohydrate recognition domain and ficolins bind to sugars mainly with *N*-acetyl group through fibrinogen-like domain (Fig. 1.3). Recognition molecules of the lectin pathway, after binding to specific PAMPs catalyse activation of MBL-associated serine proteases, MASP-1, MASP-2, MASP-3, which form complexes with them. MAp 19

and MAp 44, truncated non-enzymatic product of alternative splicing fragment of MASP-2 and MASP-1/3 respectively, regulate the lectin pathway activation by competing for the binding of MASPs to the carbohydrate recognition complexes (Schwaeble *et al.,* 2002; Wallis *et al.,* 2005; Weiser *et al.,* 1992; Skjoedt *et al.,* 2010; Degn *et al.,* 2011). Activation of MASPs triggers the downstream reaction cascade. MASP-2 is the essential effector enzyme of the lectin pathway as it cleaves C4 and C4b bound C2 fragments of complement and form a C3 convertase (C4b2a), a shared convertase complex generated during initiation of both the lectin and the classical pathway. MASP-2 has same modular domain structure as the classical pathway serine proteases C1r and C1s, but is 20-25 times more efficient than C1s to cleave C4. It can activate the lectin pathway independent of other serine proteases (Fujita, 2004; Thiel *et al.,* 1997; Rossi *et al.,* 2001).

The mechanisms of action of the lectin pathway components MASP 1 and MASP-3 is not fully understood. MASP-1 can cleave C4b bound C2, but it does not cleave C4 and can only enhance the activation of the lectin pathway initially triggered by MASP-2 complexes. It cannot activate the lectin pathway independently without the help from MASP-2 (Chen & Wallis, 2004; Møller-Kristensen *et al.,* 2006). MASP-1 can also cleave C3 directly but at a very negligible rate (Dahl *et al.,* 2001; Rossi *et al.,* 2001).

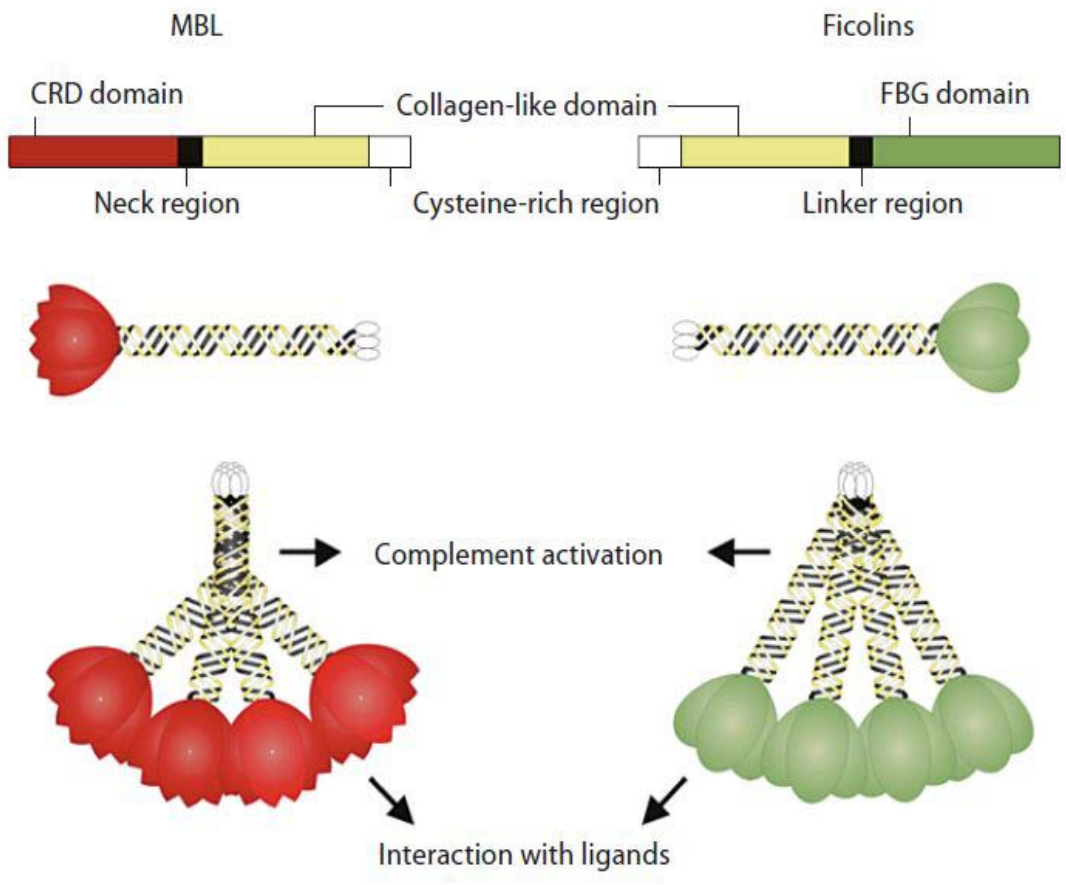


Figure 1.3: A schematic representation of MBL and ficolin domain organisation, structural subunit and higher oligomeric forms (figure modified after Garred *et al.*, 2009).

1.2.4 Terminal pathway and membrane attack complex

After initiation of complement, the end products of all three activation pathways are C3 convertases (C4b2a & $C3_{H2O}$ Bb or C3bBb) (Fig. 1.1 a) which cleave C3 in C3a and C3b. C3a is involved in the recruitment and activation of innate immune effector cells. C3b is deposited on nearby surfaces and aids to amplification of complement immune response and binding of multiple C3b molecules in close proximity of C3 convertases enables the formation of C5 convertase. C5 convertase cleaves C5 fragment into C5a and C5b. Upon C5 cleavage, the C5a fragment is released and acts as a potent mediator for inflammation and chemotaxis (Gros *et al.*, 2008; Zipfel & Skerka, 2009). C5b

attaches via its thio ester to the pathogen surface and forms a trimolecular complex with C6 and C7 which expresses a meta membrane binding site inserting the complex into the membrane. C5b67 also exposes a receptor for C8, which binds to C5b67 complex and induces the polymerisation of several subunits of C9 to form C5b-9_n a lytic pore called terminal complement complex (TCC) or membrane attack complex (MAC) which leads to leakage of ions and hydrophilic molecules and induce a permeability defect ultimately resulting in cell lysis (Fig. 1.1 b) (Carroll & Sim, 2011; Bhakdi *et al.*, 1991).

1.3 Effector functions of complement

A major function of complement is pathogen recognition, which may lead to the lysis of target cells through the terminal activation cascade. However, as a result of this proteolytic cascade, different biological products are generated, which are also capable of performing additional biological activities which provide protection against invading pathogen by opsonisation, initiating pro-inflammatory signals, removing of dead cells and promoting healing of tissues (Rawal & Pangburn, 2001). During the activation and amplification process, smaller cleavage products of C3 and C5, i.e. C3a and C5a, are continuously released. These products are called anaphylotoxins which mediate the inflammatory response by triggering pro-inflammatory signals through their corresponding receptors (C3aR and C5aR) (Rawal and Pangburn 2001). C5a is the most powerful of the anaphylotoxins and acts as strong chemoattractant for neutrophils, monocytes and macrophages (Marder *et al.*, 1985; Hartmann *et al.*, 1997). It can also induce degranulation of mast cells and basophils, releasing histamine, prostaglandins, serotonins and kinins, which cause vasodilation and capillary leakage hence increasing the influx of inflammatory mediators and leukocytes at the site of infection and

promoting phagocytosis and eliciting the immune response against invading pathogen (Schumakar *et al.,* 1991; Haynes *et al.,* 2000). C5a can also maintain the balance between anti- and pro-inflammatory responses by inducing or downregulating inflammatory cytokines. It can activate leukocytes by triggering the release of pro-inflammatory cytokines like TNF-α, IL1-β, IL-8 and IL-6. On the other hand, it can down regulate the production of IL-6 in human umbilical vein endothelial cells (Strieter *et al.*, 1992; Monsinjon *et al.*, 2003).

Another important effect or function of complement is opsonisation, a process by which a pathogen is tagged by serum components for recognition by macrophages and leukocytes. Cleavage products of different complement components, such as C3b, iC3b, C4b and C5b can facilitate phagocytosis of pathogens by opsonising their surfaces. Macrophages and leukocytes have receptors like complement receptor I (CRI, CD35) and CR3, which can recognise these opsonins on the surface and promote phagocytosis of the pathogens. Opsonisation can also help in clearance of soluble antigen-antibody complexes. C3b and C4b attach to these complexes and bind to CRI on erythrocytes as a result of which immune complexes are transported to spleen and liver and eliminated (van Lookeren champagne *et al.,* 2007; Marder *et al.,* 1985). Degradation products of C3b, iC3b, C3c and C3dg can also act as ligands for CD21 and CD35 on B-Cells and contribute in B-cell activation initiating the production of specific antibodies and differentiating memory B-cells (Roozendaal & Carroll, 2007).

Besides the role of complement in the inductive and the effector phases of immunity, complement activation may also resolve inflammation by providing a safe clearance of apoptotic cells. Morphological changes in apoptotic cell results in the breakdown of membrane integrity and loss of membrane complement regulators on cell surface

making them a target for C1q and C- reactive protein (CRP) recognition. C1q, CRP (along with factor H) binds to these cells resulting in activation of early steps of classical pathway but blocks the amplification beyond C3 convertase. This results in opsonisation of these apoptotic cells due to continuous C3b deposition, which trigger the removal of cell debris by phagocytic cell uptake (Gershov *et al.*, 2000). A role of MBL, L-ficolin and H-ficolin has recently been demonstrated in clearance of apoptotic cells (Stuart *et al.*, 2005; Kuraya *et al.*, 2005).

1.4 Regulation of complement system

The complement system mainly targets infectious agents. Continuous exposure of pathogens triggers additional immune reactions that might be harmful for host cells. Host cells are protected from bystander lysis by a number of regulators to control the complement attack. These regulators adjust the severity of complement attack and maintain a delicate balance between activation and inhibition resulting in clearance of foreign or modified cells whereas self cells are protected (Zipfel & Skerka, 2009). These regulators are classified in three major groups: fluid phase, attached to the surface of host cells and membrane integral complement receptors (Fig. 1.5). The number of these regulators may exceed the number of proteins involved in complement activation (Morgan & Harris, 1999).

1.4.1 Fluid phase regulatory proteins

Fluid phase regulatory proteins are present in plasma and body fluids. Regulators of the alternative pathway include factor H (fH) and factor H like protein 1 (FHL-1). fH dissociates the C3 convertases of the alternative pathway in two ways (Fig. 1.4). It removes C3b competitively from the C3bBb complex by binding to C3b either in fluid-

phase or on the cell surface making it unavailable for fB binding (decay accelerating function). fH also acts as a cofactor to factor I (fI) mediated degradation of C3b (cofactor function) (Jozsi & Zipfel, 2008; Carroll & Sim, 2011). Regulators of the classical and the lectin pathway include C1 inhibitor (C1INH) and C4 binding protein (C4bp). C1INH, also called SERPIN 1, blocks the serine proteases of classical and lectin pathway C1r, C1s, MASP-1 and MASP-2 (but not MASP-3) by acting as an inhibitory substrate (Davis *et al.*, 2008; Chen *et al.*, 1998). C4bp acts in a similar way in classical and lectin pathways as fH does in the alternative pathway by serving as a cofactor in the factor I mediated conversion of C4b to iC4b and C4dg and accelerates the decay of C3 convertases of classical and lectin pathway or binding C4b and decaying C4bC2 complexes and inhibiting the binding of C2 hence preventing the formation of C3 convertase (Blom *et al.*, 2004). Factor H related protein 1 (CFHR1), clusterin and vitronectin are the recently described fluid phase regulators that inhibit the terminal pathway (Schwarz *et al.*, 2008; Heinen *et al.*, 2009; Preissner and Seiffert, 1998). Another plasma enzyme, carboxypeptidase N, acts as the anaphylactic inactivator in blood cleaving the anaphylactic peptides C3a and C5a (Skidgel & Erdos, 2008; Mueller-Ortiz *et al.*, 2009).

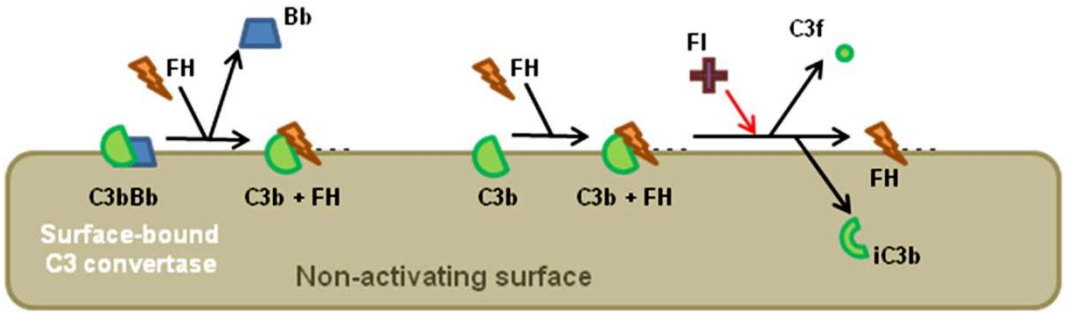


Figure 1.4: Regulatory activity of fH. Decay acceleration activity (left) and factor I cofactor activity (right) (figure from Carroll & Sim, 2011).

1.4.2 Membrane-bound regulators

Human cells expose membrane-bound proteins on the surface which prevent complement activation. CR1 (also known as CD35) and CD 46 (also known as MCP) serve as cofactors to factor I mediated conversion of C3b and C4b into small inactive fragments preventing further activation of complement (Whaley and Schwaeble, 1997; Khera and Das, 2009). CD59 (also known as protectin) binds to C8 and C9 inhibiting the binding of C5b-7 preventing the formation of the MAC (Rollin *et al.,* 1991). CD55 (also known as DAF) dissociates the already formed C3 and C5 convertases (Medof *et al.,* 1984). Complement receptor CR1 regulates the C3b containing C3 and C5 convertases by an unknown mechanism (He *et al.,* 2008)

1.4.3 Surface-attached regulators

The fluid phase regulators like fH, fHL1, C4bp, CFHR1, clusterin and Vitronectin also attach to the cell surfaces and the biomembranes of host and modified cell surfaces such as apoptotic cell surfaces and necrotic bodies. After attaching to the surfaces, these regulators control the complement activation (Manuelian *et al.,* 2003; Ferreira & Pangburn, 2007). Attachment of these regulators to the host surfaces produces an additional protective layer, known as surface zone which further limits the complement on host cells (Perkins *et al.,* 2002). On the other hand, membrane bound regulators such as CD59 and CD46 can be shed from the host cell membranes and can function as complement regulator in soluble forms (Hakulinen *et al.,* 2004)

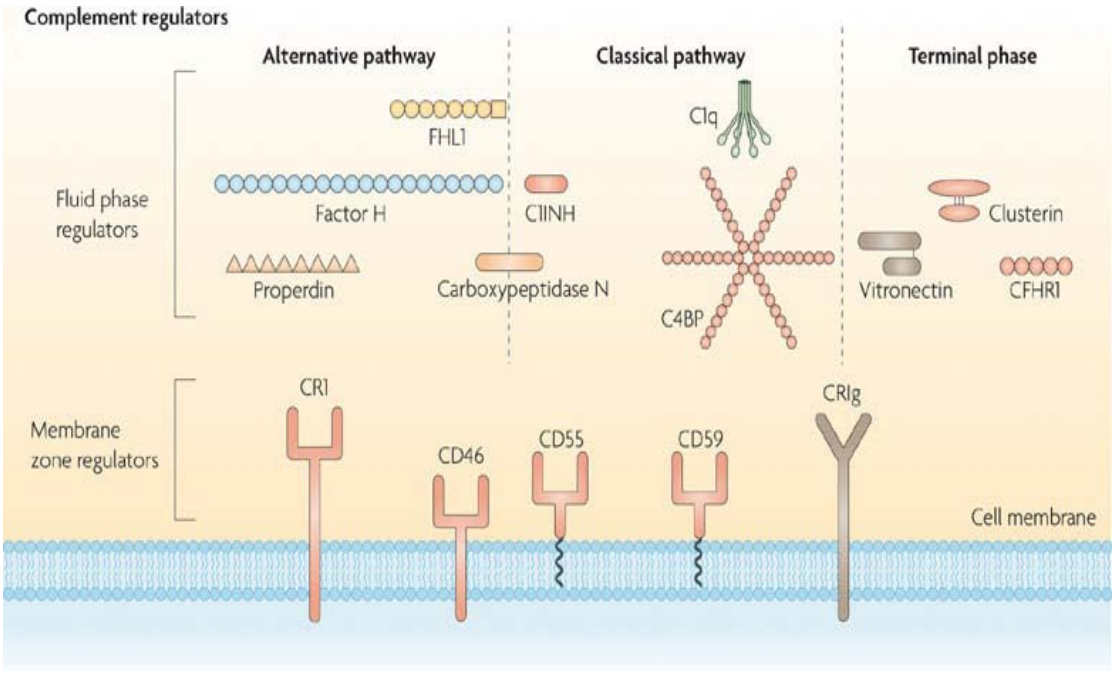


Figure 1.5: An overview of complement regulatory proteins classified into fluid phase and membrane-bound regulators (figure modified after Zipfel & Skerka, 2009)

1.5 Carbohydrate recognition molecules of the lectin pathway

Carbohydrate recognition molecules of the lectin pathway belong to either the collectin family or the ficolin family of proteins and are generally defined by the presence of an N-terminal collagenous domain and C- terminal C-type carbohydrate recognition domain or fibrinogen-like domain (in case of ficolin). Members of collectin family usually include collectin-K1, -L1, -P1, the serum proteins MBLs and the surfactant proteins SP-A and SP-D. Except MBL and collectin-K1, other molecules do not act as lectin pathway recognition molecules as they do not bind MASPs. Collectins involved in recognition of PAMPs on the surface of microbes are normally referred as lectins, a general term for carbohydrate-binding proteins with multivalent sites, which facilitate recognition and aggregation of oligosaccharides and polysaccharide compounds. Lectins, including MBLs and CL-11 have the ability to form complexes with MBL-associated serine proteases (MASPs) that help them in initiating complement against pathogens they recognise by third pathway of complement, the lectin pathway. Ficolins

are the other members of lectins, which are a family of oligomeric proteins similar to collectins. Instead of carbohydrate recognition domain of MBLs and CL-11, ficolins have different C- terminal globular fibrinogen-like domain that recognise PAMPs on microbial surfaces (Endo *et al.,* 2011; Wallis & Lynch, 2007; Hansen *et al.,* 2010).

1.5.1 Mannan-binding lectin (MBL)

Mannan-binding lectin (MBL; also known as mannose binding lectin or mannose binding protein) belongs to the family of collageneous lectins. It was initially described as mannan-binding protein due to its high affinity for mannan, but later discovered that specificity of MBL is not restricted to mannan. It can bind to array of carbohydrates on the surfaces of bacteria, fungi, viruses and multicellular or single cellular parasites that expose key ligands for MBL recognition, including mannose, N-acetyl glucosamine, N-acetyl mannose amine, gluoce and fucose (Kilpatrick *et al.,* 2002). MBL can interact with a wide range of pathogens, including numerous pathogens isolated from immunocompromised children, including *Candida albicans, Aspergillus fumigatus, Staphylococcus aureus, Escherichia coli, Klebsiella* and *Haemophilus influenzae* type b, and beta-hemolytic group A streptococci. There is no evidence of binding of MBL to *Streptococcus pneumoniae*, *Streptococcus sanguis*, *Enterococcus faecalis*, and non-type-b *Haemophilus influenzae.* Binding of MBL was also reported to be poor on the surface of anaerobic bacterial pathogens, showing an inverse proportionality between pathogenicity and MBL binding (Neth *et al.,* 2000; Townsend *et al.,* 2001). MBL can also bind to nucleic acids (Palaniyar *et al.,* 2003). It can also bind to self-derived ligands from apoptotic cells and to regulate immune response (Stuart *et al.,* 2006).

Humans have only one functional *MBL* gene whereas in most of the mammals like rats, mice, rabbit, pigs and rhesus monkeys, MBL is present in two forms expressed by two

distinct *MBL* genes, which share 50% homology in their structures. All forms of MBLs are mainly produced by hepatocytes, although extra hepatic biosynthesis has also been reported in spleen, kidney, muscle, lungs and the brain (Wagner *et al.,* 2003). All forms of MBL circulate in serum and can activate the complement system (Hansen *et al.,* 2000, Wagner *et al.,* 2003).

MBL forms oligomers of homotrimeric subunits. Each subunit is composed of three identical polypeptide chains twisted tightly to each other like a rope in the collagenous region (Fig. 1.6). Each polypeptide chain is composed of 248 amino acids subdivided into four structural domains. N terminal tail region of each polypeptide is comprised of 21 amino acids containing three cysteins which form covalent bonds between each polypeptide that are essential for effective oligomerisation. This domain is followed by a collagen like domain of 59 amino acids. The third domain is a short α-helical neck region comprising of 33 amino acids. The fourth domain comprised 118 amino acids and form the C-terminal carbohydrate recognition domain (Holmskov *et al.*, 2003; Jensenius *et al.,* 2009). Human MBL, as well as mouse MBL-A and C are present as a mixture of higher numbers of oligomers pattern. Human MBL and rodent MBL-A form large oligomers in serum, ranging from dimers to hexamers with trimers and tetramers being predominant. The formation of higher number oligomers is facilitated by three N-terminal cysteines. Rodent MBL-C forms only lower number oligomers and this lack of oligomerisation is due to the presence of only two cystein residues that could form disulfide bridges in the N-terminal region of MBL-C (Phaneuf *et al.,* 2007; Wallis & Drickamer, 1997).

1.5.2 Ficolins

Ficolins are a subfamily of oligomeric carbohydrate binding molecules. Like collectins they have the ability to recognise pathogen associated molecular patterns (PAMPs) on the surface of pathogens. Their basic structure is quite similar to that of MBLs having subunits consisting of both fibrinogen-like carbohydrate binding domains and collagen-like domains giving them the name 'ficolins'. C-terminal globular fibrinogen-like domain implied as the functional domain that recognises PAMPs just like the carbohydrate recognition domain (CRD) of collectins. Collagen like stalks provide a link to immune effectors and like collectins ficolins are thought to bind to surface of collectin receptors of phagocytes (such as CR1 and cC1q receptor/calreticulin on phagocytes) and other effector cells (Matsushita, 2010; Matsushita *et al.,* 2000; Lu *et al.,* 2002). Ficolins can bind a wide array of carbohydrate moieties on microbial surfaces specially N-acetyl polysaccharides such as N-acetyl glucose amine (GlcNAc). Mice have two ficolins, ficolin A and ficolin B. Three types of ficolins are found in humans, including L-, H- and M-ficolins. (Endo *et al.,* 2011; Runza *et al.,* 2008; Garlatti *et al.,* 2010).

1.5.2.1 L-Ficolin

L-Ficolin, (also referred as ficolin L or ficolin 2) was first reported as an oligomeric lectin capable of binding GlcNAc consisting of 35 kDa subunits, each subunit consisting of collagen and fibrinogen like domain. A polypeptide chain of L-ficolin is encoded by *FCN2* gene present on chromosome 9 (9q34) containing eight exons encoding 314 amino acids (Matsushita *et al.,* 1996; Endo *et al.,* 1996). A single polypeptide chain of L-ficolin consists of short N-terminal region with two cysteine residues (Cys7 and Cys 27), a collagen like domain of 69 amino acids with 15Gly-X-Y

(X and Y representing any amino acid) repeats followed by C- terminal fibrinogen like domain of 209 amino acids (Fig 1.6). The fibrinogen-like domain forms a globular structure similar to the carbohydrate recognition domain of MBL. Three polypeptide chains assemble to form a trimer through coiling of collagen like domains whereas an oligomeric structure is formed when four subunits crosslink via disulfide bridges involving Cys7 and Cys27 at N-terminal region resulting in formation of a tetramer with four triple helices formed by 12 subunits giving a "bouquet" like appearance (Holmskov *et al.,* 2003; Hummelshoj *et al.,* 2007). L- ficolin circulates in plasma and mainly expressed in liver. Hepatocytes are the primary source of synthesis. Protein product is secreted into serum with concentration ranging from 1.1 to 12.8 (median 3.7) μg/ml (Kilpatrick *et al.,* 1999; Endo *et al.,* 1996) or 4.15 μg/ml (Le *et al.,* 1998).

L-ficolins specifically recognises acetyl groups of different sugars. Hence it can bind to acetylated sugars including GlcNAc, N-acetyl-mannoseamine, N-acetyl-D-galactosamine (GalNAc), *N*-acetyl-cysteine, N-acetyl-glycine and acetylcholine (Krarup *et al.,* 2005). L-ficolin binding has also been shown to bind lipoteichoic acids (LTAs), a common cell wall constituent of most Gram-positive bacteria (Lynch *et al.,* 2005). It also binds peptidoglycans, a major constituent of bacterial cell wall and 1,3-*β*-D-glucan present on fungal and yeast cells as well as apoptotic cells (Ma *et al.,* 2004; Garlatti *et al.,* 2007). L-ficolin can also bind to DNA exposed by permeable late apoptotic cells and participates in the removal of these cells (Jensen *et al.,* 2007). Recognition of pathogen depends on the presence of these sugars in capsules and cell wall of microbes. L-Ficolin binding has been documented for *Streptococcus pneumoniae* serotype 11F, 11A and 11D as well as *Staphylococcus aureus* serotype 1, 8, 9, 11, and 12 (Krarup *et al.,* 2005; Krarup *et al.,* 2003). L-Ficolin also binds to and

enhances phagocytosis against Group B streptococci (*Streptococcus agalactiae*), and *Salmonella typhimurium* (Aoyagi *et al.,* 2005; Matsushita *et al.,* 1996).

1.5.2.2 H-Ficolin

H-ficolin (often referred as ficolin 3, ficolin H or Hakata antigen) was the first ficolin discovered, initially recognised as auto-antigen that reacted with autoantibodies present in sera of systemic lupus erythematosus patients (Epstein and Tan 1973). H-ficolin (*FCN3*) mRNA is expressed in liver and lung. The primary sources of H-ficolin biosynthesis in the liver are hepatocytes as well as bile duct epithelial cells (from where it is secreted into serum and bile) (Aikawa *et al.,* 1999). H-ficolin is also synthesised in lungs by ciliated bronchial epithelial cells and type II alveolar epithelial cells and secreted into bronchus and alveolus. The relative *FCN3* expression level is higher in the lung as compared to liver. H-ficolin production has also been reported in glioma cell lines (Hummelshoj *et al.,* 2007a&b; Kuraya *et al.,* 2003). The serum concentration of H-ficolin has been determined, and the values are somewhat inconsistent. One report gives an average concentration of 18.4 μg/ml (Krarup *et al.,* 2005), another 4.5 μg/ml (Kilpatrick *et al.,* 2003), 24 μg/ml (Munthe-Fog *et al.,* 2008), 26 μg/ml (Schlapbach *et al.,* 2010) and another value gives an average value of 7-23 μg/ml (Yae *et al.,* 1991). H-ficolin is present in serum as homopolymer of 35 kDa-subunits. The H-ficolin gene *FCN3* is located on chromosome 1 (1p35.3) and is composed of eight exons encoding 299 amino acids. Each monomeric chain is comprised of an N-terminal region (24 amino acids) followed by a collagen-like domain (33 amino acids) composed of 11 Gly-X-Y repeats, followed by a neck region and a C- terminal fibrinogen like domain (207 amino acids) (Fig 1.6). The C- terminal fibrinogen like domain of H-Ficolin has 84% homology with that of L-ficolin while the overall homology between L-ficolin and

H-ficolin is 48%. H-ficolin is present in serum as an octadecamer consisting of an elementary trimeric unit (Sugimoto *et al.,* 1998).

H-ficolin shows binding to GalNAc, GlcNAc and D-fucose. H-ficolin agglutinates erythrocytes coated with lipopolysaccharides (LPS) extracted from *Salmonella typhimurium* and *Salmonella minnesota.* (Sugimoto *et al.,* 1998). Role of H-ficolin in pathogen recognition is somewhat limited. H-ficolin binding to pathogens has only been documented for *Aerococcus viridans* (Tsujimura *et al.,* 2001 and 2002). No H-ficolin binding was detected with different strains of *Streptococcus aureus* and *Streptococcus pneumoniae* (Krarup *et al.,* 2005). H-ficolin also binds to apoptotic cC1q receptors on apoptotic Jurkat cells and participates in the clearance of endogenous apoptotic cells (Kuraya *et al.,* 2005).

1.5.2.3 M-ficolin

M-ficolin (often referred as Ficolin 1 or ficolin M) is a pattern recognition molecule of the lectin pathway of complement system. mRNA of M-ficolin is expressed in human peripheral monocytes, alveolar epithelial cells of lungs, spleen and bone marrow. It is the secretory protein and has been found in secretory granules in the cytoplasm of peripheral neutrophis, type II alveolar epithelial cells of lungs and monocytes (Lu *et al.,* 1996; Liu *et al.,* 2005). M-ficolin used to be considered as non-serum ficolin, but recent studies demonstrated that it can be detected in the circulation at an average concentration of 60.5 ng/ml (Honore *et al.,* 2008), with another study reporting an average serum concentration of 1.07 μg/ml (Wittenborn *et al.,* 2010). Its serum level is therefore, much lower than that of the other two human ficolins. The *FCN1* gene is located on chromosome 9 (9q34) encoding 326 amino acids. The *FCN1* gene has very similar exon organisation to that of *FCN2* gene (encoding L-ficolin). The M-ficolin

gene has an extra exon that encodes 4 additional Gly-X-Y repeats. Basic protein structure of M-ficolin is 76% and 48% homologous to L-ficolin and H-ficolin respectively (Endo *et al.*, 1996). M-ficolin shows very strong binding activity towards acetylated sugars, including GlcNAc and GalNAc (Liu *et al.*, 2005), sialic acids (Frederiksen *et al.*, 2005; Gout *et al.*, 2010) and N-acetyl BSA (Hein *et al.*, 2010). With regards to pathogen recognition, M-ficolin binding has been reported for *Staphylococcus aureus*, smooth type of *Salmonella typhimurium* (Liu *et al.*, 2005) and *Streptococcus agalactiae* (Kjaer *et al.*, 2011). M-ficolin also works as an opsonin. A polyclonal antibody against M-ficolin inhibits the phagocytosis of *Escherichia coli* by U937 cells suggesting the role of M-ficolin binding to a phagocytic receptor on the surface of circulating monocytes (Teh *et al.*, 2000). M-ficolin also binds to activated, but not resting T-lymphocytes. Activation of T-lymphocytes results in a significant up-regulation of sialic acid residues that enhance the binding with M-ficolin. This binding was decreased by treating monocytes with sialidase (Honoré *et al.*, 2010).

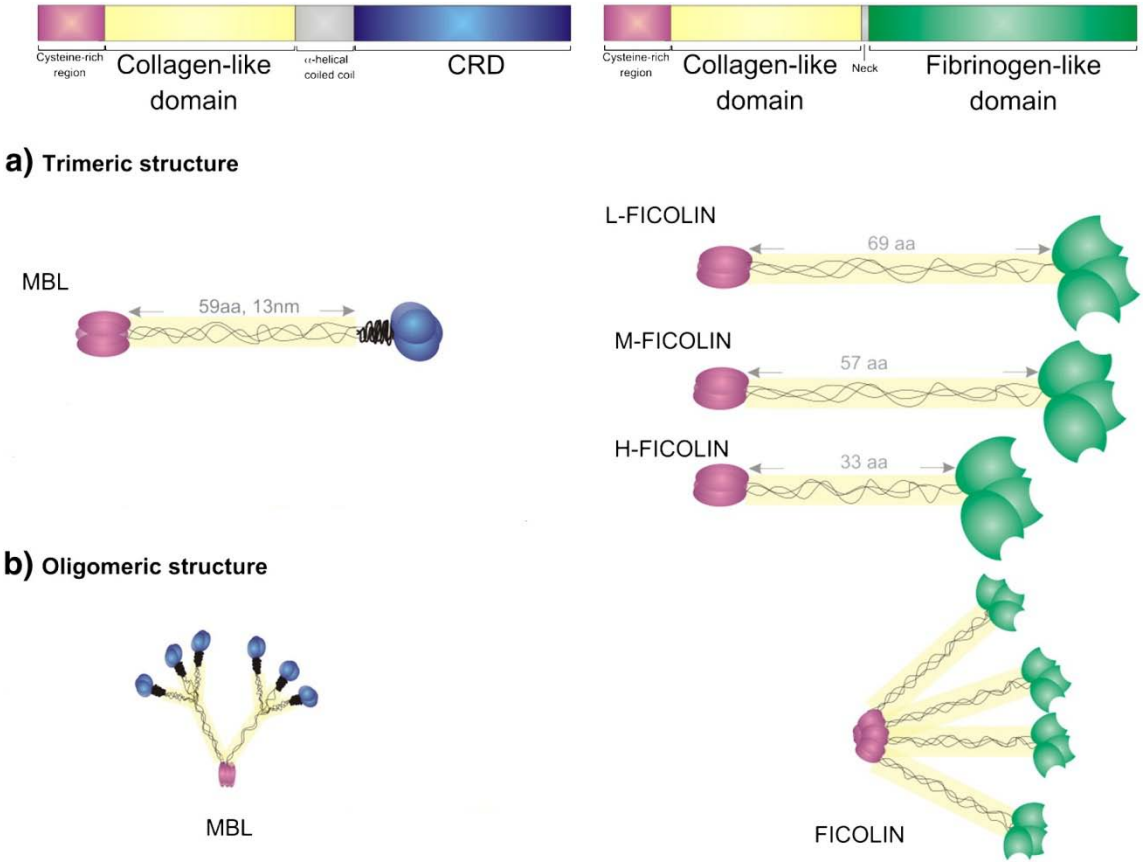


Figure 1.6: Domain structure, trimeric structure, and oligomeric structures, of the lectin pathway recognition molecules. Cysteine-rich region is shown in pink, collagen like domain in yellow, alpha helical coiled region in grey, CRD of MBL in blue and fibrinogen like domain of ficolin in green (figure modified after Yongqing *et al.,* 2012).

1.5.2.4 Murine ficolin A

Ficolin A is the only circulating ficolin in mice and rats. It is encoded by *FcnA* gene, which was first isolated from a mouse liver library. The mRNA of ficolin A is highly expressed in liver and spleen, and protein is present in plasma with a molecular mass of 37kDa (Fujimori *et al.,* 1998). mRNA is also expressed in macrophages in both adult liver and spleen and has been found localised in the lining of hepatic sinusoids in the liver and red pulp of spleen using *in situ* hybridization. Ficolin A mRNA is expressed during early embryonic stage. It starts to increase in liver as the development proceeds, peaks during birth and slightly declines during adult stage suggesting a potential role of ficolin A during prenatal and postnatal stages (Liu *et al.,* 2005b). Ficolin A is often

referred as mouse orthologue of human L-ficolin. Studies of protein structure of ficolin A show that ficolin A shares 80% amino acid identity with other ficolins, i.e. human L-ficolin, M-ficolin and mouse ficolin B (Ohashi & Erickson, 1998; Endo *et al.,* 2004; Endo *et al.,* 2011). *Fcna* gene is located on chromosome 2 (2A3) containing 1002 base pairs, consisting of 10 exons encoding 334 amino acids. First exon encodes single peptide, exons 2, 3 and 4 encode the collagen like region, exons 5 and 6 encode the neck sequence and last four exons; 7, 8, 9 and 10 encode the fibrinogen like domain (Fig. 1.7) (Fujimori *et al.,* 1998; Endo *et al.,* 2004; Endo *et al.,* 2005). Ficolin A is present in serum as a tetramer of homotrimeric chains, (i.e. 12 subunits) forming a parachute like structure (Ohashi and Erickson 1998). Ficolin A binds to *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and elastin (Endo *et al.,* 2005; Fujimori *et al.,* 1998; Girija *et al.,* 2011). At present, there is no data available about pathogen recognition of ficolin A.

1.5.2.5 Murine ficolin B

Ficolin B is one of the two murine ficolins, first characterised in 1998. The mRNA of ficolin B is highly expressed in bone marrow and weakly expressed in spleen (Ohashi and Erickson 1998). Ficolin B mRNA has also been detected in lysosomes of mouse peritoneal exudate macrophages and expression is up regulated upon activation of these macrophages (Runza *et al.,* 2006). Ficolin B expression has also been reported in liver tissue during development at a developmental stage where the liver fulfils bone marrow functions (hepatolienal phase), but after birth, the expression in liver tissue regresses dramatically, indicating that ficolin B is not made by hepatocytes (Liu *et al.,* 2005b). Ficolin B is encoded by *Fcnb* gene located close to the chromosomal region where *Fcna* gene is located (Chromosome 2A3). The exon structure of *Fcnb* gene resembles

that of the *Fcna* gene (Fig. 1.7). Exon 5 is missing in the *Fcnb* gene. However, exon organisation of *Fcnb* gene shows a lot of similarities to the organisation of the human *FCN1* gene. Ficolin B is therefore considered mouse orthologue of human M-ficolin. The first exon encodes a single peptide. Exons 2, 3 and four encode the collagen like domain. Exon 5 encodes the neck region, whereas exons 6, 7, 8 and 9 encode the fibrinogen-like domain (Endo *et al.*, 2004 and 2011). Ficolin B can recognise acetylated sugars, including GlcNAC and GalNAc and can (like its human orthologue) bind specifically to silalic acid residues (Endo *et al.*, 2005; Girija *et al.*, 2011). Like ficolin A, pathogen recognition studies have not been documented for ficolin B yet. Murine ficolin B was first considered as the only ficolin that is not capable of associating with MASPs and activate the lectin pathway of complement (Endo *et al.*, 2005). However, recent studies reported that rat ficolin B could bind to MASP-2 and can activate MASP-2 on target surface to activate the lectin pathway of complement although with two fold lower activity than murine ficolin A (Girija *et al.*, 2011).

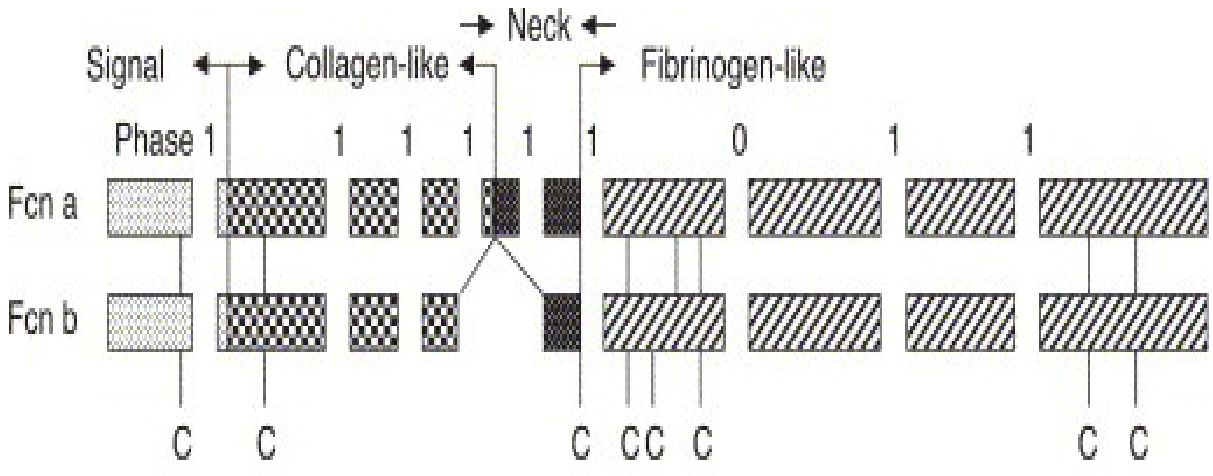


Figure 1.7: Exon-entron structure of genes encoding ficolin A and ficolin B. The phases of entron insertion are indicated by 0 and 1. Cystein residues conserved in amino acid sequences are represented by C (figure modified after Endo *et al.,* 2004).

1.5.3 Collectin 11

Collectin 11 (CL-11) or Collectin kidney (CL-K1) is a newly described member of the collectin family. It was first identified and characterised by Keshi *et al*. in 2006. CL-11 mRNA was detected in all human organs with a very high degree of expression in kidney tissue, a reason why it was initially named Collectin kidney (CL-K1) (Keshi *et al.,* 2006). Later studies confirmed the high level of expression of CL-11 in human adrenal glands, the liver and the kidney (Hansen *et al.,* 2010). Abundant CL-11 mRNA expression was detected in nearly all murine tissue tested with the highest levels of expression in heart and a relatively high level of expression in liver, testis, white adipose tissues, brain and kidney (Motomura *et al.,* 2008). CL-11 is present in many species. Its structure is highly conserved amongst different species and the degree of identity at amino acid level ranges from 72-98%. CL-11 is encoded by *COLLEC11* gene on human chromosome 2. Like other collectins, CL-11 is composed of four domains, the N- terminal domain comprising 15 amino acids, followed by 72 amino acid collagen-like region, a 34 amino acid α-helical coiled coil and 125 amino acid

long carbohydrate recognition domain (CRD). The carbohydrate recognition domain of CL-11 shares 54% homology with the CRD of MBL. CL-11 is present in both human and mice serum with serum concentration of approximately 2.1 μg/ml in humans. In human serum CL-11 has been isolated as a 34 kDa protein under reducing conditions. Under non-reducing conditions, CL-11 runs as a 100 and a 200kDa band indicating that CL-11 is present in serum in form of dimeric of trimeric subunits (Hansen *et al.*, 2010). CL-11 shows strong binding affinity to L-fucose, D-mannose, and α-methyl-D-mannose and weak binding with *N*-acetyl-D-mannosamine and D-glucose (Hansen *et al.*, 2010; Keshi *et al.*, 2006). CL-11 can recognise a broad range of microorganisms, including bacteria, fungi and viruses and specific CL-11 binding has been documented for *Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and influenza A virus. CL-11 also complexes with MASP-1 and/or MASP-3 indicating its potential role in complement activation. Interaction with MASPs and its pathogen recognition abilities provides a strong indication that CL-11 might play a role in the innate immune defence as a pathogen recognition molecule of the lectin pathway along with MBLs and ficolins (Hansen *et al.*, 2010).

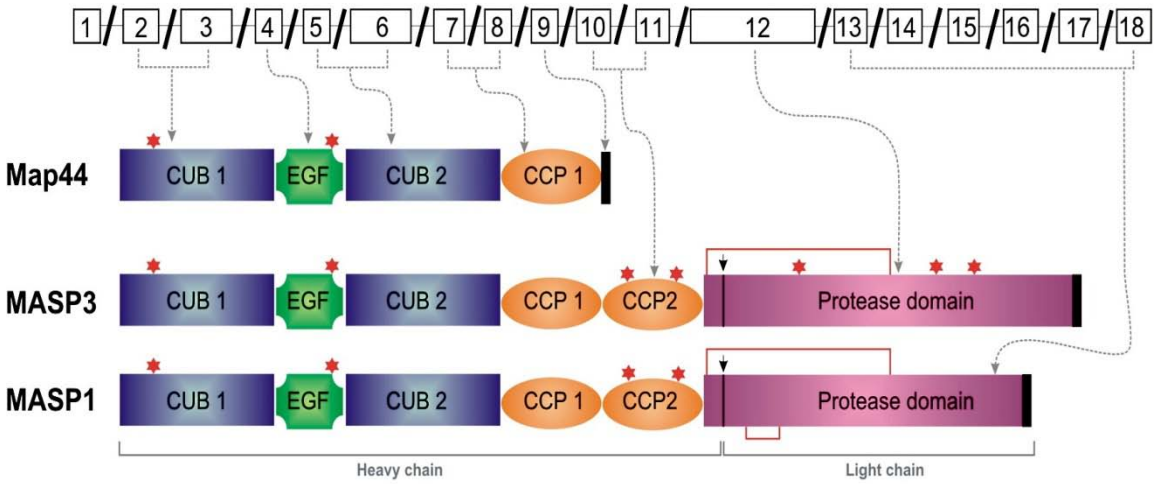
1.6 Serine proteases of the lectin pathway

The carbohydrate recognition molecules of lectin pathway associate with all members of recently described serine protease super family called MASPs, which comprise the proteolytic enzymes of the lectin activation pathway. These include MASP-1, MASP-2 and MASP-3 (Fujita *et al.*, 2004). PRMs of the lectin pathway are also found associated with non-enzymatically active truncated gene products such as MAp19 alias small MBL-associated proteins (sMAP) and Map44 alias MBL/ficolin associated protein 1. Map19 is a truncated alternative sliced product of *MASP2* gene and later a truncated

product of *MASP1/3* gene. Both of these products are non-proteolytic and compete with MASP-1, -2 and -3 for the binding to lectin pathway recognition complexes to regulate lectin pathway activation of complement (Schwaeble *et al.,* 2002; Takahashi *et al.,* 1999; Iwaki *et al.,* 2006; Degn *et al.,* 2009; Skjoet *et al.,* 2010). This lectin pathway specific serine proteases share structural resemblance to the serine protease of the classical pathway, i.e. C1r and C1s. MASPs are normally present in serum in their zymogen form and are converted into their active form to drive further downstream activation events (Fujita *et al.,* 2002; Wallis *et al.,* 2010)

All MASPs and C1r and C1s share an identical domain organisation composed of six domains (Fig 1.8). Serine protease domain is at C- terminus. The other five domains are non-catalytic modules involved in the interaction with the carbohydrate recognition domains. The domain structure includes an N-terminal CUB domain followed by an epidermal growth factor like domain (EGF), another CUB domain (CUB II) and two domains described for complement control protein (CCP) (Gal *et al.,* 2007).

A.



B.

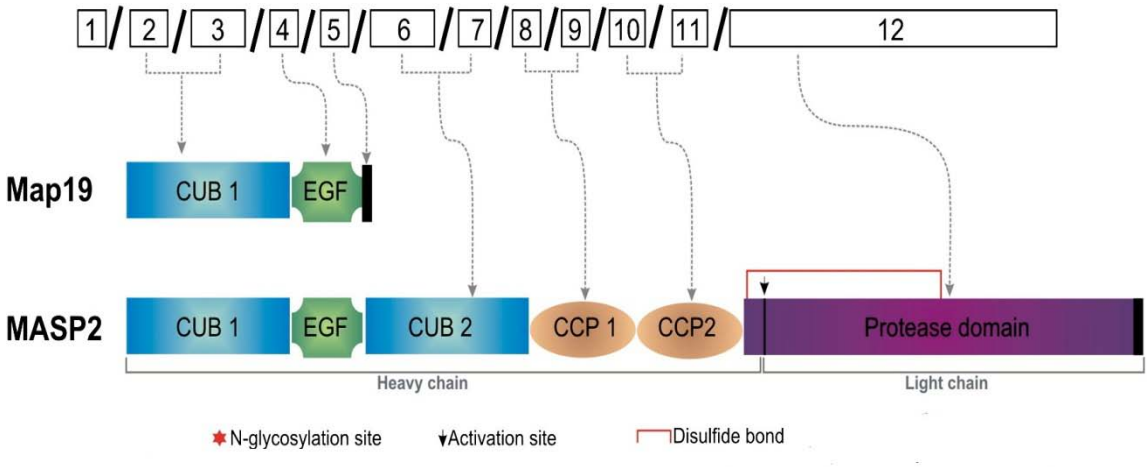


Figure 1.8: Genomic organisation of *MASP1/3* gene (a), *MASP2* gene (b), and resulting protein structures (figure modified after Yongqing *et al.*, 2012).

1.6.1 MASP-1

MASP-1 was the first serine protease of the lectin pathway isolated in 1992 and was regarded as only serine protease associated with MBL (Matsushita and Fujita, 1992). MASP-1 is encoded by the *MASP1/3* gene located on chromosome 3q27-q28 in humans and chromosome 16B2-B3 in mice by alternative splicing (Fig. 1.8) (Dahl *et al.*, 2001; Stover *et al.*, 2003). mRNA of MASP-1 is highly expressed in hepatocytes

(Seyfarth *et al.,* 2006) but also in extra hepatic sites (Stover *et al.,* 2003; Lynch *et al.,* 2004). Initially, the serum concentration for MASP-1 was reported to be in the range of 6.27 μg/ml (Terai *et al.,* 1997). It was, however, later discovered that this value includes other derivates of the *MASP1* gene, and the actual concentration is around 1μg/ml (Degn *et al.,* 2010). Before the discovery of MASP-2, MASP-1 was regarded as the key enzyme of lectin pathway activation (Thiel *et al.,* 1997). One factor that limits the role of MASP-1 as the major serine protease of the lectin pathway is that unlike MASP-2 (which cleaves both C4 and C2), MASP-1 can only cleave C2 but not C4. Thus, it has a supporting role in augmenting lectin pathway activation, but its absence does not affect the formation of the lectin pathway C3 and C5 convertases C4b2a and $C4b2a(3b)_n$ (Matsushita *et al.,* 2000; Rossi *et al.,* 2001; Møller-Kristensen *et al.,* 2006). Recent studies have proposed that MASP-1 may be involved in the activation of MASP-2 (Takahashi *et al.,* 2008), but the question remains unanswered why MASP-1 would be important for activation of MASP-2, if MASP-2 can auto activate itself (Gal *et al.,* 2005). Some studies have speculated that MASP-1 can directly cleave C3 (Matsushita *et al.,* 2000). However, later studies further clarified that it can only cleave $C3_{H2O}$ but not active C3 (Ambrus *et al.,* 2003). Even then, this cleavage occurs in negligible amount and is not sufficient to activate complement system on its own (Rossi *et al.,* 2001; Ambrus *et al.,* 2003). Most recent reports demonstrated the role of MASP-1 as an essential enzyme for the alternative pathway activation cascade as it is essential to convert factor D from its zymogen form into its active form. This was demonstrated by showing that $MASP1/3^{-/-}$ mice lacked alternative pathway functional activity as fD in $MASP1/3^{-/-}$ mice serum was only present in its enzymatically inactive zymogen form (Takahashi *et al.,* 2008; Takahashi *et al.,* 2010; Banda *et al.,* 2010).

1.6.2 MASP-2

MASP-2 was first described in 1997 as an enzyme with structural homology to MASP-1 and the serine proteases of the classical pathway, i.e. C1r and C1s. It was shown to form complexes with MBL and to activate antibody independent complement pathway (Thiel *et al.,* 1997). Structural and functional similarities of MASP-2 and C1s were later studied in detail, and it was shown that both proteases share the same substrates, i.e. C4b and C4b bound C2 to form C3 the convertase complex C4b2a (Vorup-Jensen *et al.,* 2000). Later work, however, showed that MASP-2 can cleave other substrates, including prothrombin, factor X and factor XII. The activation of MASP-2 differs from that of C1s. MASP-2 can be autoactivated, while C1s needs to be cleaved by C1r (Krarup *et al.,* 2007). The *MASP2* gene is located on human chromosome 1p36.3-2 and on chromosome 4 in mice. MASP-2 biosynthesis and its expression is exclusively restricted to hepatocytes (Stover *et al.,* 1999; Stover *et al.,* 2004; Endo *et al.,* 2002) and has been detected at serum concentration of 0.5 μg/ml (Thiel *et al.,* 1997) or 170-1196 ng/ml (Møller-Kritensen *et al.,* 2003). MASP-2 is synthesised as a proenzyme. It can form complexes with MBLs, CL-11 and ficolins. When MBLs, CL-11 and ficolins bind to carbohydrate moieties on microbial surfaces, the conformational change in the carbohydrate recognition component catalyses the auto-activation of MASP-2 resulting in the break of the arginine-isoleucine peptide bond at the N-terminal of the serine protease domain and subsequently cleaves C4 and C4b bound C2 leading to the formation of the lectin pathway C3 convertase C4b2a (Presanis *et al.,* 2004). MASP-2 is composed of three N-terminal non-catalytic domains comprising CUB1-EGF-CUB2 and three catalytic domains comprising CCP-1-CCP-2-SP. Two main functions are attributed to non-catalytic domains. This part of MASP-2 is responsible for the formation of homodimers through binding interaction between CUB1 to the EGF

domain of the opposed and antiparallel oriented second binding partner to form a MASP-2 zymogen homodimer with both binding partners attached in antiparallel orientation. The second function of the non-catalytic domain is to form the MASP-2 homodimer with the carbohydrate recognition interaction site of the complement in a calcium dependent manner which also occurs through CUB-1 and EGF domains. EGF has binding site having affinity for calcium ions, which protects against proteolysis and keeps the domain orientation stable (Thielens *et al.,* 2001; Wallis *et al.,* 2000). In catalytic domain SP is responsible for proteolytic activity of MASP-2 whereas CCP1 and CCP-2 helps to increase the stability of SP domains (Harmat *et al.,* 2004).

1.6.3 MASP-3

MASP-3 is the most recently discovered lectin pathway specific serine protease first described by Dahl *et al.,* as an alternative splice product of the *MASP1/3* gene (which also encodes MASP-1). MASP-3 shows a high degree of similarity with MASP-1. The first 5 domains are 100% identical whereas as SP domains share only 32% similarity between MASP-1 and MASP-3. Very little is known about enzymatic activity of MASP-3. It does not autoactivate and shows no enzymatic activity towards C4, C2 and C3. It was therefore, initially suggested that MASP-3 might downregulate MASP-2 by competing for its binding sites (Dahl *et al.,* 2001; Zundel *et al.,* 2004; Moller-Kristensen *et al.,* 2007). Unlike MASP-1 and MASP-2 (which are primarily expressed in the liver), expression of MASP-3 mRNA has been detected in a wide range of tissues, including pancrease, skeletal muscle, spleen, thymus, prostate and ovary in addition to liver (Lynch *et al.,* 2005; Seyfarth *et al.,* 2006). MASP-3 is present in serum at a concentration of approximately 5.2 μg/ml (Degn *et al.,* 2010), but some reports calculate an average concentration of 6.4 μg/ml (Skjoedt *et al.,* 2009). The biological

functions of MASP-3, however, have remained largely unknown. Several studies have identified substrate specificities of MASP-3 with components unrelated to the complement system (Cortesio & Jiang, 2006). Interestingly, a most recent study described a role of MASP-3 in the initiation of the alternative pathway by catalyzing the conversion of C3bB to C3bBb, the alternative pathway specific C3 convertase (Iwaki *et al.,* 2011).

1.7 Formation of lectin pathway specific carbohydrate recognition complexes with MASPs

Circulating MBLs, ficolins and CL-11 are forming complexes with MASPs and trigger the lectin activation pathway of complement. MASPs are normally present as zymogens and circulate in the inactive form bound to lectins. When lectins bind to their ligands on the surface of pathogens, MASP-1 and MASP-2 are auto-activated through cleavage between CCP and C-terminal protease domain forming heavy H-chain and light L-chain connected via disulfide bridges (Takahashi *et al.,* 1999; Zundel *et al.,* 2004; Hansen *et al.,* 2010). MBL can form complexes with either MASP-1, -2 or -3 and the truncated expression product of *MASP2* gene called MAp19 or the truncated expression product of *MASP1/3* gene called MAp44. The formation of the lectin pathway C3 and C5 convertases is strictly MASP-2 dependent. All MASPs are present as homo dimers bound to each other in a head to tail configuration held together by binding of the CUBI to the EGF domain of the anti parallel binding partner. This binding interaction is stabilized by Ca^{+2} bound near the end of the terminal of EGF-like domain (Wallis *et al.,* 2004; Feinberg *et al.,* 2003). MASPs bind to the collagenous domains of MBL through CUB1-EGF-CUB2 domain. The MBL binding site has been located near the Ca2+ binding site near the C terminal end of the CUB1 domain. This

means that each MASP dimer has binding sites for two MBL subunits, meaning that an MBL dimer or trimer can bind to a single MASPs dimer (Chen and Wallis 2001). Like MBL, CL-11, all human ficolins and murine ficolin A can form complexes with MASPs and activate the lectin pathway (Matsushita *et al.,* 2000; Schwaeble *et al.,* 2011). Mouse ficolin B does not form complexes with MASPs (Endo *et al.,* 2005), whereas rat ficolin B has recently been reported to be associated with MASP-2 and activating lectin pathway (Girija *et al.,* 2011). CL-11 can form complexes with MASP-1 and/or MASP-3 (Hansen *et al.,* 2010) as well as with MASP-2 (see Ali *et al.,* 2012 addendum 2 Fig. S1).

1.8 *Streptococcus pneumoniae*

1.8.1 General characteristics

Streptococcus pneumoniae, also referred to as pneumococcus is a Gram positive bacterium which is usually present in pairs (diplococci), but also capable of forming short chains. Cells of *S. pneumoniae* are either of oval or spherical shape with a diameter ranging from 0.5-1.25 μm. *S. pneumoniae* do not form spores and are non motile. The cell wall is surrounded by a polysaccharide capsule that acts as a virulence factor of the organism. *S. pneumoniae* is an aero-tolerant anaerobic bacterium and grows best at 5-10% CO_2 in a medium as a source of catalase like blood or serum at 37^{o}C. *S. pneumoniae* can be identified by α-hemolysis on blood agar. In the liquid medium, the pneumococcal cells grow diffusely as single cells, diplococci and short chains. The optimal temperature range for growth is 25-42 ^{o}C. *S. pneumoniae* are Optochin sensitive, a property used to differentiate these bacteria from other streptococci (Weiser *et al.,* 1994; Sternberg 1881; Mims 1998).

1.8.2 History

Streptococcus pneumoniae was first isolated and grown by George Sternberg and Louis Pasteur in 1880 working simultaneously and independently. Louis Pasteur found the bacteria in the saliva of a patient with rabies, whereas Sternberg isolated bacteria from his own saliva (Sternberg, 1881). Due to its diplococcus nature and its nature as an etiological agent for pneumonia, the organism was termed *Diplococcus pneumoniae* in 1924 (Winslow *et al.,* 1920) which was the accepted terminology until 1974, after which it was re-classified as *S. pneumoniae* due to its growth in chains in liquid media.

It has been commonly known as pneumococcus due to its role as the etiological agent of pneumonia. *S. pneumoniae* played an important role in the discovery of a variety of scientific principles, including vaccination, the development of antibiotic drugs such as Penicillin and has played a role as model organism to demonstrate that genetic material consists of DNA (Avery *et al.,* 1944; Lederberg, 1994).

1.8.3 Impact of pneumococcal disease on human health

Streptococcus pneumoniae infections still remain one of the most important causes of morbidity and mortality in adults and children throughout the world (Varon *et al.,* 2010). It is the most common respiratory pathogen causing variety of invasive and non-invasive diseases. Invasive diseases refer to the presence of pathogen in sterile components of the body, i.e. blood, pleural fluid and cerebrospinal fluid causing pneumonia, septicemia, osteomyelitis, endocarditis, peritonitis, cellulitis, brain abscesses and meningitis, whereas non-invasive disease includes otitis media and sinusitis (Fig. 1.9) (Weiser, 2010; Daniel & Musher, 2003). *S. pneumoniae* is the most frequent cause of community-acquired pneumonia and remains the leading cause of morbidity and mortality claiming for over a million deaths each year of which the major part includes children in developing countries (Scott, 2008; Lim *et al.,* 2001; Williams *et al.,* 2002). *S. pneumoniae* can cause the disease in all age groups, but individuals at high risk are children below the age of five years and senior individuals over 65 years old. The prevalence of infection is high in individuals with underlying diseases such as HIV infection, asthma, cancer and preceding viral infections specially influenza virus infection (Klugman *et al.,* 2007; Mullholland, 1999). *S. pneumoniae* has been the main cause of child death in developed countries, including the United Kingdom and the United States (Lim *et al.,* 2001). Improved living standards in 20th

century largely decreased the mortality caused by pneumonia even before introducing antibiotic treatment and vaccination. Introduction of antibiotics in 1930s further decreased the mortality significantly (Grove *et al.,* 1968; Mulholland *et al.,* 2007). However, pneumococcal pneumonia still remains the major cause of child death in developing countries of Asia and Africa with a mortalility rate of 60-100 per 1000 children under five years of age (Rundan *et al.,* 2004; UNICEF, 2007). The difference in incidence rates in developed and developing countries is thought to be poor socio-economic standards and malnutrition (Greenwood, 1999).

1.8.4 Serotypes of *Streptococcus pneumoniae*

Based on the differences in capsular polysaccharides and their unique serological profiles, more than 90 serotypes classified into 46 serogroups have been described so far (Henrichsen, 1995; Park *et al.,* 2007; Calix & Nahm, 2010). Chemical difference in capsular polysaccharides and recognition of these structural differences by specific antibodies produced after immune response elicited in rabbits against the antigen of each different type are used as the basis to distinguish serotypes (Shapiro, 1991). *Streptococcus pneumoniae* serotypes are classified by two classification systems, the Danish system and the American system. The Danish system classifies *S. pneumoniae* on the basis of cross reaction between different types. Serologically cross reactive serotypes are allocated in common serogroup. In the American system, *S. pneumoniae* are sequentially classified based on the order of their discovery, ignoring the antigenic cross reactivity among different types (Lund, 1970; Henrichsen, 1979; Henrichsen 1995).

The basic technique used for serotyping of *S. pneumoniae* is called quelling reaction and relies on the swelling of the capsule after a homologous antibody binds to it. A loop

full of colonies is mixed with equal quantity of specific anti serum and examined under 1000x for capsular swelling. This technique has been used to identify more than 90 described serotypes (Lund, 1960; Heineman, 1973; Henrichsen, 1995). Recently, several new approaches have been developed to identify the serotypes. The PCR based systems analyse the DNA sequences of capsular polysaccharide synthesis gene locus (*cps*), containing the genes for the capsule biosynthesis. (Brito *et al.,* 2003; Kong *et al.,* 2005; O'Halloran and Cafferkey, 2005). Multiplex immune assay for capsular polysaccharides involves the identification of vaccine-related serotypes by using as set of monoclonal antibodies (Yu *et al.,* 2008). A new serotyping system has been developed recently combining the PCR- and mAb-based approaches using the flow cytometric bead array technology (Yu *et al.,* 2011)

1.8.5 Virulence factors

Streptococcus pneumoniae produces a variety of virulence factors surrounding the cell (i.e. Capsular polysaccharide), surface proteins and enzymes, or intracellularly produced toxins (i.e. pneumolysin) all of which play a role in the pathogenesis of infection. The ability to produce virulence factors differs between the strains of *S. pneumoniae*. This variation in producing virulence factors corresponds to the ability of strains to cause different types of diseases (Mitchell & Mitchell, 2010). Virulence factors interfering with complement proteins will be discussed here in detail whereas other important virulence factors are listed in the description of the pathogenesis of pneumococcal infections.

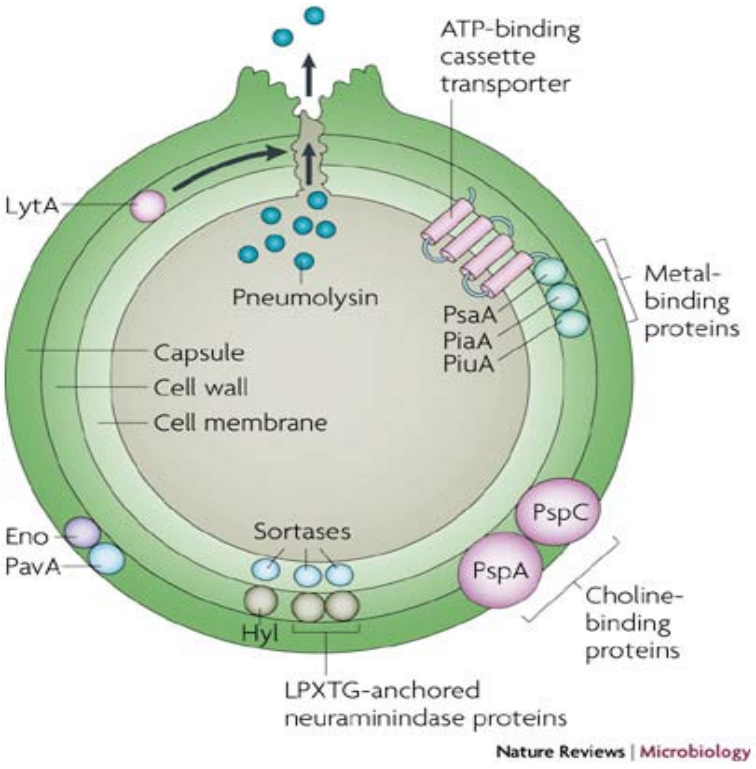


Figure 1.9: Major virulence factors of *Streptococcus pneumoniae* (figure taken from Kadioglu *et al.*, 2008).

1.8.5.1 The capsule

The most important virulence factor is the capsular polysaccharide (CPS) which forms a 200-400 nm thick outer most layer of *S. pneumoniae* cells (Sorensen *et al.*, 1988; Kadioglu *et al.*, 2008). A total of 90 serotypes have been described that serologically differ from each other depending on the variation of capsular polysaccharides (Henrichsen 1995). The capsule is attached covalently to the peptidoglycan of the cell wall in all serotypes except serotype 3 (Sorensen *et al.*, 1990). The major role of the capsule in mediating virulence is the prevention of phagocytosis. All the serotypes have the ability to reduce opsonophagocytosis regardless of differences in the capsule (Jonsson *et al.*, 1985). The degree of inhibition of phagocytic activity varies between serotypes depending on the nature of capsular polysaccharide (Melin *et al.*, 2010).

Antibodies against cell wall constituents including, teichoic acids and cell surface proteins attached to surface of *S. pneumoniae* and bind complement (Winkelstein *et al.,* 1981; Musher, 1992). Another factor responsible for reduced phagocytosis is the net negative charge of the capsule at physiological pH, which electrostatically repels bacteria from phagocytic cells (Lee *et al.,* 1991; Magnusson, 1989). The capsule also adds to the virulence of *S. pneumoniae* due to its adhesive function (López & García, 2004). The capsule is essential for colonisation and helps to escape from the mechanical removal by mucus (Nelson *et al.,* 2007). It also limits autolysis and reduces exposure to antibiotics (Van der Poll and Opal 2009). The virulence of *S. pneumoniae* differs significantly between capsular serotypes (Austrian, 1997). One factor contributing to the variation in capsular polysaccharide impacts the accessibility of bacterial surface adhesions, which interact with host ligands, affecting the virulence of bacterial cells (Carlos *et al.,* 2011). The ability of particular strain or serotype to cause disease often depends on the thickness of the capsule. Non-capsulated strains are less virulent as compared to encapsulated strains (MacLEOD & Kraus, 1950; Hostetter *et al.,* 1986), however, capsule itself is non-inflammatory (Mitchell *et al.,* 1997). Virulent clinical isolates recovered from sterile sites are encapsulated. Spontaneous non-encapsulated derivatives of these strains have been found to be almost completely avirulent (Lee *et al.,* 1991; Briles *et al.,* 1992). However, some non-encapsulated strains have been found associated with superficial infections like conjunctivitis (Martin *et al.,* 2003). The degree of complement deposition has been shown to be correlated with the capsular type. The capsule impairs C3 deposition on the bacterial surface and conversion of surface bound C3b to iC3b (Abeyta *et al.,* 2003; Hyams *et al.,* 2010; Melin *et al.,* 2010b).

1.8.5.2 Pneumolysin (PLY)

Pneumolysin is a broadly studied virulence factor of *Streptococcus pneumoniae* found virtually in all pneumococcal isolates. Pneumolysin is produced as a soluble 53-kDA protein and expressed during the late log phase. Pneumolysin is released when cells undergo autolysis (Benton *et al.,* 1997; Berry *et al.,* 1989). Pneumolysin is released as monomer and kills target cells by forming pores on membranes containing cholesterol. Pneumolysin pores are assembled of single monomers to form large oligomeric rings (Tilley *et al.,* 2005). Besides the cytolytic activities, these oligomers are involved in a range of potentially harmful activities at sublytic concentrations including inhibition of ciliary beating on respiratory epithelium, inhibition of phagocyte respiratory burst, and induction of cytokine synthesis and $CD4^+$ T-cell activation, and chemotaxis (Hirst *et al.,* 2004; Kadioglu *et al.,* 2004). The role of pneumolysin in pathogenesis has been explored using a pneumolysin-negative mutant, PLN-A, of *S. pneumoniae* in animal models of infection. Virulence was reduced in PLN-A strain via intranasal and systemic routes of infection (Berry *et al.,* 1989). The inflammatory response to pneumolysin-negative mutant, PLN-A, is significantly reduced when compared to wild-type bacteria. After intranasal infection with both the wild-type strain and mutant strain, cell recruitment at the site of infection was delayed, and reduced in response to pneumolysin mutant (Kadioglu *et al.,* 2000). In the absence of pneumolysin, colonisation of *S. pneumoniae* is greatly affected. The number of bacteria after infection with pneumolysin-negative mutant was significantly lower in lungs, trachea and nasopharynx as compared to the wild-type strain. The neutrophil response was most significantly affected. Response of T and B lymphocytes was also delayed in and around bronchioles (Kadioglu *et al.,* 2002). Pneumolysin also plays a central role in

early-onset of acute lung injury by causing an impairment of the pulmonary microvascular barrier function and severe pulmonary hypertension. Delivery of recombinant pneumolysin through airways in murine lungs was followed by increased capillary permeability and severe lung edema, whereas the intra-vascular delivery of recombinant pneumolysin resulted in increased pulmonary vascular resistance and lung microvascular permeability. Hence, pneumolysin has a central role in the early onset of acute lung injury by causing an impairment of the pulmonary microvascular barrier function and severe pulmonary hypertension (Witzenrath *et al.,* 2006). Although this work attributes these effects as a result of direct cytotoxic actions on pulmonary endothelial cells, later work showed that lung injury could be due to pro-apoptotic and pro-inflammatory activity of pneumolysin rather than cytotoxic activity (Garcia-Suairez *et al.,* 2007). Pneumolysin also reported to activate the classical pathway at sublytic concentrations despite the absence of antibody (Mitchell *et al.,* 1991). When pneumolysin is released during pneumococcal autolysis, it binds to the Fc portion of IgG resulting in potent activation of the classical pathway. This mechanism leads to increased bacterial virulence by depleting complement components away from the bacterial surface restricting the opsonophagocytosis of bacteria (Alcantara *et al.,* 2001).

1.8.5.3 Pneumococcal surface protein A (PspA)

PspA is the extracellular choline-binding protein present in cell wall and is generally exposed on the whole cell surface (Jedrzejas, 2006; McDaniel *et al.,* 1984). It has been found in all the strains of *Streptococcus pneumoniae* discovered to date (Crain *et al.,* 1990). The molecular weight varies between the strains raging from 67-99 kDA. It has three structural domains: The N-terminal consisting of repeated α-helices, which extend the cell wall and protrude outside of the capsule (which interacts with all

antibodies, and has been described as functional part of this protein) (Jedrzejas *et al.*, 2001 and 2000). The outer domain of PspA is highly electronegative with anti-complementary properties preventing the host complement system from attaching to *S. pneumoniae* (Jedrzejas *et al.*, 2000; Briles *et al.*, 1997; Ren *et al.*, 2004). Studies on the role of PspA in complement inhibition have been inconclusive and conflicting. Some studies have shown that PspA interferes with complement C3 activation on pneumococcal cell surface and thus protecting the bacteria from complement mediated phagocytosis (Tu *et al*., 1999; Ren *et al*., 2003; Ren *et al.*, 2004). Some studies showed that PspA inhibits complement by interfering with C4 deposition on the surface of *S. pneumoniae* (Li *et al.*, 2007; Li *et al.*, 2009).

Recently, PspA has been considered to be used in alternative vaccine approaches. It is present in all serotypes and elicits a serotype independent antibody response (Nabors *et al.*, 2000; Jedrzejas *et al.*, 2001). PspA is an essential virulence factor of *S. pneumoniae* and has been shown to be highly immunogenic having determinants that elicit the immune response against *S. pneumoniae* (McDaniel *et al.*, 1987; Crain *et al.*, 1990; Jedrzejas *et al.*, 2001). Besides antigenic heterogeneity within different strains, PspA elicits cross protection against strains with different capsular types (Kolberg *et al.*, 2001; McDaniel, 2007; Moreno *et al.*, 2010). An intranasal PspA vaccine has promising protective effects against secondary pneumonia after influenza. In this case, PspA-specific IgGs have a critical role in protection against *S. pneumoniae* (Ezoe *et al.*, 2011). Intranasal immunisation with recombinant fusion proteins composed of PspA fused with flagellin was shown to elicit more efficient protective mucosal immune responses against pneumococcal infection in mice than immunisation with PspA alone (Nguyen *et al.*, 2011).

1.8.5.4 Pneumococcal surface protein C (PspC)

PspC also known as choline binding protein A (CbpA) or SpsA is another choline binding surface protein. It is the only molecule homologous to PspA, similar in its structure and function (Mook-Kanamori *et al.,* 2011; Briles *et al.,* 1997). Like PspA, PspC is also a surface exposed protein attached to the cell wall through specific choline binding motifs (Jedrzejas 2001). It has been described as one of the important virulence factors of *Streptococcus pneumoniae* with a crucial role in adherence, colonisation and immunogenicity of *S. pneumoniae. In vitro* binding to epithelial cells and silaic acid residues is compromised when using *pspC* mutant and shows reduced nasopharyngeal colonisation as compared to wild-type strain (Rosenow *et al.,* 1997). *pspC* mutants of serotype 2 and 3 were found less virulent in sepsis models (Iannelli *et al.,* 2004). PspC shows a high degree of genetic variabity between strains and the contribution of PspC to pneumococcal virulence is strain dependent not only in lung infection but also during systemic infection (Ogunniyi *et al.,* 2007; Kerr *et al.,* 2006).

An important property of PspC in evading complement is its ability to bind factor H (FH), a negative regulator of the alternative pathway, hence preventing the alternative pathway activity against *S. pneumoniae* (Duthy *et al.,* 2002**;** Dave *et al.,* 2001; Hammerschmidt *et al.,* 1997). This can be achieved by three potential mechanisms. First through the dissociation of factor B from the alternative pathway C3 convertase (C3bBb) hence reducing the C3b deposition on the surface of bacteria; Secondly, factor H may acts as a cofactor in the degradation of C3b through factor I-dependent cleavage of C3b bound to the bacterial surface to iC3b; and thirdly, the formation of the C3 convertase on the surface of bacteria might be inhibited due to factor H binding to C3b, which can dissociate C3b from factor B and decay the C3 convertase C3bBb (Dave *et*

al., 2004). PspC can also bind to the fluid phase complement inhibitor C4b-binding protein, blocking the activation of classical complement pathway (Dieudonne-Vatran *et al.,* 2009).

1.8.6 Host defences against pneumococcal infection

A successful host immune defence against *Streptococcus pneumoniae* involves a series of mechanisms required for the innate and the adaptive immune response to cooperate (Andersen & Feldman 2011). At the mucosal region of the nasopharynx, *S. pneumoniae* is attacked by mucosal antibodies especially secretory IgA (sIgA) as well as by mucosal antibodies of the IgG class directed against the antiphagocytic capsule providing type-specific protection against *S. pneumoniae* by interfering with binding of *S. pneumoniae* to the nasopharyngeal mucosa. This facilitates opsonisation of *S. pneumoniae* enabling phagocytosis through antigen presenting cells (APCs) and neutrophils (Kurono *et al.,* 1991; Bogaert *et al.,* 2004). Antibodies against the pneumococcal proteins PspA, PspC and pneumolysin may also be present but provide a less efficient protection as compared to sIgA. This protection mechanism is not serotype restricted (Ogunniyi *et al.,* 2007).

Lactoferrin present in human body fluids like saliva and nasal secretions is a bactericidal iron scavenger which depletes the iron necessary for the bacterial metabolism. Unbound lactoferrin (apolactoferrin) has direct bactericidal activities for *S. pneumoniae,* which are independent of iron scavenging. Unbound lactoferrin can induce the disruption of the bacterial cell wall, leading to lysis by an unknown mechanism (Raphael *et al.,* 1989; Arnold *et al.,* 1980; Senkovich *et al.,* 2007). Lactoferrin is also carried by neutrophils (which may have a role in phagocytosis and killing of *S. pneumoniae*) (Esposito *et al.,* 1990).

Another important component of mucosal innate immunity is the complement system. The complement activation cascade results in the subsequent cleavage of several complement factors each leading to different biological activities, including opsonisation and phagocytosis, leukocyte recruitment and formation of the membrane attack complex. Host neutrophils control pneumococcal infection by killing the pathogen through opsonophagocytos, a process requiring opsonisation of bacteria by the complement system (Bogaert *et al.,* 2010; Lysenko *et al.,* 2007). Complement activation leads to covalent deposition of the complement component C3 on the surface of bacteria, which interacts with complement receptors to promote phagocytosis (Dalia and Weiser 2011; Lambris *et al.,* 2008).

Another important innate immune defence mechanism of the respiratory tract is mediated through C- reactive protein (CRP), a protein mainly produced by liver cells during the acute phase of infection. CRP is also found in respiratory tract secretions where it is mainly expressed in respiratory epithelial cells. It can bind to several bacteria, including *S. pneumoniae* (Gould & Weiser 2001; Winkelstein & Tomasz 1977). After binding to cell surfaces of *S. pneumoniae*, CRP can either activate the classical pathway of complement through complement subcomponent C1q or promote phagocytosis and macrophage cytokine production by binding to Fcγ receptor on macrophages and dentritic cells (Suresh *et al.,* 2006; Mold & Du 2006; Thomas-Rudolph *et al.,* 2007).

Certain innate and antibody-independent mechanisms are utilised by the host to fight against pneumococcus infection. Pneumolysin normally augments bacterial virulence; however, at sublytic concentrations it helps the host in eliciting an effective immune response against *S. pneumoniae*. Small numbers of *S. pneumoniae* in the respiratory

tract result in low and sublytic levels of pneumolysin which bind to epithelial cell of host and form sublethal pores on the surface of bacteria due to their low concentration. Sublethal pores on epithelial cells lead to Ca^{++} influx, which results in several intracellular cascades initiating the activation of various transcription factors that activate pro-inflammatory cytokines, especially IL-8, which promote neutrophil infiltration and help in controlling early colonisation (Ratner *et al.,* 2006; Koga *et al.,* 2008; Mathias *et al.,* 2008).

Pneumolysin also interacts with Toll-like receptors, the pattern recognition molecules which primarily function as a part of the innate immune system, but also link innate and adaptive immunity by promoting the upregulation of costimulatory molecules on antigen-presenting cells (APCs) and the secretion of pro-inflammatory cytokines. After binding to TLR-4 on respiratory epithelium or on phagocytes present in airways, pneumolysin initiates a signalling cascade inducing the production and release of pro-inflammatory cytokines IL-8, IL-6, IL-1β and TNF-α which assist the chemo-attraction and infiltration of neutrophils. Through mediating chemotaxis endothelial cells control the colonisation of bacteria in tissues (Malley *et al.,* 2003; MacLeod *et al.,* 2007).

These pro-inflammatory responses provide protection against *S. pneumoniae* infiltration. However, hyperactivation due to poorly controlled immune responses can lead to overproduction of cytokines leading to inflammation-mediated disruption of bronchoalveolar-epithelial barrier adding to extra-pulmonary dissemination of *S. pneumoniae* (Garcia-Suarez Mdel *et al.,* 2007; Martner *et al.,* 2008)

1.8.7 Pathogenesis of pneumococcal infection

Streptococcus pneumoniae is normally carried in the upper respiratory tract, and is transferred between individuals by direct person-to-person contact via airborne transmission. Under specific conditions, hosts become more susceptible to infections and the commensal bacteria become pathogenic and cause disease, which may be local or invasive and disseminate to other organs through the blood stream (Fig. 1.10) (Bogaert *et al.*, 2004).

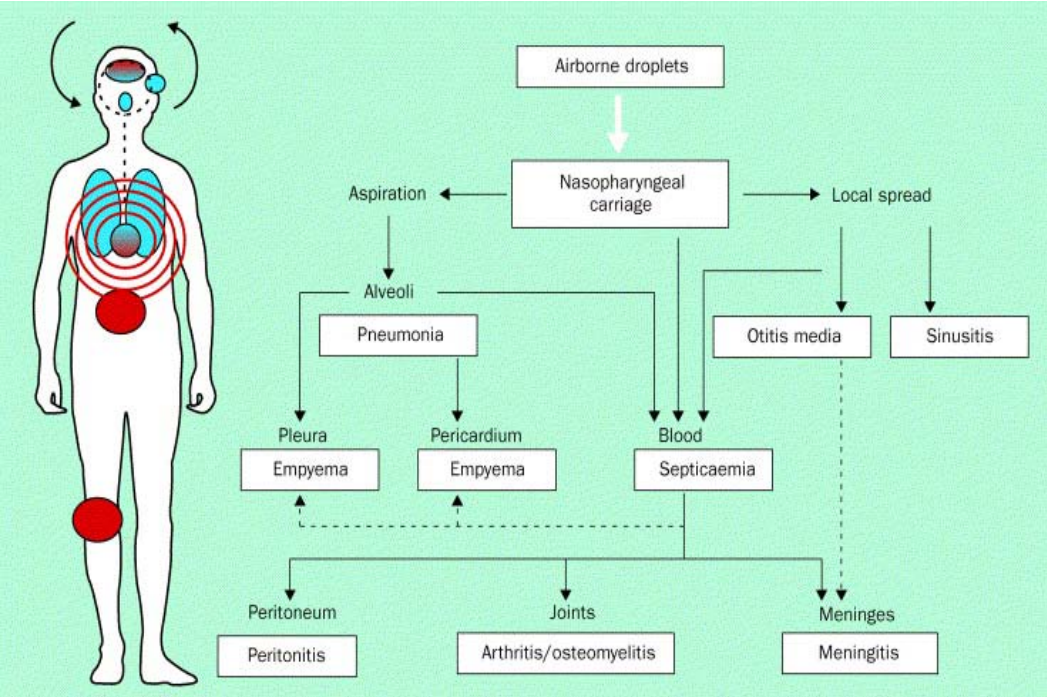


Figure 1.10: Pathogenic routes followed during *Streptococcus pneumoniae* infection starting from nasopharynx through airborne droplets and spreading to other organs. Organs infected through airborne showed in blue, and organs infected through blood showed in red (figure taken from Bogaert *et al.*, 2004).

1.8.7.1 Colonisation in the URT

Streptococcus pneumoniae enjoys a commensal relationship with its principle host under normal circumstances and resides in the upper respiratory tract where it colonises in mucosal surfaces lining the nasopharynx. Colonisation of the URT is normally

asymptomatic and referred to as "carrier state" (carrying the organism without manifestation of disease). Disease occurs when resident organism in the URT gains access to normally sterile part of the body, including lungs, the middle ear or the blood stream. Colonisation in humans starts early on the day of birth with multiple strains of *S. pneumoniae* being carried simultaneously during childhood. Carriage rates are higher in the first two years, exceeding 50% and declining thereafter reaching 10% during first few years of life (Musher *et al.,* 2003; Bogaert *et al.,* 2004). The transfer of organism between individuals occurs through sneezing and coughing. Adherence, nutrition and replication are the major factors deciding the successful colonisation of bacteria. To achieve that, bacteria have to break the host's natural barriers including the barriers formed by the respiratory mucosa, the host immune system and by other pathogens colonising and competing for the same niche.

The first barrier that *S. pneumoniae* encounters is the mucus secretions on respiratory mucosal surfaces (Coonrod *et al.,* 1991; Nelson *et al.,* 2007). The entrapment by mucus is inhibited by *S. pneumoniae* in three ways. First, the negatively charged capsule that repulses the sialic acid residues as it is also negatively charged. The polysaccharide capsule of almost all pneumococcal polysaccharides is negatively charged (Nelson *et al.,* 2007). The second strategy to escape mucous entrapment is the expression of exoglycosidases including neuraminidase A (NanA), beta-galactosidase A (BgA), beta–Nacetylglucosaminidase (StrH) and neuraminidase B (NanB). These exoglycosidases deglycosylate mucous glycoconjugate (removal of terminal sugars) resulting in decreased mucous viscosity limiting the chances of mucous entrapment (Fig 1.11 a) (Davis *et al.,* 2008; King *et al.,* 2006; Tong *et al.,* 2001). Third factor that helps to escape from mucous entrapment is pneumolysin (PLY), a pore-forming toxin

that inhibits epithelial cell ciliary beating and thus facilitates the binding of *S. pneumoniae* to epithelial cells without interference from mucus (Feldman *et al.,* 2002). Other challenges faced by *S. pneumoniae* are the presence of lysozyme on mucosal cell surfaces, a muramidase that damages the cell wall of bacteria by catalysing hydrolysis of peptidoglycan, major constituent of the cell wall of *S. pneumoniae* and the presence of mucosal antibodies.

In the nasopharynx, the capsule of *S. pneumoniae* limits the opsonisation mediated by secretory IgA (sIgA) (Fasching *et al.,* 2007). The pneumococcal IgA1 protease cleaves capsule bound sIgA at the hinge region and further reduces IgA mediated phagocytosis. The remaining Fab portion of IgA also facilitates *S. pneumoniae* adhesion to epithelial cells by binding to PCW and exposing the choline-binding proteins (Cbps) decreasing the negative charge of the capsule which in turn supports adhesion (Fig. 1.11 b) (Weiser *et al.,* 2003; Sabharwal *et al.,* 2009). Apolactoferrin-mediated killing of *S. pneumoniae* in the nasopharynx is prevented by PspA, which binds to the active site of human apolactoferrin and inhibits its bactericidal activity (Shaper *et al.,* 2004).

1.8.7.2 Binding to Epithelium

The thick pneumococcal capsule might be advantageous to bacteria to escape from mucous entrapment as well as from immunoglobulin and complement binding and thereby prevent opsonophagocytosis. Phase variation helps bacteria to overcome this problem by varying the amount of polysaccharide from opaque (thick capsule) to a transparent (thin capsule). After breaching mucosal barriers through opaque variants, transparent variants become prominent exposing adhesion molecules for binding host epithelium (Cundell *et al., 1995;* Weiser *et al.,* 1994; Li-Korotky *et al.,* 2008). *Streptococcus pneumoniae* binds to glycoconjugates present on the surface of epithelial

cells by using products encoded by *spxB, ami, msrA,* and *plpA* genes which might bind directly to glycoconjugates or indirectly by inducing upregulation of their binding molecules on the epithelial lining *(*Fig. 1.1 c) (Cundell *et al.,* 1995; Cundell & Tuomanen, 1994; Wizemann *et al.,* 1996). An array of bacterial adhesins is involved in the attachment of *S. pneumoniae* to host epithelial cells. Phosphorylcholine (ChOP), a part of cell wall C polysaccharide, and capsular polysccharide, is a non-protein virulence factor mediates the attachment initially by interacting with platelet-activating factor (PAF) receptor on the epithelium (Kadioglu *et al.,* 2008; Preston & Dockrell, 2008). PspA and PspC can also act as adhesin by interacting with epithelial polymeric immunoglobulins receptors (Hammerschmidt *et al.,* 1997; Rajam *et al.,* 2008). Some serotypes possess pilus-like structure involved in the adhesion to unknown receptors present on human buccal cells in the host nasopharynx (Barocchi *et al.,* 2006; Bagnoli *et al.,* 2008). A novel protein adhesin, plasminogen- and fibronectin-binding protein B (Pfb B) has been described recently that significantly increase *S. pneumoniae* binding to epithelia cells (Papasergi *et al.,* 2010). *S. pneumoniae* also expresses three surface neuraminidases, NanA, NanB and NanC, which expose the potential binding sites for bacterial adhesin on host cell by cleaving terminal sialic acids from glycan chains on epithelial cells and enhancing biofilm formation by free sialic acid that decreases vulnerability of bacteria faced at this stage due to reduced capsular size (Soong *et al.,* 2006; Trappetti *et al.,* 2009). After binding to the epithelium, *S. pneumoniae* establishes colonisation and utilises a range of virulence mechanisms to fight the innate and adaptive immune defence. This includes the establishment of resistance against lysozyme by enzymatic modifications of the peptidoglycans of the cell wall (Davis *et al.,* 2008), minimising the effect of complement activation pathways by PspA, PspC and pneumolysin (McCool & Weiser 2005; Lu *et al.,* 2008), and provide additional

protection through the cleavage of sIgA by zinc metalloproteinase (Wani *et al.,* 1996; Kadioglu *et al.,* 2008) and formation of biofilms (Hall-Stoodley & Stoodley, 2009).

1.8.7.3 Biofilm Formation

Biofilms are surface-associated microbial communities, surrounded by a hydrated and self generated extracellular polymeric substance (EPS) matrix. This is a tool used by bacteria to achieve successful colonisation and to persist *in vivo* by providing insulation against cellular and humoral defence mechanisms of the host and against other antibacterials. Bacteria remain sequestered in the biofilm either on the epithelial surface or intracellularly and re-emerge when the host becomes immunocompromised causing a secondary infection resulting in invasive disease (Hall-Stoodley & Stoodley, 2009; Grau *et al.,* 2009; Murdoch *et al.,* 2009).

1.8.7.4 Invasive Disease

The likelihood of invasive pneumococcal diseases increases when the host is colonised by a certain strain of *Streptococcus pneumoniae* without having established immunity against it. It depends on both the virulence of bacteria and efficiency of anti-pneumococcal host defences (Bogaert *et al.,* 2004; Feldman & Anderson, 2009). Invasion of *S. pneumoniae* in the host epithelial and endothelial cells involve endo- and transcytosis. Two mechanisms have been described for epithelial transmigration of bacteria. First one involves the binding of pneumococcal phosphorylcholine (ChoP) (a component of teichoic and lipotechoic acids that extend outward from the bacterial cell wall and cell membrane, respectively) to platelet activating factor (PAF) on activated epithelial and endothelial cells of the host (Fig. 1.11 d). After binding, *S. pneumoniae* follows the PAF receptor recycling pathway which carries the bacterium to the basement membrane leading to invasive disease (Cundell *et al.,* 1995; Rijneveld *et al.,*

2004; Radin *et al.,* 2005; Mook-Kanamori *et al.,* 2011). A second mechanism of invasion involves pIgR recycling pathway, analogous to the PAF receptor pathway (Fig 1.1 d). PspC binds to pIgR (referred to as secretory component, an Fc receptor expressed on epithelial cells, which facilitates the secretion of immunoglobulin A and immunoglobulin M). In this pathway, bacteria are transferred to basal membrane of epithelial cells after binding of PspC to pIgR (Elm *et al.,* 2004; Zhang *et al.,* 2000). *S. pneumoniae* can also cross epithelial and endothelial layers by inter- or pericellular migration via plasminogen (Fig. 1.11 e). Plasminogen binds to bacteria through specific receptors on bacteria, i.e. enolase, Gly3Ph and Cbpe. Plasminogen facilitates the binding of *S. pneumoniae* to the epithelial surface. After binding to epithelial cells, plasmin cleaves proteins present in intercellular adherene junction which in turn facilitates the migration of bacteria to the basal membrane (Bergmann *et al.,* 2001; Attali *et al.,* 2008). *S. pneumoniae* also express hyaluronidase which degrades hyaluran, a major component of the extracellular matrix, to facilitate the invasive disease (Jedrzejas *et al.,* 2007).

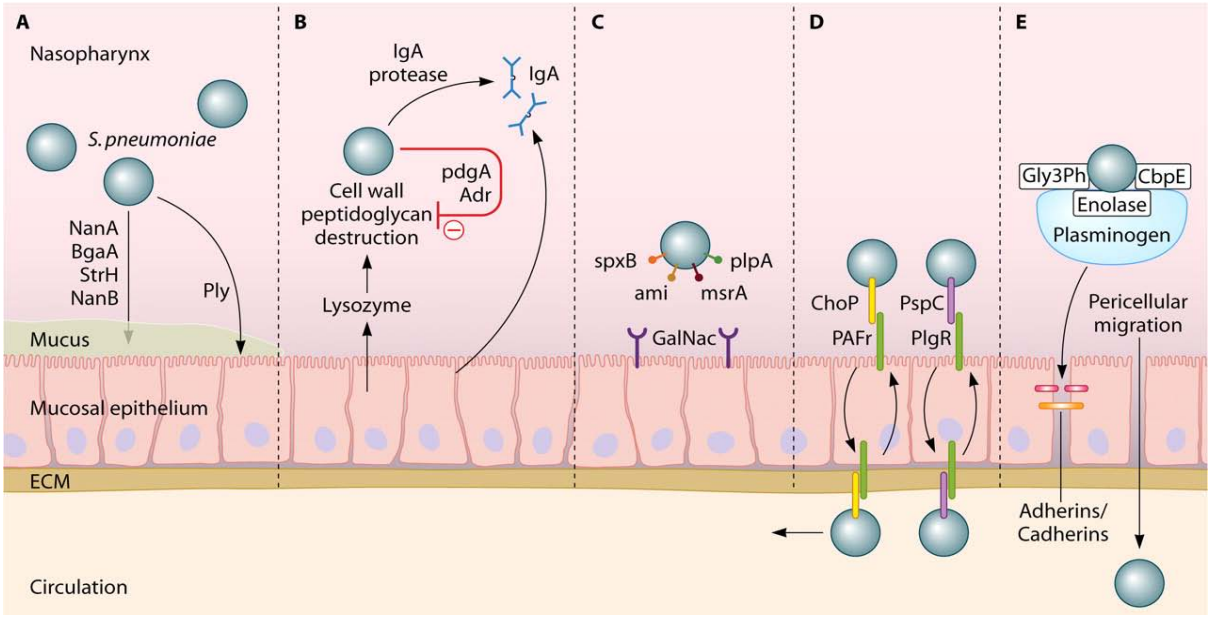


Figure1.11: Colonisation and invasion of *Streptococcus pneumoniae* in upper respiratory tract: (a) Mucus breakdown by enzymes produced by *S. pneumoniae*. (b) Evasion from lysozyme mediated by deacetylation of *S. pneumoniae* the cell surface peptidoglycans. (c) Binding to epithelial cells. (d) Intracellular translocation and (e) Inter- and pericelluar translocation of *S. pneumoniae* (Figure taken from Mook-Kanamori *et al.*, 2011).

1.8.8 The role of complement activation in pneumococcal infection

Complement activation is mediated via three pathways; the classical pathway, the alternative pathway and the lectin pathway. Recent studies have described the role of the classical and alternative pathways of complement in providing protection against pneumococcal infection. The effect of C1q deficiency on the outcome of pneumococcal infection has been studied in different mouse models. C1q deficient mice were highly susceptible to pneumococcal infection as compared to wild-type mice in models of pneumonia and systemic infection (Brown *et al.*, 2002). Moreover, immune response to pneumococcal infection was severely impaired against pneumococcal infection in C1q deficient mice in an experimental model of pneumococcal meningitis (Rupprecht *et al.*, 2007). Similarly, C1q deficient mice were more susceptible to pneumococcus mediated otitis media (Tong *et al.*, 2010). *In vitro,* opsonophagocytosis was severely affected

when human serum was depleted of C1q (Yuste *et al.,* 2010). All these studies underline the importance of the classical complement activation pathway in providing protection against pneumococcal infection.

The classical pathway of complement activation is usually activated by antigen-antibody complexes. Natural IgM binds with low affinity to pneumococcal C polysaccharide (i.e. teichoic acid), and activates the classical pathway on *Streptococcus pneumoniae* (Mold *et al.,* 2002). IgM deficient (i.e. $\mu^{-/-}$) mice are more susceptible to infection with higher bacterial loads in blood and in lung tissues as compared to the wild-type controls (Brown *et al.,* 2002). The classical pathway is also thought to be activated via binding of acute phase proteins, such as CRP to the surface of *S. pneumoniae* followed by the binding of C1q to CRP or the direct binding of C1q to the surface of *S. pneumoniae* (Janeway, 2005). Another way in which C1q could mediate antimicrobial activity even in the absence of complement activation is by binding to the C-type lectin SIGN-R1, which usually captures microbial polysaccharides in the spleen. C1q is directly activated upon binding to pneumococcal polysaccharides, triggering antimicrobial activity in the spleen. Classical pathway dependent activation against *S. pneumoniae* was reduced in SIGN-R1 deficient mice (Kang *et al.,* 2006). In SIGN-R1 deficient mice, splenic macrophages were unable to activate splenic B cells to produce specific immunoglobulins against *S. pneumoniae* (Koppel *et al.,* 2008).

The alternative pathway of complement activation is seen to be less efficient in fighting *S. pneumoniae* infections than compared to the classical pathway. Mice deficient in factor B showed higher mortality and increased bacterial burden in blood and lungs as compared to wild-type mice following infection with *S. pneumoniae* in models of pneumonia and systemic models of infection. However, the outcome of disease was

less severe when compared to mice deficient in the classical pathway (Brown *et al.,* 2002). Similarly, alternative pathway deficiency leads to increased survival of *S. pneumoniae* in middle ear in a model of acute otitis media using factor B deficient mice (Tong *et al.,* 2010). C3 opsonisation and opsonophagocytosis of *S. pneumoniae* were impaired in the middle ear lavage of factor $B^{-/-}$ mice. However, others claim that complement activation in the middle ear depends to a large proportion on the alternative pathway, and to a less extent on the classical pathway (Li *et al.,* 2011). The kinetics of C3b deposition on the surface of *S. pneumoniae* was much slower in factor D-deficient sera as compared to sera of wildtype mice indicating the important role of alternative pathway activation in the early stage of infection (Xu *et al.,* 2001).

Clinical studies have addressed the association of complement deficiencies with pneumococcal disease. A Swedish cohort of 40 patients with homozygous C2 deficiency (due to a deletion of the *C2* gene) was analysed for their clinical history of pneumococcal infection: 58% of these patients developed invasive pneumococcal infection (Jonsson *et al.,* 2005).

A genetic association study was performed to monitor the association between MBL genotypes and pneumococcal disease. No association between MBL deficiency and the susceptibility to community-acquired pneumonia was detected (Endeman *et al.,* 2008). Another study showed that polymorphisms of the *MBL* gene are not associated with an increased risk of invasive pneumococcal disease (Moens *et al.,* 2006). A further case-control study analysing a large cohort indicated that the risk of invasive pneumococcal disease may be slightly increased in MBL deficiency (Roy *et al.,* 2002). No association between L-ficolin polymorphisms and invasive pneumococcal disease has been described (Chapman *et al.,* 2007).

1.9 Aims and objectives of this thesis

This study was designed to achieve following goals.

- To characterise the role of murine ficolin A in activating the lectin pathway in fighting pneumococcal infection by using a combination of *in vitro* assays and experimental model of *Streptococcus pneumoniae* infection in a mouse strains with and without a gene targeted deficiency of *Fcna* gene.
- To define the role of MASP-2 mediated lectin pathway of complement activation using a MASP-2 inhibitory antibody in an experimental model of *S. pneumoniae* infection.
- To investigate the mechanism resulting in the loss of detection of C4b deposition on the surface of *S. pneumoniae*.
- To assess the role of various bacterial virulence factors that might affect the classical and the lectin pathway specific C3 convertases and C5 convertases C4b2a or C4b2a$(C3b)_n$.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Media

1kb plus DNA ladder	Invitrogen
36.5 % formaldehyde solution	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Barbital	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Blood Agar Base	Oxoid
Brain heart infusion (BHI) medium	Oxoid
Calcium chloride	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Ceftriaxone	Sigma-Aldrich
Deoxyribo nucleotide PCR grade (dNTPs)	Promega
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich
EDTA	Sigma-Aldrich
Eosin	BDH laboratories
Ethanol	Fisher Scientific
Ehidium bromide	Sigma-Aldrich
Foetal calf serum	Harlan

Formalin	Fisher Scientific
Glacial acetic acid	Fisher Scientific
Haematoxylin	BDH laboratories
Heparin	Sigma-Aldrich
Horse blood	Oxoid
Isopentane	Fisher Scientific
Isopropanol	Fisher Scientific
Magnecium chloride	Sigma-Aldrich
Mannan	Sigma-Aldrich
N-acetyl BSA	Promega
Oligo $(dT)_{23}$ anchored primers	Sigma-Aldrich
Oligonucleotides	Eurofin
Optochin disks	Sigma-Aldrich
Phenol/Chloroform for RNA extraction	Sigma-Aldrich
Phosphate Buffer Saline	Oxoid
Proteinase K	Qiagen
QuantiTect SYBR Green Master Mix	Qiagen
RNase H	Promega
RNase-free DNaseI	NEB
RNasout	Promega

Sigma Fast p-Nitrophenyl Phosphate tablet	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium carbonate	Sigma-Aldrich
Sodium chloride	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Spectinomycin	Sigma-Aldrich
Superscript II reverse transcriptase	Invitrogen
Taq DNA polymerase	Thermo
Triazol	Invitrogen
Tris-HCl	Sigma-Aldrich
Triton X-100	BDH laboratories
Trizma base	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Zymosan	Sigma-Aldrich
β-mercaptoethol	Sigma-Aldrich

2.1.2 Buffers and solutions

2.1.2.1 Buffers used in ELISA

Coating buffer	15 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
Tris buffered saline (TBS)	10 mM Trizma base 140 mM NaCl pH 7.4
MBL- binding buffer	20 mM Trizma base 10 mM $CaCl_2$ 1 M NaCl 0.05% (v/v) Triton X-100 0.1% (w/v) HSA pH 7.4
BSA-TBS blocking buffer	TBS with 1% (w/v) BSA pH 7.4

Barbital buffer saline (BBS)	4 mM barbital 145 mM NaCl 1 mM $MgCl_2$ 2 mM $CaCl_2$ pH7.4

2.1.3 Kits

Wizard genomic DNA purification kit	Promega

2.1.4 Antibodies

Chicken anti-human C4 monoclonal antibody	Agrisera
Goat anti-mouse IgG (whole molecule) Alkaline phosphatase antibody	Sigma-Aldrich
Goat anti-rabbit IgG (whole molecule) Alkaline phosphatase antibody	Sigma-Aldrich
Goat anti rat IgG (whole molecule) Alkaline phosphatase antibody	Sigma-Aldrich
Rabbit anti-human C3c polyclonal antibody	Dako
Mouse anti-human C4 monoclonal antibody	Santa Cruz

Mouse anti-human C4dg monoclonal antibody	Quidel
Mouse anti-human C4bp monoclonal antibody	Quidel
Mouse anti-human CL-11 monoclonal antibody	Provided by Dr. Soren Hansen, Department of Cancer and Inflammation Research, University of Southern Denmark.
Mouse anti-human MBL	Hycult
murine-specific MASP-2 inhibitor, (AbD 04211)	Omeros Corporation Seatle USA.
Rabbit anti ficolin A	Provided by Prof. T. Fujita, Department of Immunology, Fukushima Medical University, Japan
Rabbit anti H-ficolin	Provided by Dr. H. Shiraki, Department of Immunology, Fukushima Medical University, Japan
Rabbit anti L-ficolin	Provided by Prof. T. Fujita, Department of Immunology, Fukushima Medical University, Japan
Rabbit anti M-ficolin	Provided by Prof. T. Fujita, Department of Immunology, Fukushima Medical University, Japan

Rat anti-mouse C4	Hycult
Rat anti-mouse MBL-A monoclonal antibody	Hycult
Rat anti-mouse MBL-C monoclonal antibody	Hycult
Rat anti-mouse CL-11 monoclonal antibody	Provided by Dr. Soren Hansen, Department of Cancer and Inflammation Research, University of Southern Denmark.

2.1.5 Bacteria

Streptococcus pneumoniae serotype 2 D39, the model strain used in all infection experiments, along with serotype 6B, 9V, 19A, 9C, 18C and 3 were provided by Professor Peter Andrew, University of Leicester. Nine patient isolates, 3 each from serotype 6B, 18C and 3, used to assess strain variation in binding of lectin pathway carbohydrate recognition molecules and complement activation assays, were kindly provided by Prof. Hermínia de Lencastre, ITQB, Portugal (Sá-Leão *et al.*, 2009).

pspA mutants, 117-1, 117-2 and 117-3 were kindly provided by Dr Hasan Yesilkaya, University of Leicester. *pspC* mutant was kindly provided by Dr. Aras Kadioglu, University of Leicester. Genetic properties and relevant phenotype of mutants used in this study are listed in Table 2.1.

Table 2.1: Genetic properties and relevant phenotype of mutants

Strain	Serotype	Derivation	Relevant Properties	Relevant phenotype
117-1^{+}	2	D39	*pspA::aadA* *	PspA^{-}
117-2^{+}	2	D39	*pspA::aadA*	PspA^{-}
117-3^{+}	2	D39	*pspA::aadA*	PspA^{-}
H42	2	D39	*ΔpspC*; Em^{r}	PspC^{-}

$^{+}$ In all strains *pspA* has been mutated but the location of spectinomycin casette differs.
* spectinomycin resistant
Em^{r}, Erythromycin resistant

All the mutants listed above will be subsequently discussed by their strain name and relevant genotype i.e. 117-1 (*pspA* mutant), 117-2 (*pspA* mutant), 117-3 (*pspA* mutant) and H42 (*pspC* mutant).

2.1.6 Mouse sera

Serum was obtained from C57BL/6 mice deficient in different complement components and used in complement activation assays. Mice deficient in different complement components will be discussed in whole thesis by their relevant phenotype e.g. ficolin A$^{-/-}$, MASP-2$^{-/-}$, MBL-A/C$^{-/-}$. Ficolin A deficient serum was obtained from ficolin A$^{-/-}$ mice (Endo *et al.,* 2010). MASP-2 deficient serum was obtained from MASP-2$^{-/-}$ mice (Schwaeble *et al.,* 2011). MBL-A/C$^{-/-}$ mice were purchased from MMRRC, Bar Harbor, Maine. For establishment of MBL-A/C/ficolin A$^{-/-}$ mice, MBL-A/C$^{-/-}$ mice were backcrossed with ficolin A$^{-/-}$ mice to produce a strain deficient in three lectin pathway carbohydrate recognition molecules. Wild type control sera used in all assays was obtained from C57BL/6 mice purchased from Charles River UK.

2.2.1 *In vitro* Studies

2.2.1.1 Preparation of formalin fixed *Streptococcus pneumoniae*

Bacteria used in all the *in vitro* assays were fixed with 0.5% formalin. Bacteria were inoculated in 10 ml of brain heart infusion (BHI, oxoid) and left overnight at 37^{o}C. Next day, bacteria were spun at 3000xg for 10 minutes. Then bacterial pellet was washed with phosphate buffer saline thrice and suspended in 0.5% formalin (Sigma Aldrich) in PBS for 1-3 hours at room temperature. After fixation with formalin, bacteria were spun again and washed twice with PBS and re-suspended in the coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) adjusting the OD_{550nm} of suspension to 0.6 (Lynch *et al*., 2004).

2.2.1.2 Preparation of sera

Blood was collected from mice by cardiac puncture and immediately transferred on ice to prevent complement activation. After 1-3 hours of incubation on ice, serum was separated by spinning the blood at 7000 rpm for 7 minutes at 4^{o}C using a cooled centrifuge and stored in -80^{o}C freezer until used.

2.2.1.3 Enzyme Linked Immune Sorbent Assays (ELISAs)

ELISAs were used in different experiments, including solid phase binding assays to detect the binding of lectin pathway carbohydrate recognition molecules to *Streptococcus pneumoniae*, and complement activation assays for the detection of levels of C3b and C4b on the surface of *S. pneumoniae*.

2.2.1.3 Solid Phase binding assays

To check the binding affinity of carbohydrate recognition molecules to *Streptococcus pneumoniae*, Nunc Maxisorb microtiter ELISA plates were coated with 100 µl formalin fixed *S. pneumoniae* (OD_{550nm}=0.6). Different positive controls were used for each carbohydrate recognition molecule tested. 10 µg/ml of mannan (Sigma-Aldrich) was used as positive control for MBL-A, MBL-C and human MBL, 10 µg/ml of N-acetyl BSA (Promega) for ficolin A and M-ficolin, 5 µg/ml of FCN-2 specific mAb GN4 (Hycult) for L-ficolin, 5 µg/ml FCN-1 specific mAb (Hycult) for H-ficolin and 10 µg/ml of zymosan (Sigma-Aldrich) was used as positive control for CL-11 binding. All these sugars and monoclonal antibodies used as positive control were suspended in the coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and 100 µl was added to each well. After overnight incubation at 4^oC, residual protein binding sites were saturated by incubating the plates with 300 µl of 1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4) (blocking buffer) for 2 hours at room temperature. Plates were then washed three times with 250 µl of TBS with 0.05% Tween 20 and 5mM $CaCl_2$ (wash buffer). Serum to be tested was added to the plates in duplicates after serial dilution in blocking buffer and incubated for 90 minutes at 37 ^oC. For the detection rficolin-A and rC-11 binding to *S. pneumoniae*, rficolinA and rCL-11 was serially diluted in blocking buffer and added to each well. The last well of every column received only block buffer without any serum and was used as negative control. After incubation with serum, plates were washed again three times with wash buffer and proteins were detected by rat anti-mouse MBL-A diluted 1:1000, rat anti-mouse MBL-C diluted 1:1000, rabbit anti-mouse ficolin A diluted 1:1000, rat anti-mouse CL-

11 diluted 1:500, mouse anti-human MBL diluted 1:5000, rabbit anti-human L-ficolin diluted 1:2000, rabbit anti-human H-ficolin diluted 1:2000 and rabbit anti-human M-ficolin diluted 1:2000 and mouse anti-human CL-11 diluted 1:500. Following incubation at room temperature for 90 minutes, plates were washed again thrice and primary antibodies were detected by alkaline phosphatase (AP) conjugated goat α-mouse, goat α-rabbit or goat α-rat (Sigma-Aldrich), diluted 1:10000 in wash buffer. After incubation at room temperature for 90 min, plates were washed again and AP was detected by adding 100 µl of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich). After incubation at room temperature, absorbance was measured at 405nm using BioRad microtitre plate reader.

2.2.1.4 C3 deposition assay

To measure C3 deposition on the surface of *Streptococcus pneumoniae*, Nunc Maxisorb microtiter ELISA plates were coated with 100 µl/well of formalin fixed *S. pneumoniae* (OD_{550}nm =0.6), 10 µg/ml of mannan(Sigma-Aldrich), 10 µg/ml of zymosan and 10 µg/ml of N-acetyl BSA diluted in coating buffer and incubated overnight at 4^{o}C. Residual protein binding sites were blocked next day by 1% BSA in TBS buffer. After washing three times with wash buffer, serum was diluted two folds in BBS (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4) with dilution starting from 1/80. Plates with serum were incubated at 37 ^{o}C for 90 minutes and washed again. Bound C3b was detected by adding 100 µl of rabbit anti-C3c (Dako) diluted 1:5000 in wash buffer and incubated for 90 minutes at 37 ^{o}C. Plates were washed thrice followed by addition of 100 µl of AP conjugated goat anti-rabbit (Sigma-Aldrich) diluted 1:10,000 and incubated at room temperature for 90 min. Plates were washed again, and

extent of C3b deposition was determined by adding 100 µl of colorimetric substrate ρ–nitrophenyle phosphate (pNPP) (Sigma-Aldrich). After incubation at room temperature, absorbance was measured at 405nm using BioRad microtitre plate reader.

2.2.1.5 Lectin pathway specific C4 cleavage assay

Two different assays were used for detecting serum endogenous C4 and exogenous C4 from humans.

To measure the lectin pathway specific serum endogenous C4 deposition on the surface of bacteria, Nunc Maxisorb microtiter ELISA plates were coated with 100 µl/well of formalin fixed *Streptococcus pneumoniae* (OD550nm =0.6) and 10 µg/ml of mannan (Sigma-Aldrich) and incubated overnight at 4^{o}C. Residual protein binding sites were blocked by blocking buffer as discussed above. After washing the plates three times with wash buffer, serum was diluted two folds in the MBL binding buffer (20mM Tris, 10mM CaCl2, 1M NaCl, pH 7.4) with dilution starting from 1:80 was added to plates except the wells from last row of each column, which did not receive any serum and were used as negative control. After incubation with serum at 37^{o}C for 90 minutes and washing thrice, endogenous C4c was detected by adding 100 µl of specific rat anti-mouse C4 (Hycult) diluted 1:500 in wash buffer and incubated at room temperature for 90 minutes. Plates were washed again three times followed by addition of 100 µl of AP conjugated goat anti-rat (Sigma-Aldrich) diluted 1:10,000 in wash buffer and incubated at room temperature for 90 minutes. After washing the plates thrice, extent of C4 deposition was determined by adding 100 µl of colorimetric substrate ρ–nitrophenyle phosphate (pNPP) (Sigma-Aldrich) and incubated at room

temperature. Absorbance was measured at 405nm using BioRad microtitre plate reader.

In another assay, exogenous C4 was detected by modifying above assay. After incubation with serum, 1 μg/ml of human C4 (provided by Dr. Nick Lynch, University of Leicester, UK) diluted in BBS was added to each well and incubated at 37^{o}C for 90 minutes. After washing the plates thrice, exogenous human C4 deposition was checked by specific chicken anti-human C4 (Agrisera) diluted 1:2000 and mouse anti-human C4 (Santa Cruz) diluted 1:1000 which were detected by specific AP conjugated goat anti-chicken and goat anti-mouse (Sigma-Aldrich).

2.2.2 *In vivo* Studies

2.2.2.1 Mice

All the mice used in infection study were 8-10 weeks old female C57BL/6 background. Ficolin A$^{-/-}$ mice used in study were provided by Dr. Teizo Fujita, Fukushima Medical University Japan (Endo *et al.*, 2010). These mice lack the ability to mediate the lectin pathway mediated via ficolin A and have completely functional MBL-A, MBL-C and CL-11 mediated lectin pathway. MBL-A/C$^{-/-}$ mice were purchased from MMRRC, Bar Harbor, Maine .Wild type mice of C57BL/6 background were purchased from Charles River UK Ltd. MF1 mice used for passage of bacteria and virulence testing of infectious dose were obtained from Harlan Olac, Bicester, UK. Mice obtained from outside source were housed in DBS for seven days prior to infection to acclimatise to the new conditions. All the procedures used in study were in accordance with guidelines from UK Home Office.

2.2.2.2 Preparation of non-passaged bacteria

Streptococcus pneumoniae serotype 2, strain D39 used in this study was provided by Professor Peter Andrew. Bacteria from bead stocks were streaked on blood agar plates, and Optochin disks were placed in the plates to confirm Optochin sensitivity. Plates were incubated overnight at 37 ^{o}C in CO_2 gas jar. Next afternoon, a sweep of colonies from plates was inoculated in 10ml BHI broth (Oxoid) and incubated statistically for 16-18 hours. Next day, BHI with bacterial growth was centrifuged at 3000rpm, and pellet was re-suspended in 1ml of BHI with 20% v/v heat inactivated foetal calf serum (Sigma) (BHI serum broth). 700 µl of re-suspended pellet was added to 10ml of fresh BHI broth with final OD_{500} of 0.6 and incubated statically at 37^{o}C. After OD_{500} of bacterial growth reached 1.6, bacteria were stored in -80^{o}C into 500 µl aliquots. After at least 24 hours of storage at -80^{o}C viability of the stocks was determined by Miles and Misra count and checked for optochin sensitivity (Miles *et al.,* 1938).

2.2.2.3 Animal passage of *Streptococcus pneumoniae*

An aliquot of non-passaged *Streptococcus pneumoniae* D39 was thawed and streaked on the blood agar plate. After overnight incubation at 37^{o}C, a sweep of colonies was inoculated in 10 ml BHI and incubated statically at 37^{o}C. Next day, bacteria were centrifuged at 3000 rpm and pellet was re-suspended in 5ml phosphate buffer saline (PBS, pH 7.4). 100 µl of *S. pneumoniae* suspension was injected *i.p.* in 8-10 week old MF1 mice. After 22-28 hours of infection mice, symptoms reached ++ starry coat and blood was collected by cardiac puncture after anaesthetising the mice with 5% v/v isoflurane and 1.6-1.8 L O_2 /min in an anaesthetic box. 50 µl of blood was inoculated in BHI and incubated statically at 37^{o}C. Next day, cloudy suspension was separated from

loose sediments of RBCs and centrifuged at 3000rpm for 10 min. Pellet was re-suspended in 1ml of BHI serum broth. 700 µl of re-suspended pellet was inoculated in 1ml BHI serum broth and OD_{500} was adjusted at 0.6 and incubated at 37^{o}C until OD_{500} of suspension reached 1.6 (in 5-6 hours) and separated into 500 µl aliquots in 1.5ml sterile eppendorf tubes. Stocks were then stored in -80^{o}C freezer. After 24 hours of freezing, viability of stocks was determined and optochin sensitivity was checked by streaking onto blood agar base (BAB plates) and adding optochin disks.

2.2.2.4 Virulence testing of passaged stocks of *Streptococcus pneumoniae* D39

Before using in infection studies, virulence testing of the passaged stocks of bacteria was performed in MF1 mice. An aliquot of passaged bacteria was thawed at room temperature and centrifuged at 13000rpm at room temperature for 2 minutes, and pellet was washed by re-suspending in 500 µl sterile PBS and centrifuged again. Pellet was then diluted in to achieve the $1x10^{6}$ CFU per 50 µl. 5 MF1 mice were anaesthetised with 2.5% v/v isoflurane and 1.6-1.8 L O_2/min and intranasally infected with $1x10^{6}$ CFU in 50 µl PBS. Mice were then monitored for signs of disease. On reaching +/+ lethargic stage, mice were euthanised by cervical dislocation. Dose was considered to be of standard lab virulence when mean time of survival of all five mice was within 48-72 hours.

2.2.2.5 Infection of mice

After successful virulence testing, dose was used in infection experiment. 8-10 week old female ficolin $A^{-/-}$ and ficolin $A^{+/+}$ mice were used in this model of infection. Mice were lightly anaesthetised with 2.5% v/v isoflurane and 1.6-1.8 L O_2/min. 25 µl PBS

containing 1×10^6 CFU of *Streptococcus pneumoniae* D39 was administered by intranasal route. The inoculum dose was confirmed by viable count after plating on blood agar plates. After the infection, mice were transferred to special cages assigned for infected animals and were placed on separate infectious racks. Mice were closely monitored every three hours after clinical symptoms appeared around 36 hours (hunched appearance, starry coat, lethargy, moribund). Mice showing terminal symptoms (++ lethargic) were humanely culled by cervical dislocation according to Home Office guideline before reaching the moribund state. Survival was recorded for 7 days (168 hours).

To investigate the effect of α-MASP-2 mAb treatment on mortality in a mouse model of *S. pneumoniae* infection, mice were treated by *i.p.* injection with 1mg/kg body weight of α-MASP-2 mAb (AbD 04211) and control antibody that specifically inhibits human but not murine MASP-2. Antibodies were injected either before or after infection. Groups receiving antibiotic were pre-treated with *i.p.* injection of 20mg/kg of ceftriaxone 16 hours before infection and every 12 hours after infection. Time points for the injection of antibodies and antibiotic are discussed in detail in chapter 4 as per requirement of each experiment.

2.2.2.6 Determination of blood and lung bacterial burdens

The course of infection was monitored by following the time-course of bacteraemia. Blood was collected from mice via tail bleed at pre-chosen time points. Mice were incubated in the 37^{o}C incubator for 20 to 30 minutes to induce vasodilatation of veins. 20 μl of tail blood sample was collected from each mouse. For determining the

recoverable CFU counts, 10 μl of blood was serially diluted in PBS tenfold and plated onto agar supplemented with 5% (v/v) horse blood (oxoid) and incubated overnight at 37^{o}C under anaerobic conditions. Next day, numbers of colonies were counted and CFU/ml was calculated. For determination of bacterial burdens in lungs, 2 mice at time point 0, and 5 mice at time points 12, 24, 48 and 60 hour post infection were sacrificed. Lungs were removed aseptically into 5 ml of sterile PBS. Lungs were weighed and finally homogenised in a Stomacher-Lab blender (Seward Medical, London, UK). 10 μl of lung homogenate was serially diluted in PBS and CFU/mg was calculated as mentioned above.

2.2.2.7 Freezing of lungs for mRNA extraction

Two mice from each group at the time of infection followed by five mice at time points 6, 12, 24, 36 and 48 hours of infection were sacrificed and lungs for total RNA extraction were removed aseptically. Isopentane (Fisher Scientific) was pre-cooled on dry ice, and lungs were flash frozen and stored in -80^{o}C freezer.

2.2.2.8 Histology

After infection with 1x10^{6} CFU *Streptococcus pneumoniae* D39, 2 mice from each group were sacrificed at pre-chosen time points, and lungs were excised aseptically for histopathological analysis. Excised lungs were immediately fixed in formalin for 24 hours and transferred to 70% alcohol until embedded in paraffin wax and sectioned onto poly-L-lysine coated glass slides (Thermo-Fisher Scientific). For H & E staining, slides were dehydrated in 70% ethanol for 1min and then washed with distilled water for another 1min. Sections were then immersed in Haematoxylin stain (BDH) for 1 min and washed again with distilled water for 1min. Slides were then immersed in Eosin

stain for 30sec and rinsed in distilled water. Stained slides were subsequently dehydrated in 70%, 90% and 100% ethanol. Dehydration was followed by rinsing the slides in xylene solution for 1 min and allowed to air dry and covered with a cover slip. All the sectioning and staining procedures were performed at Department of Pathology, Leicester Royal Infirmary.

2.2.2.8 Statistical analysis

Statistical significance of differences in bacterial loads, relative cytokine expression and average illness scores at different time points post infection were determined using unpaired t test. Survival data was analysed by Kaplan-Meier plots and compared by logrank test. All statistical analysis were done using Graphpad Prism, Version 5.0 (Graphpad Software, San Diego, Calofornia.). Differences were considered significant at P values of <0.05.

2.2.3 Molecular Biology Techniques

2.2.3.1 RNA based methods

2.2.3.1.1 RNA isolation from from mouse lungs

Total RNA was extracted from lungs to analyse the expression of different inflammatory cytokines during the course of infection. Total RNA was isolated using Trizol reagent, which is a mono-phasic solution of phenol and guanidine isothiocyanate, which helps inactivate RNase and maintains the integrity of the RNA while distracting cells and dissolving cell components during homogenisation. 100 mg of lung tissue (that was previously flash frozen in isopentane and stored in -80^{o}C) was cut on dry ice and homogenised in 1ml of Trizol reagent (Invitrogen) using Stomacher-

Lab blender (Seward Medical, London, UK). After homogenization, homogenate was centrifuged for 15 min at 12000xg, and supernatant was collected and transferred to sterile microcentrifuge tube. 200 µl of chloroform was added to supernatant and shaked vigorously for 15 seconds and incubated for 2-3 minutes at room temperature. After incubation, tubes were centrifuged for 15 min at 12000xg. After centrifugation, the homogenate was separated into three layers, the lower layer was phenol, middle layer chloroform and a colourless upper aqueous phase contained the RNA which was carefully transferred to RNase-free microcentrifuge tube. RNA was precipitated from this aqueous layer by mixing with 500 µl of isopropyl alcohol, incubated for 15 min at 30^{o}C and then centrifuged for 15 min at 12000xg. Supernatant was discarded and pellet was collected, washed with 75% ethanol and centrifuged at 7500xg for 5 mins at 2-8 ^{o}C. The RNA pellet was air dried and dissolved into DEPC treated water. RNA purity was calculated by measuring the absorbance ratio A_{260}/A_{280} using NanoDrop 3300 spectrophotometer. RNA was considered pure when A_{260}/A_{280} was between 1.6 & 1.8. RNA concentration was calculated as (OD_{260} X 40 X Dilution factor = Yµg/µl). RNA was quantitated by measuring the absorbance at A_{260}.

2.2.3.1.2 Purification of RNA

RNA purification was done to isolate contaminating DNA by digesting the RNA with RNase-free DNaseI. 10 µg of RNA sample was digested with 2 µl DNase I (Promega), 5 µl DNase buffer in a final volume of 50 µl DEPC water (RNase-free water) and was incubated at 37°C for 30 minutes. After digestion, DEPC water was added to make the final volume of mixture 200 µl. After digestion 200µl of phenol/ chloroform/isoamyl alcohol (Sigma) was added to the mix, vortexed vigorously and centrifuged at 10,000xg

for 10 minutes. The upper aqueous layer was transferred to a clean Eppendorf tube and precipitated with 500 µl of 100% ethanol and 1/10 volume of 3M NaAcetate and mixed. After centrifugation at 10000xg for 15 minutes, the supernatant was discarded. The pellet was air dried and re-suspended in 25µl of DEPC treated water (Lynch *et al.*, 2005). Purity of RNA was calculated by measuring A_{260}/A_{280} using NanoDrop 3300 spectrophotometer samples with reading between 1.6 and 1.8 were considered pure. RNA concentration was calculated by using the following formula.

$$\text{RNA } (\mu g/\mu l) = OD_{260} \times 40 \times \text{dilution factor}$$

2.2.3.1.3 Synthesis of cDNA by Reverse Transcriptase PCR (RT-PCR)

RT-PCR was performed to synthesise DNA from total RNA. Superscript II ^{TM} Reverse Transcriptase (Invitrogen) was used that eliminates RNase H activity, which degrades mRNA during first strand reaction and helps in obtaining full-length cDNA. One µg of RNA was taken in DEPC water in a final volume of 10.5 µl. Volume of RNA sample and water was adjusted depending on the concentration of each RNA sample. One µl of Oligo(dT) anchored primers (Sigma) were added to the total RNA solution. The reaction mixture was incubated for 10 minutes at 70°C in PCR machine. After incubation was completed, the temperature was dropped down to 45°C. One µl superscript II (Invitrogen), 2 µl RT buffer (10x), 2 µl $MgCl_2$, 2 µl of 0.1M DTT, 1 µl dNTPs (10mM) and 0.5 µl RNaseOUT (Promega) were added to each sample and incubated for 60 minutes at 45°C. Reaction was stopped at 70 ^{o}C for 10 mins and tubes were placed on ice. To digest the template of RNA, 1 µl RNase H (Promega) was added to mixture and incubated at 37^{o}C for 30 minutes. DEPC water was added to adjust the final volume to 50 µl and samples were stored at -20^{o}C.

2.2.3.1.4 Quantitative Real Time-PCR (qRT-PCR)

Quantitative PCR was used to analyse cDNA using Light Cycler (Roche diagnostics, Mannheim). This is the technique used to monitor the fluorescence emission during the reaction indicating the amplicon production of each PCR cycle in real time as opposed to end point detection. cDNA was amplified by SYBR green master mix (Qiagen). The master mix contains fluorescent dye, SYBR green I. SYBR green I, unbound with cDNA emits a minor fluorescence. This fluorescence is extremely increased when it binds to double-stranded DNA. After the annealing step of each cycle, more and more SYBR green I bind to newly replicated double-stranded DNA during extension step, resulting in a huge increase in fluorescence emission at the end of each cycle. So amplification of cDNA is directly proportional to increased fluorescence levels at the end of each amplification cycle.

2.2.3.1.4.1 Analysis of gene expression by quantitative qRT-PCR

Levels of lung mRNA expression of MIP-2, IL-6, IL-1β, IL-10, IL-17, IFN-γ and TNF-α, were determined by quantitative real-time PCR using the Light Cycler. Before that, the concentration of GAPDH (Glucose-6-phosphate dehydrogenase) cDNA was determined for each sample and used as an endogenous control to normalise the expression levels of each cytokine by estimating the amount of starting material for each sample. Each sample was processed in 15 µl of PCR reaction containing 1 µl of original cDNA synthesis reaction (corresponding to 30 ng of total RNA), 0.5 µM of each primer, and 7.5 µl of QuantiTect SYBR Green Master Mix (Qiagen). Reaction mixture was spun at 3000rpm for one minute and subjected to forty-five cycles of amplification with a constant annealing temperature of 58 ^{o}C for each cycle throughout

the reaction in qRT-PCR machine. The samples were cycled according to experimental protocol descried in Table 2.2.

Table 2.2: Quantification protocol for amplification of cDNA of different genes using Light Cycler.

Program	Denaturation			Type	None	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC/Sec)	2 Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	95	900	20	0	0	0	None
Program	Cycling			Type	None	Cycles	45
Segment Number	Temperature Target (C)	Hold Time (Sec)	Slope (C^o/Sec)	2 Target Temp (C)	Step Size (C)	Step Delay (Cycles)	Acquisition Mode
1	95	15	20	0	0	0	None
2	58	20	20	0	0	0	None
3	72	15	15	0	0	0	Single
Program	Melt			Type	Melting curves	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC /Sec)	2 Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	95	2	20	0	0	0	None
2	65	10	1	0	0	0	None
3	95	0	0.1	0	0	0	Continuous
Program	Cool			Type	None	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC /Sec)	2^o Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	40	30	20	0	0	0	None

2.2.3.1.4.2 qRT-PCR Data analysis

After detection of fluorescence signal at the end of each cycle, mRNA expression data was analysed by using 'Fit Points' option in LDCA software supplied with the machine. Error in calculation of the standard curve was minimised by setting the noise band above the baseline after which the values of relative expression for all samples were displayed by program. Fig 2.1 is an example of data analysis from light cycler showing analysis of GAPDH.

All the qRT-PCR experiments were performed in two runs. Inter-assay variation was typically less than 10%. If variation exceeded 10%, samples were re-run.

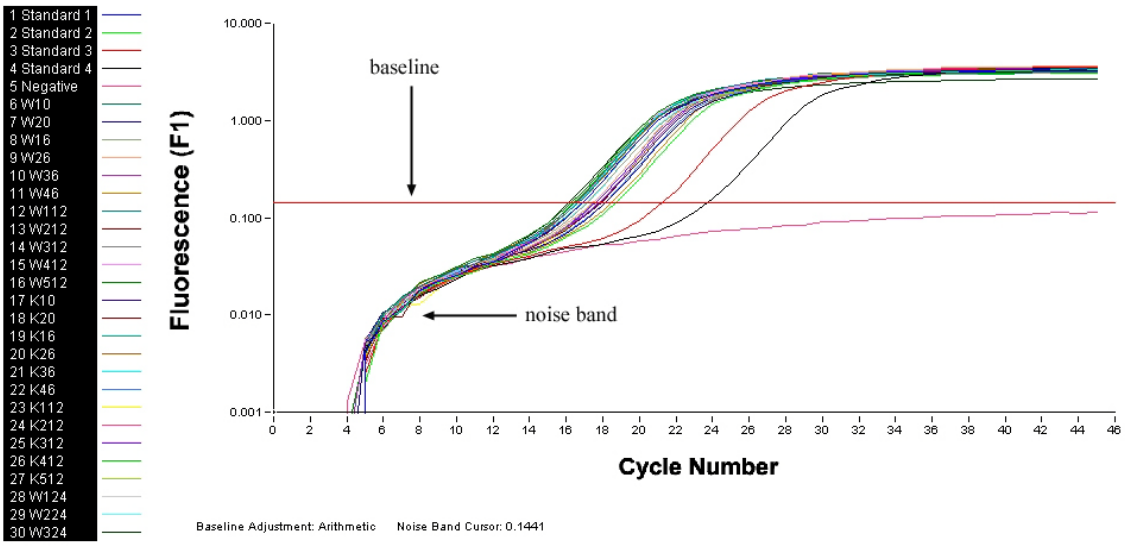


Figure 2.1: GAPDH amplification curves using the Light Cycler. Each colored line represents a sample. Curves were analysed by fit points methods by adjusting the baseline to remove the noise band background.

2.2.3.1.4.3 Optimisation of Real Time PCR

2.2.3.1.4.3.1 Standard Curve

After preparation of cDNA, 5 µl of each sample from ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice was mixed together to make pooled cDNA. Concentrated pooled cDNA was serially diluted 5-folds and was quantified along with all the samples tested for each cytokine analysis to produce a standard curve for that specific cytokine. The crossing points of the samples were determined, and the corresponding concentrations were automatically displayed on the screen (Fig 2.2). This standard curve was used to determine relative cytokine expression levels in different samples.

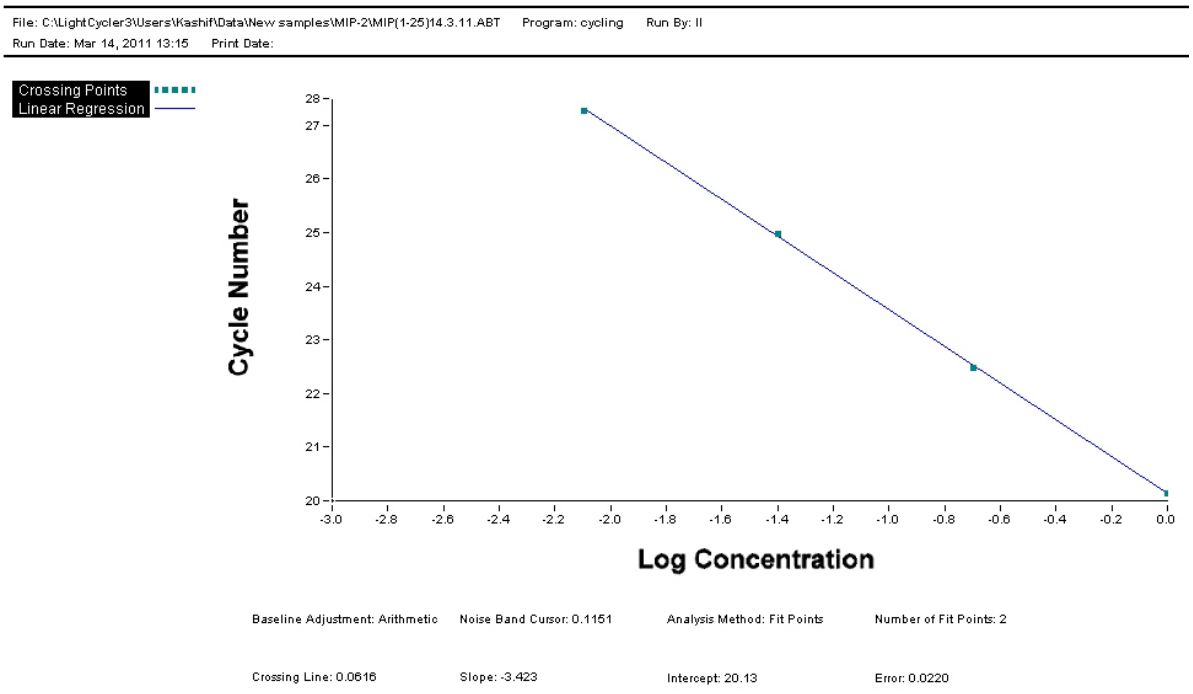


Figure 2.2: Standard curve of MIP-2 expression levels generated by the Light Cycler and used for calculation of relative expression levels of other samples.

2.2.3.1.4.3.2 Melting Curve Analysis

Melting curve analysis was used to confirm the specificity of product. To carry out melting curve analysis, the temperature was increased very slowly from a low

temperature (65°C) to a high temperature (95°C), and fluorescence intensity was plotted against the temperature. Figure 2.3 shows the effect of the temperature increase on the fluorescence. Green outline shows the decrease in fluorescence when temperature is increased. The red outline shows denaturation of dsDNA and release of SGI. Arrow shows the melting temperature and indicates the point of inflection on the melting curve and is indicative of the temperature at which half of the DNA is double stranded, and half is single stranded. Figure 2.4 shows a plot of the negative derivative of the rate of change of fluorescence (–dF/dT, the rate of change of fluorescence) vs. temperature. Tm can easily be identified by the peak at 85°C. A single peak of the melting temperature corresponds to specificity of each product.

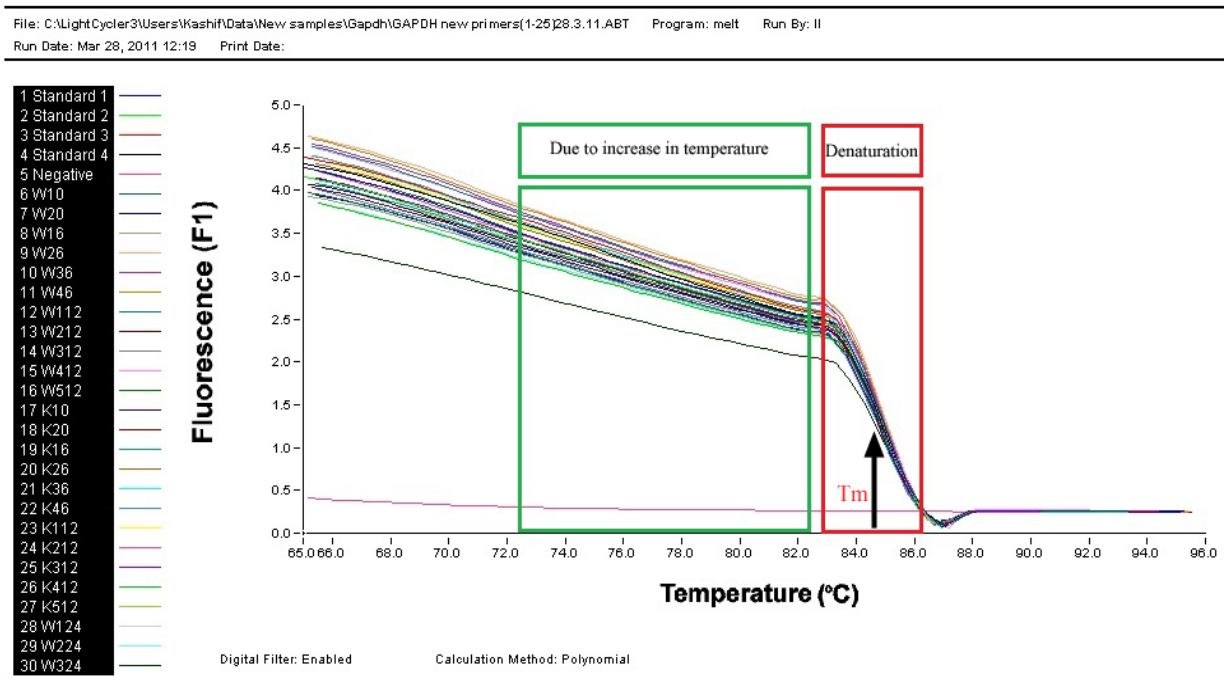


Figure 2.3: Melting curve analysis of GAPDH

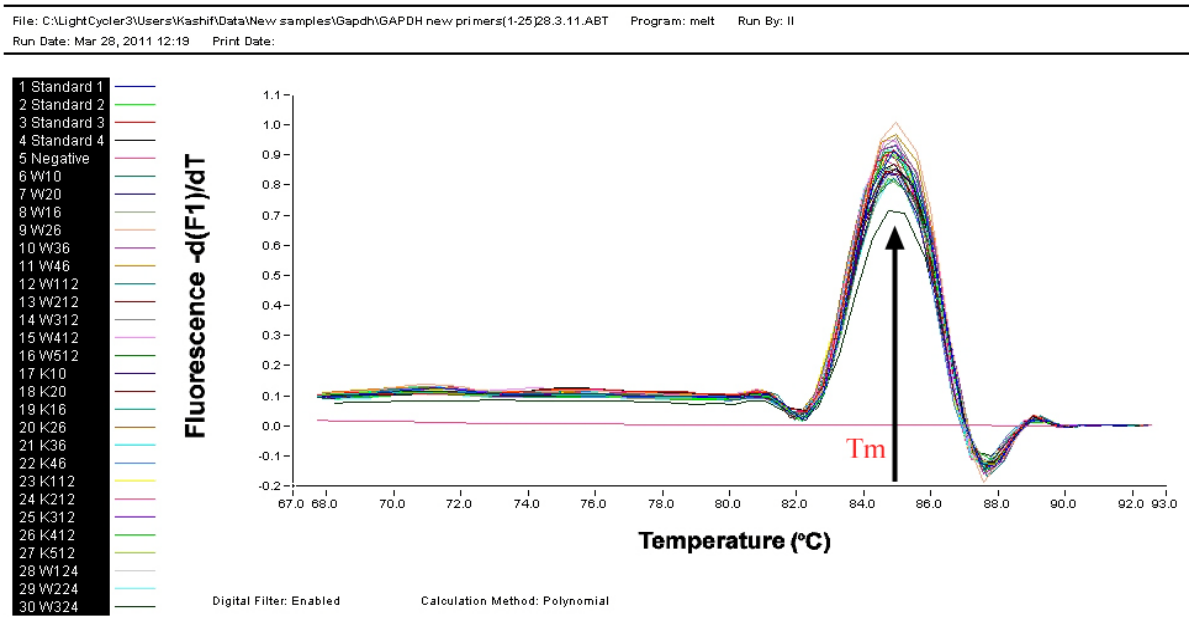


Figure 2.4: The negative derivative of the rate of change of fluorescence vs. temperature (-dI/dT) of TNF-α cDNA copies. Single peak of melting temperature and a linear negative control line (red) shows the purity of amplified product.

Table2.3: Sequence of oligonucleotides used in quantitative real time PCR

Primer	**Sequence** (5' to 3')	**Product size**
IL-1β_F	CACTCATTGTGGCTGTGGAGA	247 bp
IL-1β_R	AGGTGGAGAGCTTTCAGCTCA	
TNFα_F1	CCTCACACTCAGATCATCTTCTCA	237 bp
TNFα_R2	GTGGGTGAGGAGCACATAG	
GAPDH_F2	GTGCTGCCAAGGCTGTG 3	211bp
GAPDH_R1	AGACAACCTGGTCCTCAGTGTA	
IL6_F	CAAAGCCAGAGTCCTTCAGA	95 bp
IL6_R	CACTCCTTCTGTGACTCCA	
IL10_F	CTTGCACTACCAAAGCCACA	86 bp
IL10_R	TAAGAGCAGGCAGCATAGCA	
INFγ_F	CCTGCGGCCTAGCTCTGA	81bp
INFγ_R	CAGCCAGAAACAGCCATGAG	
MIP2_F	ATCCAGAGCTTGAGTGTGAC	90bp
MIP2_R	AAGGCAAACTTTTTGACCGCC	
IL-17 F	GTGAAGGCAGCAGCGATCATC	128bp
IL-17 R	CTTCTGGAGCTCACTTTTGCGC	

2.2.3.2 DNA based methods

2.2.3.2.1 Isolation of genomic DNA

Genomic DNA was isolated from the mouse ear snips using the Wizard genomic DNA Purification Kit (Promega). 0.3cm ear snips were taken from mice and digested overnight with 60 µl of 0.5M EDTA (pH 8.0) solution, 250 µl of Nuclei Lysis solution (comes with kit) and 10 µl of proteinase K (Qiagen). Next day, 1.5 µl of Rnase A solution (4mg/ml) was added to the nuclear lysate and mixed by inverting the tube 2-5 times and incubated at 37^{o}C for 30 minutes. Samples were allowed to cool at room temperature and 100 µl of protein precipitation solution (Promega) was added to the mixture at room temperature. The mixture was vortexed vigorously at high speed for 20 seconds and chilled on ice for five minutes. Samples were then centrifuged at 13000rpm for 10 minutes, which precipitated the protein in white pellet and left DNA in solution form. Supernatant containing the DNA was carefully removed and transferred to a clean 1.5 ml labeled Eppendorf tube. 300 µl of room temperature (RT) isopropanol was added to each tube and mixed by inverting several times followed by centrifugation at 13000 rpm for 20 minutes. Supernatant was discarded and 300 µl of 70% RT ethanol was added to the pellet by gently inverting the tube several times to wash the genomic DNA. Tubes were spun again at 13000rpm, and ethanol was carefully removed using a Gilson pipette. Tubes were inverted on clean absorbent paper, and the pellet was air dried until all the ethanol was removed. 50 µl of H_2O (Nuclease-free water) was added to dissolve the pellet and incubate at 65^{o}C for 1 hour with intermittent shaking. Isolated genomic DNA was then stored at 4^{o}C.

2.2.3.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a method of amplifying certain fragments of DNA without the need of living cell. The basic PCR reaction takes place *in vitro*, where the primer strand is added from outside in the form of deoxyoligonucleotide and Taq polymerase enzyme is added to help polymerisation. Repeating reactions maintains the unlimited supply of amplified DNA. PCR takes place in several steps. Initial denaturation at a high temperature separates the DNA in complimentary strands this is followed by 35 cycles of denaturation, annealing when primers bind specifically to DNA strand; and extension step, in which new complimentary DNA is synthesised by taq DNA polymerase. In final extension, synthesis of DNA is allowed to complete. Taq polymerase is the DNA polymerase which is isolated from *Thermus aquaticus* growing in hot springs. The enzyme acts best at 72^{o}C and the denaturation temperature of 90^{o}C does not destroy its enzymatic activity.

2.2.3.2.2.1 Genotyping of ficolin $A^{-/-}$ and ficolin $A^{+/+}$mice with PCR

Genotyping of ficolin $A^{-/-}$ mice was performed using multiplex PCR for identification of ficolin $A^{-/-}$ mice before using them in infection studies. PCR was optimised first by adjusting the annealing temperature and concentration of DNA template to be used. Annealing temperature of 64^{o}C was chosen after testing at different annealing temperature and instead of using concentrated DNA, 2 µl of isolated genomic DNA diluted 1:10 was used to amplify the fragments. *In vitro* amplification was performed in a programmable thermal cycler. The PCR mix for reaction of 15µl contained the following reagents.

Genomic DNA diluted 1:10	2 µl
Reaction buffer (10x)	1.5 µl
$MgCl_2$ (2.5mM)	1.5 µl
dNTP mix. (10 mM)	0.3 µl
FcnA_F1	1.5 µl
L3865	1.5 µl
Neoµ1	1.5 µl
Taq-DNA polymerase	0.12 µl
Nanopure distilled water	5.08 µl

PCR program used for genotyping was,

1. Initial denaturation 95^oC 90 seconds
2. Denaturation 95^oC 30 seconds
3. Annealing 62^oC 30 seconds
4. Elongation 72^oC 30 seconds
5. Final elongation 72^oC 30 seconds

Three primers used for identification of homozygous, heterozygous and wild-type mice (Table 2.4),

Table 2.4: Primers for ficolin A genotyping.

Primer name	**Primer sequence**
FcnA_F1	5'-GTT AGA GAG CTG GCT ACT CCG ATG A-3'
L3865	5'-TCT CCA CCT TCC TCT TCC TCC TCT A-3'
Neoµ1	5'-CAT CGC CTT CTA TCG CCT TCT TGA -3'

3. Role of the lectin pathway recognition molecule ficolin A in fighting pneumococcal infection

3.1 Results

3.1.1 *In vitro* Studies

3.1.1.1 Binding of carbohydrate recognition molecules of the lectin pathway to *Streptococcus pneumoniae* D39

Activation of the lectin pathway of complement is initiated through various lectin pathway recognition sub components including MBL and recently described C-type lectin, CL-11, which recognise a wide range of carbohydrate structures on microbial surfaces. There are only two different ficolins in mice, i.e. ficolin A and ficolin B. Ficolin A is the only circulating ficolin in mice. Together with mannan binding lectins (MBL-A and MBL-C) and CL-11, ficolin A functions in host defence by driving complement activation via MASP-2, the effector enzyme of the lectin pathway of complement activation. Ficolin B is found in neutrophils as well as in monocytes and macrophages where it is associated with cell surfaces and functions primarily through its agglutination activity supporting opsonophagocytosis (Runza *et al.*, 2006). To define the potential role of these carbohydrate recognition molecules in activating lectin pathway, their binding affinity was tested on *Streptococcus pneumoniae* D39. These binding assays revealed that serum ficolin A and CL-11 show strong binding to *S. pneumoniae* D39 (Fig 3.1 & 3.4 respectively), this was further confirmed using recombinant ficolin A and CL-11 by replacing murine serum with recombinant ficolin A and CL-11. Recombinant ficolin A showed very strong binding to *S. pneumoniae*

D39, which was even higher than the binding to N- acetyl BSA used as positive control (Fig 3.2). Recombinant CL-11 also showed very strong binding to *S. pneumoniae* D39 and binding levels were similar to zymosan (which was used as positive control for CL-11 binding) (Fig 3.5). MBL-A did not bind to *S. pneumoniae* D39 whereas MBL-C showed weak binding (Fig 3.2).

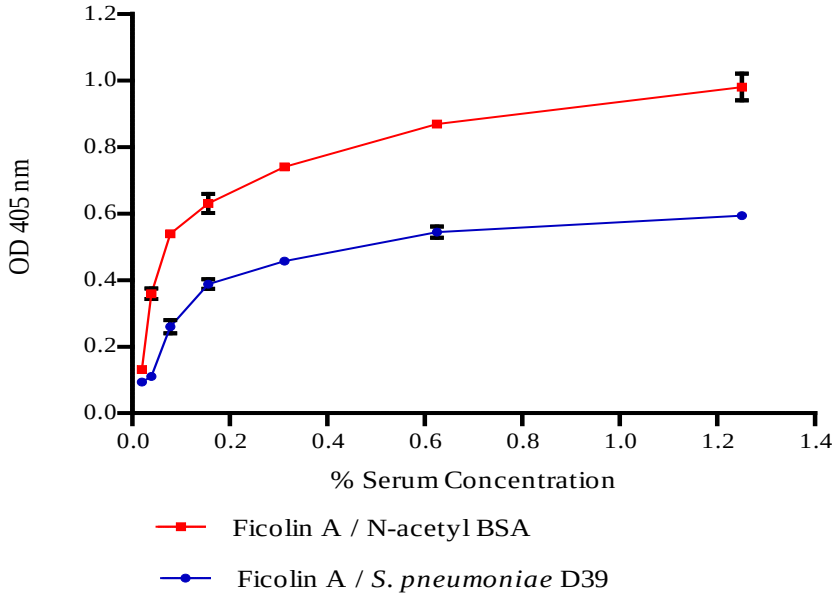


Figure 3.1: Ficolin A binding assay to determine binding of ficolin A with *Streptococcus pneumoniae* D39 by ELISA: Following coating of microtitre plates with N-acetyl BSA (+ve control) and formalin fixed bacteria, incubation with normal mouse serum, ficolin A binding was assessed using a specific rabbit anti-ficolin A (provided by T. Fujita). Results are mean (±SEM) of duplicates and are representative of three independent experiments (shown in addendum 1 Fig. 7.1).

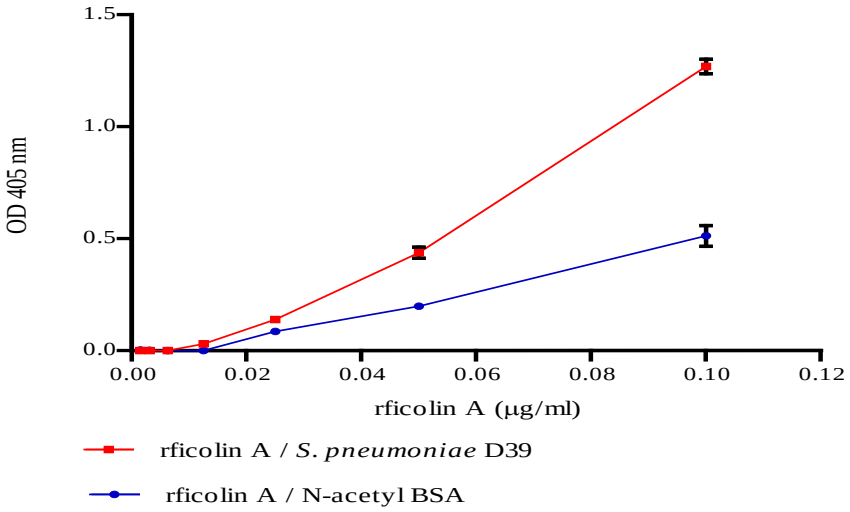


Figure 3.2: Ficolin A binding assay to determine binding of recombinant ficolin A with *Streptococcus pneumoniae* D39 by ELISA: Following coating of microtitre plates with N-acetyl BSA (+ve control) and formalin fixed bacteria, incubation with rficolin A, recombinant ficolin A binding was assessed using a specific rabbit anti-ficolin A (provided by T. Fujita). Results are mean (±SEM) of duplicates and are representative of three independent experiments (shown in addendum 1 Fig. 7.2).

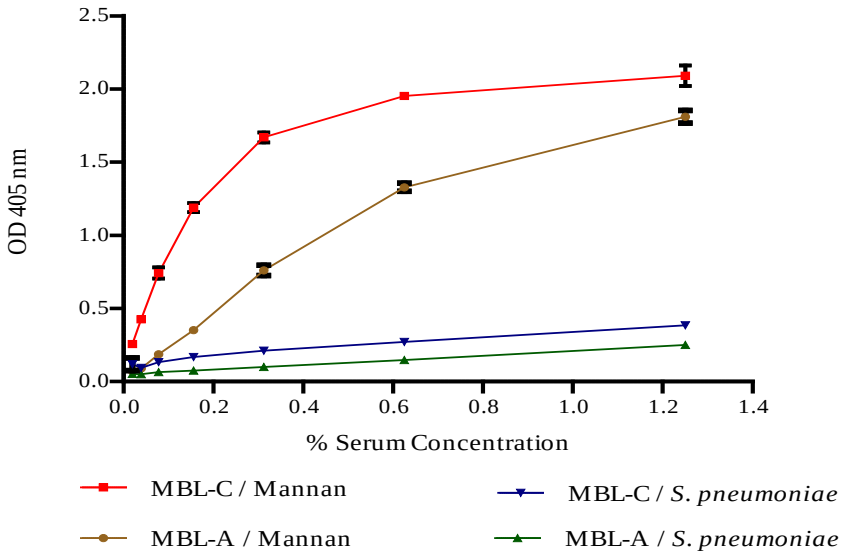


Figure 3.3: MBL-A and MBL-C binding to *Streptococcus pneumoniae* D39: Following coating of microtitre plates with mannan (+ve Ctr) and formalin fixed bacteria, incubation with normal mouse serum, MBL-A and MBL-C binding was assessed using specific rat anti-mouse MBL-A and MBL-C (Hycult). Results are mean (±SEM) of duplicates and are representative of three independent experiments (shown in addendum 1 Fig. 7.3).

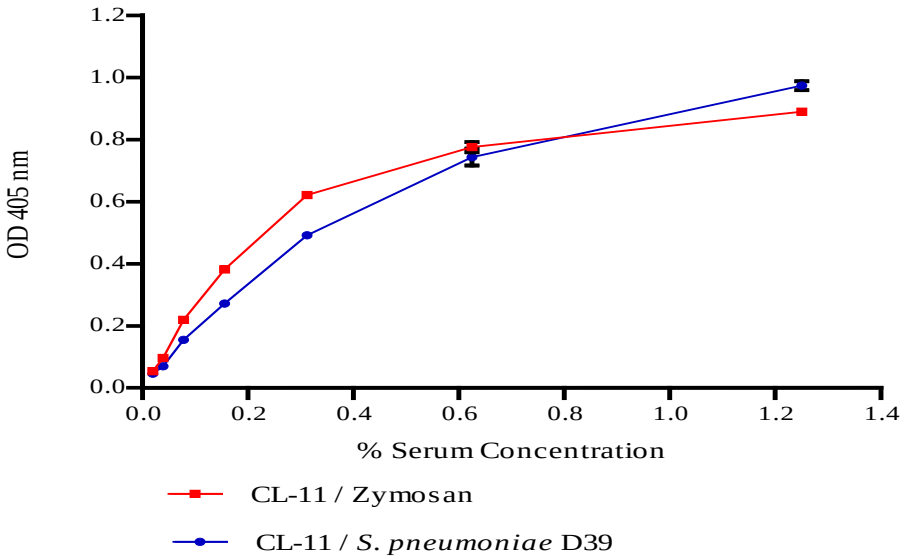


Figure 3.4: CL-11 binding assay to determine binding of murine CL-11 to *Streptococcus pneumoniae* D39 by ELISA: Following coating of microtitre plates with zymosan (+ve Ctr) and formalin fixed bacteria, incubation with normal mouse serum, CL-11 binding was assessed using a specific rat anti-mouse CL-11 (provided by Soren Hansen). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

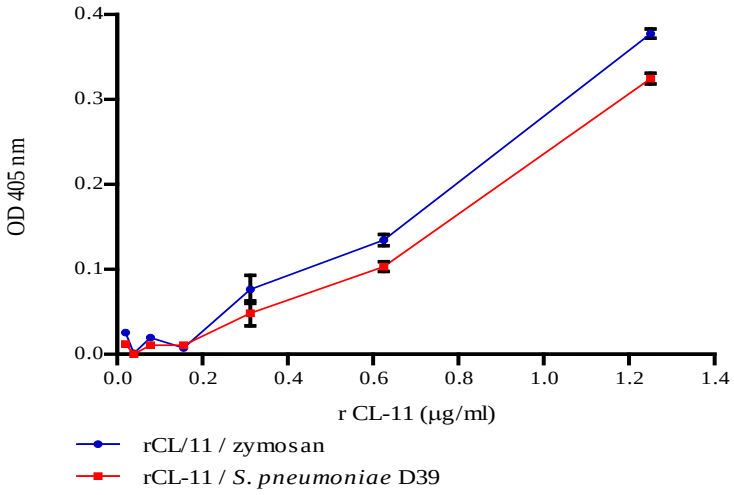


Figure 3.5: CL-11 binding assay to determine binding of recombinant human CL-11 to *Streptococcus pneumoniae* D39 by ELISA: Following coating of microtitre plates with zymosan (+ve Ctr) and formalin fixed bacteria, incubation with recombinant human CL-11, CL-11 binding was assessed using a specific rat anti-human CL-11 (provided by Soren Hansen). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.1.1.2 Binding of mouse lectin pathway carbohydrate recognition molecules to serotypes and strains of *Streptococcus pneumoniae*

Ficolin A showed strong binding to *Streptococcus pneumoniae* D39. This binding was further assessed in different serotypes and strains of *S. pneumoniae* to check any variation in binding within these serotypes or strains. Six serotypes were randomly selected including serotype 6B, 18C, 3, 9V, 8 and 19A. Interestingly, the variation of binding intensity of ficolin A with these serotypes was high indicating variations in surface compositions among serotypes (Fig. 3.6). Serotype 6B, 8 and 19A showed very high binding to ficolin A whereas selected strains of serotype 18C, 3 and 9V did not show any binding. To find if this variation is serotype or strain dependent, ficolin A binding was further assessed in different strains of serotype 6B (which showed very high binding), 18C and 3 (which did not show any binding with ficolin A). Three clinical isolates were selected from each serotype (kindly provided by Prof. Hermínia de Lencastre, ITQB, Portugal) and tested for ficolin A binding. This assay revealed variation still exists between strains of same serotype showing that variation in the binding of ficolin A is strain dependent not serotype dependent. Isolate Pt547 from serotype 6B and Pt5634 from 18C showed very high affinity for ficolin A (Fig 3.7).

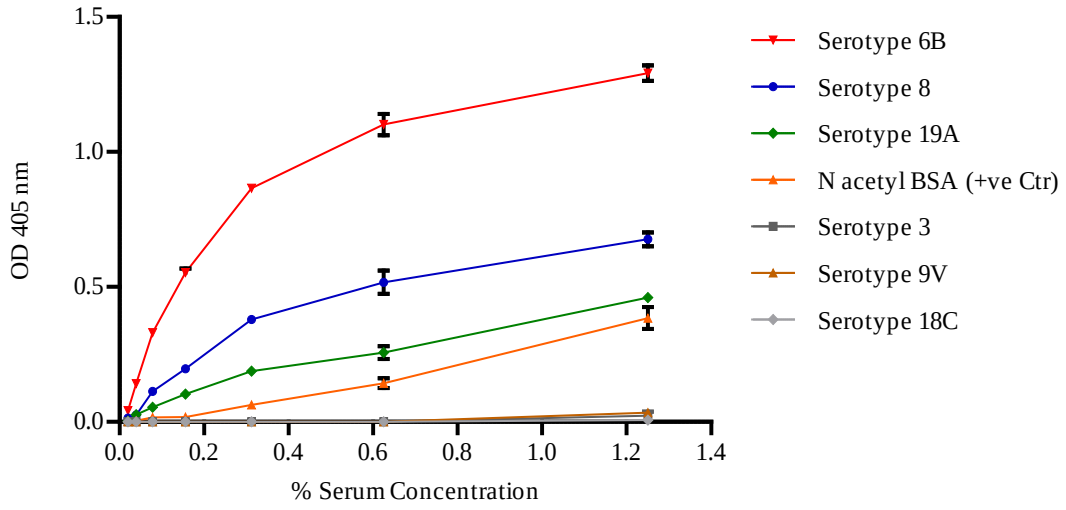


Figure 3.6: Ficolin A binding assay on *Streptococcus pneumoniae* serotype 6B, 8, 19A, 18C, 9V and 3 by ELISA: Following coating of microtitre plates with N-acetyl BSA (+ve control) and formalin fixed bacteria, incubation with normal mouse serum, ficolin A binding was assessed using a specific rabbit anti-ficolin A (T. Fujita). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

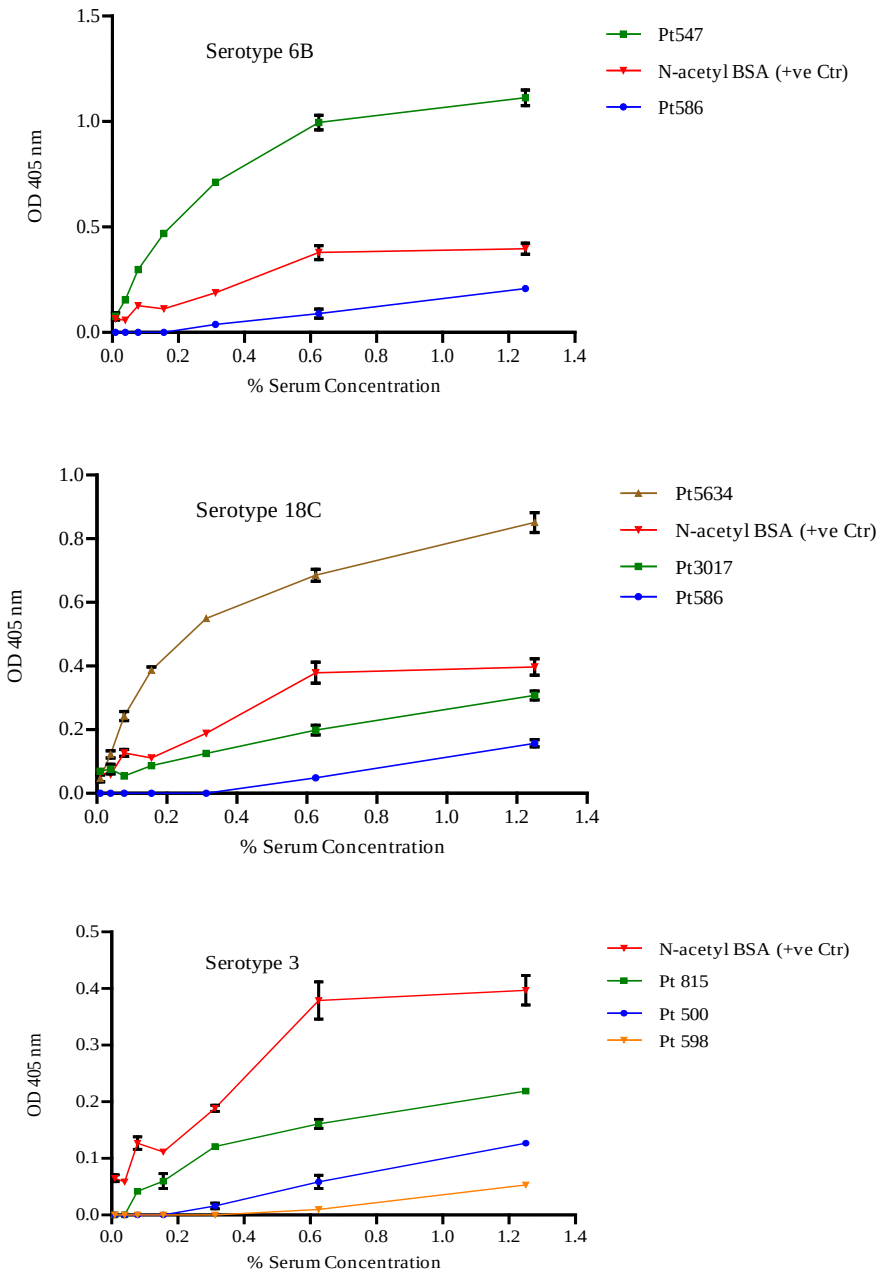


Figure 3.7: Ficolin A binding assay on strains of *Streptococcus pneumoniae* seroytype 6B, 18C , and 3 by ELISA: Following coating of microtitre plates with N-acetyl BSA (+ve control) and formalin fixed bacteria, incubation with normal mouse serum, ficolin A binding was assessed using a specific rabbit anti-ficolin A (provided by T. Fujita Japan). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

CL-11 binding was checked on the same isolates to see any variation. Results showed that CL-11 consistently shows strong binding to all the strains of *S. pneumoniae.* There was no variation in binding affinity within the strains or serotypes (Fig. 3.8). MBL-A showed no or weak binding to the strains of serotype 6B, 3 and 18C with slight variation. MBL-C showed weak binding to all the strains with slight variation (Figure 3.9).

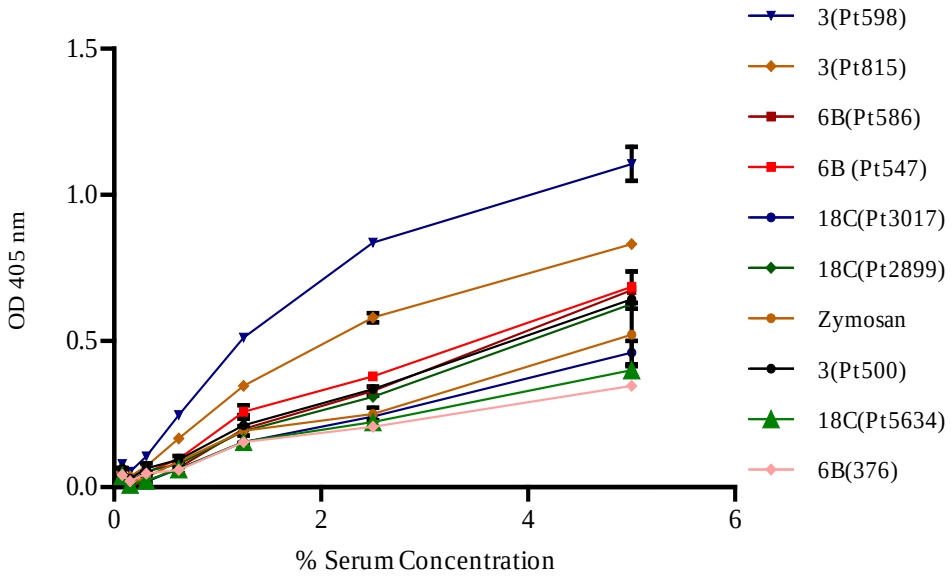


Figure 3.8: CL-11 binding assay on strains of *Streptococcus pneumoniae* seroytype 6B, 18C, and 3 by ELISA: Following coating of microtitre plates with zymosan (+ve control) and formalin fixed bacteria, incubation with normal mouse serum, CL-11 binding was assessed using a specific rat anti-mouse CL-11 (Soren Hansen). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

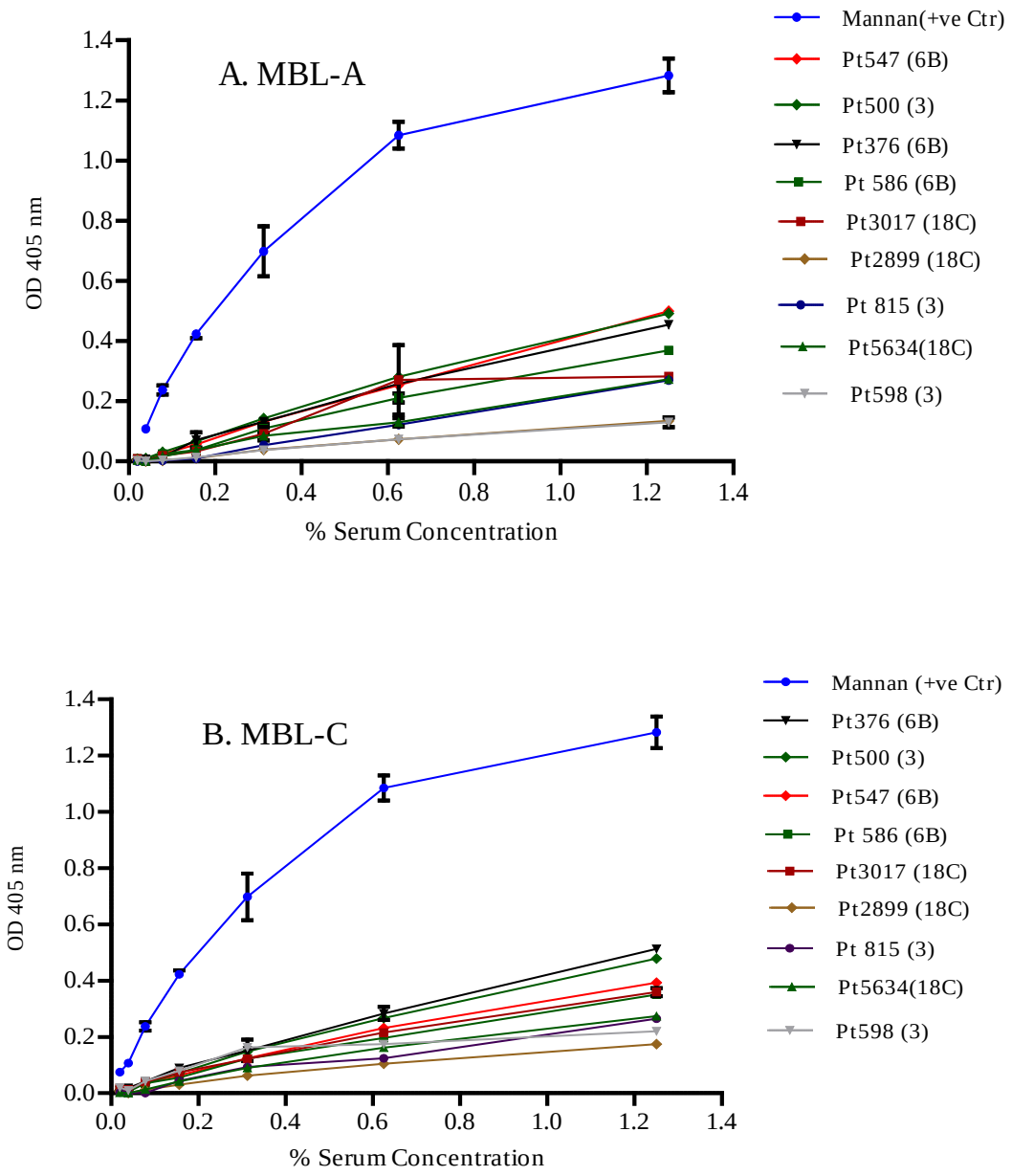


Figure 3.9: Binding MBL-A and MBL-C to different strains of *Streptococcus pneumoniae* serotypes 6B, 18C, and 3: Following coating of microtitre plates with mannan (+ve control) and formalin fixed bacteria, incubation with normal mouse serum, MBL-A and MBL-C binding was assessed using a specific rat anti-mouse MBL-A and MBL-C (Hycult). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.1.1.3 Binding of human lectin pathway carbohydrate recognition molecules to various serotypes and strains of *Streptococcus pneumoniae*

Lectin pathway in humans is activated by MBL, ficolins and CL-11. There are three different human ficolins, i.e. L-ficolin, H-ficolin and M-ficolin. Binding of all these carbohydrate recognition molecules was tested on different strains of *Streptococcus pneumoniae*. The results showed that among all human lectin pathway recognition molecules, L-ficolin (Fig. 3.11) and CL-11(Fig. 3.14) strongly bound to all strains *S. pneumoniae* without any variation within strains or serotypes. MBL (Fig. 3.10), H-ficolin (Fig. 3.12) and M-ficolin (Fig. 3.13) did not show binding to any strain or serotype of *S. pneumoniae*. Binding of all the carbohydrate recognition molecules in humans was consistent with all strains tested unlike mouse ficolin A which showed significant variations within their binding to different strains.

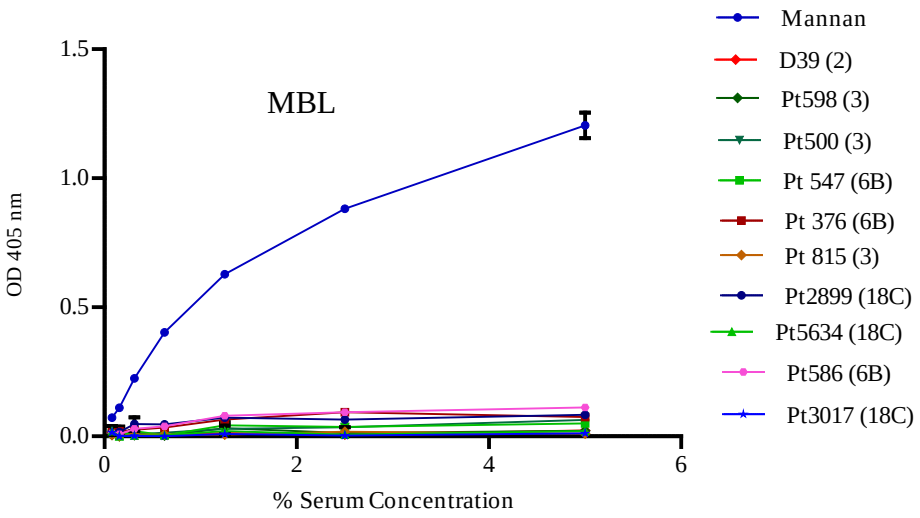


Figure 3.10: Binding of MBL to different strains of *Streptococcus pneumoniae* serotypes 6B, 18C, and 3 using human serum: Following coating of microtitre plates with mannan (+ve control) and formalin fixed bacteria, incubation with human serum, MBL binding was assessed using a specific mouse anti-human MBL (Hycult). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

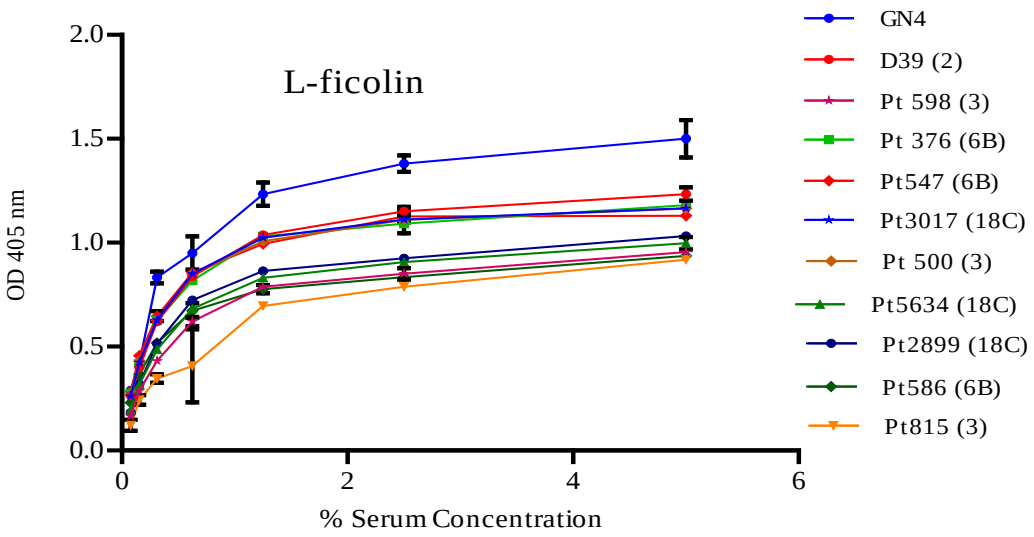


Figure 3.11: Binding of L-ficolin to different strains of *Streptococcus pneumoniae* serotypes 6B, 18C, and 3 using human serum: Following coating of microtitre plates with GN4 (monoclonal antibody against L-ficolin from Hycult used as positive control) and formalin fixed bacteria, incubation with human serum, L-ficolin binding was assessed using a specific rabbit anti L-ficolin (provided by Teizo Fujita). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

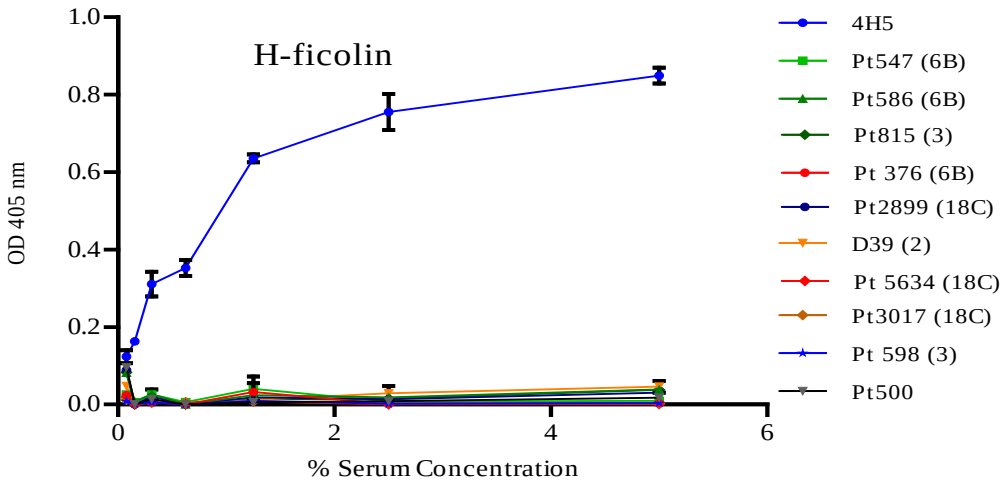


Figure 3.12: Binding of H-ficolin to different strains of *Streptococcus pneumoniae* serotypes 6B, 18C, and 3 using human serum: Following coating of microtitre plates with 4H5 (monoclonal antibody against H-ficolin from Hycult used as positive control) and formalin fixed bacteria, incubation with human serum, H-ficolin binding was assessed using a specific rabbit anti H-ficolin (Provided by H. Shiraki). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

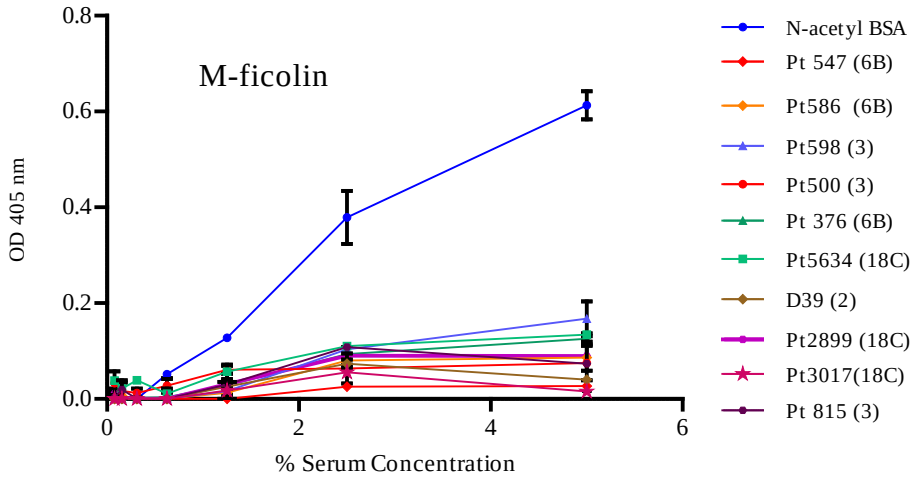


Figure 3.13: Binding of M-ficolin to different strains of *Streptococcus pneumoniae* serotypes 6B, 18C, and 3 using human serum: Following coating of microtitre plates with N-acetyl BSA (+ve control) and formalin fixed bacteria, incubation with human serum, M-ficolin binding was assessed using a specific rabbit anti M-ficolin (provided by Prof. Teizo Fujita). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

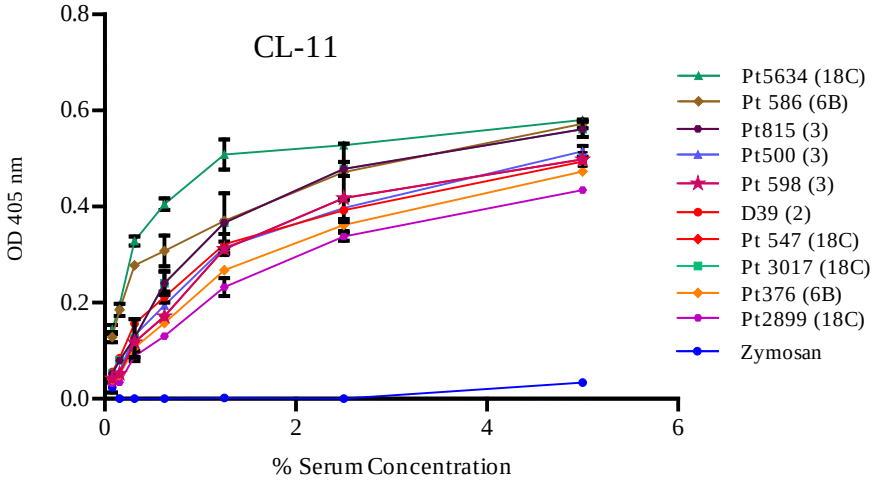


Figure 3.14: Binding of CL-11 to strains of *Streptococcus pneumoniae* seroytype 6B, 18C, and 3 using human serum: Following coating of microtitre plates with zymosan and formalin fixed bacteria, incubation with human serum, CL-11 binding was assessed using a specific mouse anti-human CL-11 (provided by Soren Hansen). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.1.1.4 C3 deposition assay on different serotypes of *Streptococcus pneumoniae*

Ficolin A showed some degree of variation in the affinity binding with different serotypes of *Streptococcus pneumoniae.* To see if the differences in binding affect the turnover of complement activation via lectin pathway activation on the surface of *S. pneumoniae*, a C3 deposition assay was performed on each of these serotypes. Serotypes showing strong binding with ficolin A, i.e. 6B, 8 and 19A (Fig. 3.6) showed higher C3 deposition (Fig. 3.15). Serotypes showing weak binding with ficolin A, i.e. Serotype 18C and 3, showed lower C3 deposition on their surface with an exception of serotype 9V, which showed higher C3 deposition despite weak binding activity. These results imply that ficolin A is the recognition molecules that predominantly control the activation of the lectin pathway against *S. pneumoniae*. Level of C3 deposition on the surface correlates with the level of ficolin A binding.

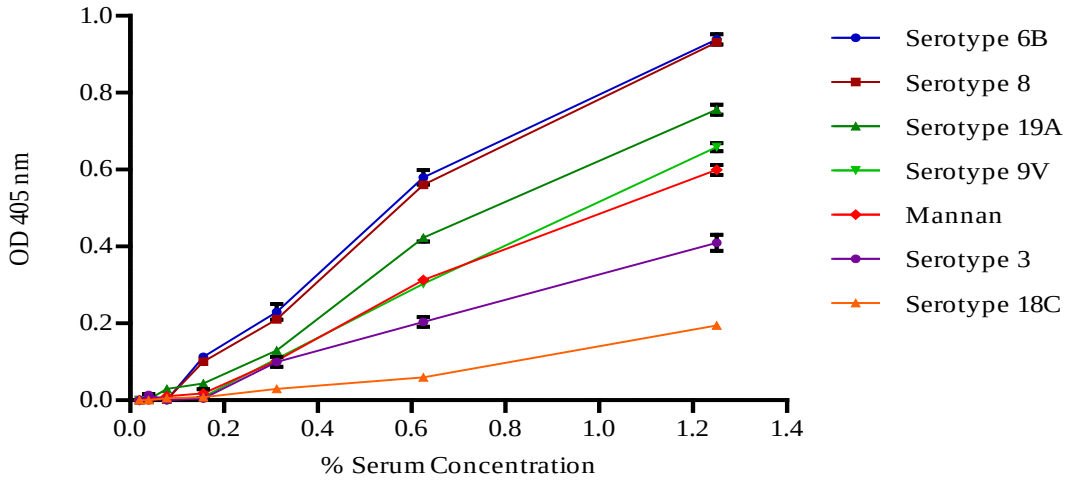


Figure 3.15: C3 deposition assay on *Streptococcus pneumoniae* serotypes 6B, 8, 19A, 9V, 3 and 18C: Following coating of microtitre plates with mannan and formalini fixed bacteria, incubation with normal mouse serum, C3b deposition was detected using specific rabbit anti-human C3c (Dako). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.1.1.5. C3 deposition assay on *Streptococcus pneumoniae* D39 in mouse sera of gene targeted mice deficient in various lectin pathway recognition subcomponents.

To assess the relative contribution of different lectin pathway recognition molecules in activating the lectin pathway against *Streptococcus pneumoniae,* C3 deposition was detected on *S. pneumoniae* D39 using sera deficient in ficolin A, MBL A/C, MBL-A/C/ficolin A and compared with wild-type and MASP-2 deficient sera. No C3 deposition was detected in MASP-2 deficient serum confirming that MASP-2 deficient serum is completely deficient of the lectin pathway functional activity. MBL-A/C$^{-/-}$ showed higher C3 deposition indicating neither MBL-A nor MBL-C is involved in the activation of the lectin pathway on *S. pneumoniae*. The level of C3 deposition was reduced in ficolin A$^{-/-}$ serum showing that ficolin A is a relevant carbohydrate recognition molecule in activating lectin pathway against *S. pneumoniae.* Remarkably, C3 deposition was still detected in ficolin A, MBL-A and MBL-C triple deficient serum, which is deficient in all carbohydrate recognition molecules except CL-11(Fig. 3.16). This implies that CL-11 on its own is capable to maintain residual lectin pathway activation by activating MASP-2 and to cause deposition of C3 on *S. pneumoniae.* These results underline that CL-11; a recently described C-type lectin, activates the lectin pathway against *S. pneumoniae* in the absence of ficolin A, MBL-A and MBL-C (Fig 3.16). My experiments showed that CL-11 shows very strong binding to *S. pneumoniae* (Fig. 3.4). The role of CL-11 in activating lectin pathway was further confirmed in later experiments.

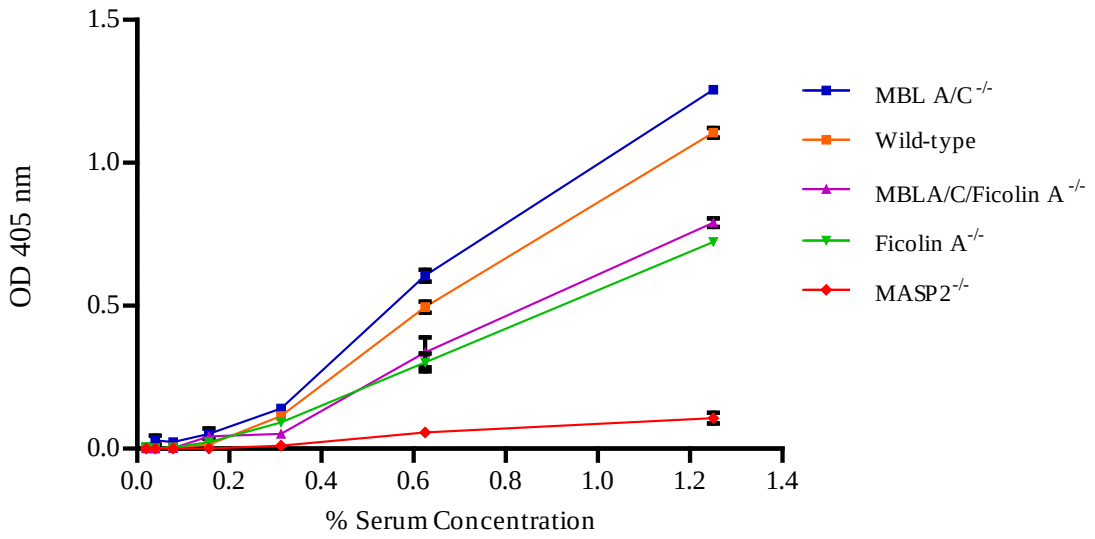


Figure 3.16: C3 deposition assay on *Streptococcus pneumoniae* D39 using sera from gene targeted mice deficient in various carbohydrate recognition molecules: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with sera, C3 deposition was detected using specific rabbit anti-human C3c (Dako). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.1.1.6. Role of CL-11 in activating lectin pathway of complement

C3 deposition assay on *Streptococcus pneumoniae* D39 using serum from mice deficient in different lectin pathway recognition molecules showed that C3 deposition occurs in serum deficient of MBL-A, MBL-C and ficolin A. Our hypothesis was that this C3 activation is initiated by CL-11, which also shows strong binding to D39. To further support this hypothesis CL-11 binding was checked on different carbohydrates, and this binding was compared with C3 deposition on these sugars using all the deficient sera previously tested on *S. pneumoniae* D39. CL-11 showed strong binding to N-acetyl BSA and zymosan but did not bind to mannan (Fig 3.17).

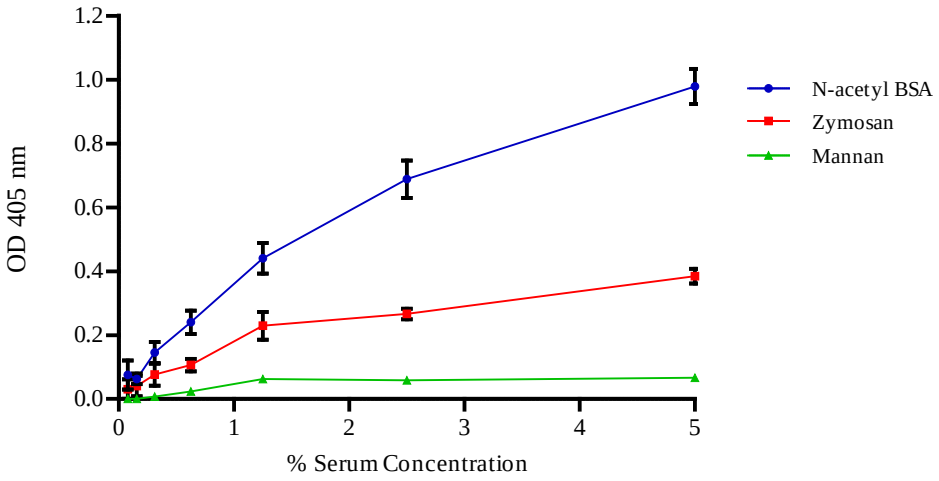


Figure 3.17: CL-11 binding to N-acetyl BSA, zymosan and mannan by ELISA: Following coating of microtitre plates with 1µg/well N-acetyl BSA, zymosan and mannan, incubation with normal mouse serum, CL-11 binding was assessed using a specific rat anti-mouse CL-11 (from Soren Hansen). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

Binding of CL-11 with above sugars was correlated with C3 deposition on those carbohydrates in MBL-A/C/ficolin A triple knock out serum. On mannan, C3 deposition is MBL-A and MBL-C dependent. C3 deposition was higher on mannan in wild-type and wasn't affected in ficolin $A^{-/-}$ mice serum, which was mediated by MBL-A and MBL-C. However, no C3 deposition occurred in MASP-$2^{-/-}$, MBL-A/$C^{-/-}$ and MBL-A/C/ficolin $A^{-/-}$ serum. Although CL-11 was fully functional in MBL-A/C double knockout and MBL-A/C/ficolin $A^{-/-}$ mice, no C3 deposition occurred on mannan using these sera due to lack of binding of CL-11/or ficolin A with mannan (Fig 3.18 A). However, reduced levels of C3 deposition were detected on zymosan and N-acetyl BSA using MBL-A/C/ficolin $A^{-/-}$ serum. CL-11 showed strong binding to zymosan and BSA so this C3 deposition detected on the surface of zymosan and N-

acetyl BSA in triple knock out serum is due to CL-11 activating the MASP-2 (Fig 3.17 B&C).

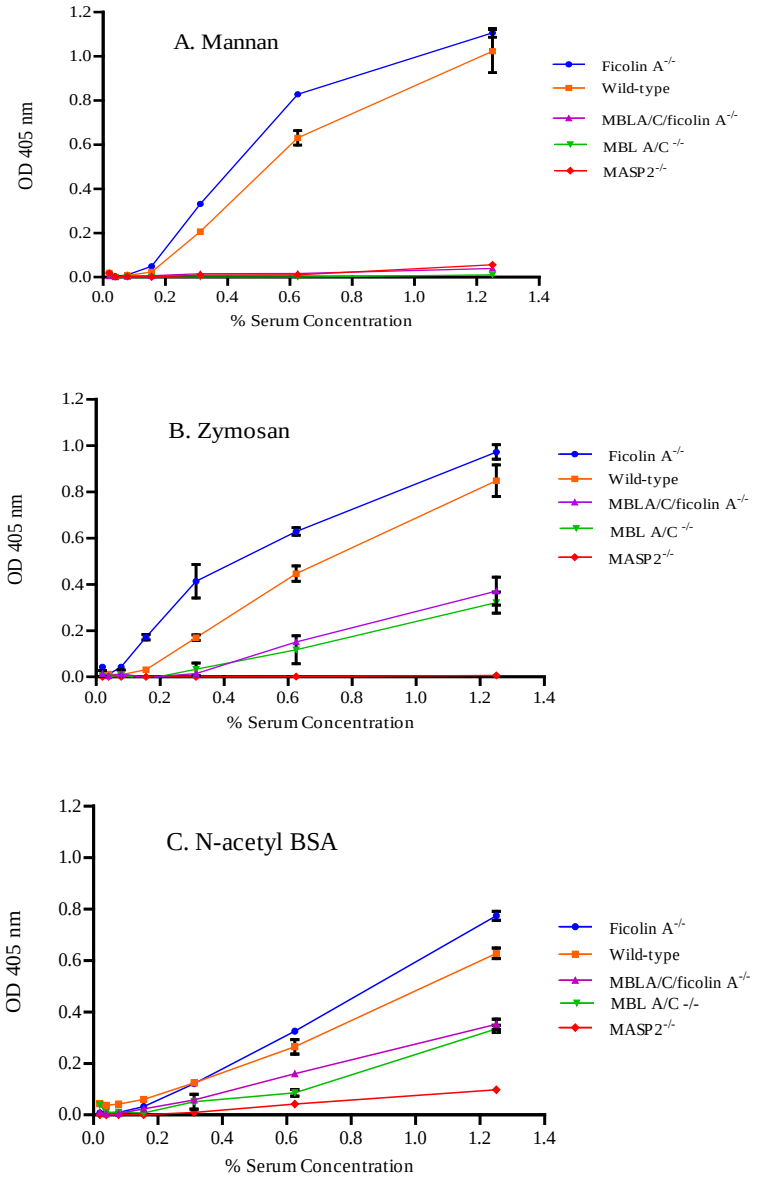


Figure 3.18: C3 deposition assay on mannan (A), zymosan (B), and N-acetyl BSA (C), using sera of transgenic mice deficient in different carbohydrate recognition molecules: Following coating of microtitre plates with mannan, zymosan and BSA, incubation with sera, C3b deposition was detected using specific rabbit anti-human C3c (Dako). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

3.1.2 *In vivo* Studies

3.1.2.1 Genotyping of ficolin $A^{-/-}$ mice

Ficolin A deficient mice used in this study were imported from Fukushima Medical University Japan. Dr. Y. Endo of Prof. Fujita's research team, generated ficolin $A^{-/-}$ mice by producing a targeting construct to disrupt the *FcnA* gene by homologous recombination. Chimeric mice were generated by implanting C57BL/6J blastocysts with targeted ES cells. Heterozygous mice were produced by mating C57BL/6J female with male chimera. Mice from F2 heterozygous generation were intercrossed to produce homozygous ficolin $A^{-/-}$ mice (Endo *et al.*, 2010).

Genotyping of ficolin $A^{-/-}$ mice was performed using a multiplex PCR analysis of genomic DNA prepared from mouse ear snips. Three oligonucleotides were used to identify wild-type and the targetted alleles. The oligonuceotide FcnA_F1 and L3865 amplify the wild-type specific allele genotyping a 935bp fragment whereas FcnA_F1 and Neoμ1 amplify the targeted allele as a 1300bp fragment. DNA from heterozygous mice is amplified in two fragments of 935bp and 1300 bp (Fig. 3.19).

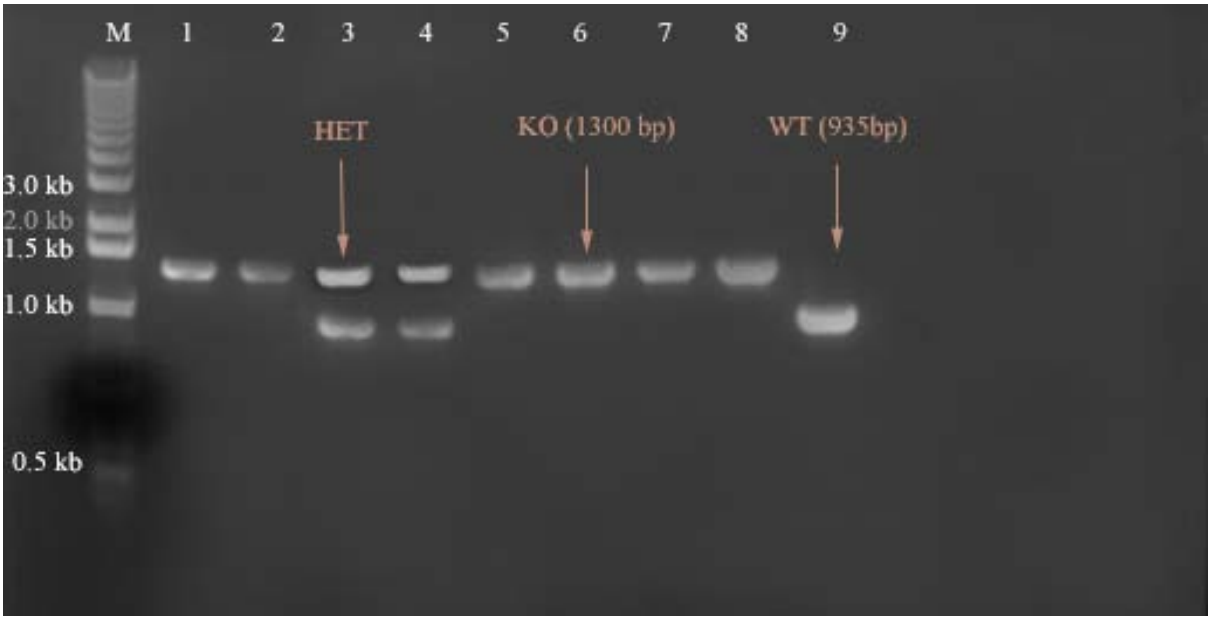


Figure 3.19: Genotyping of ficolin $A^{+/+}$, heterozygous and ficolin $A^{-/-}$ mice: Lane M represents the 1kb DNA ladder. Lane 1, 2, 5, 6, 7 & 8 represents the DNA amplified from ficolin $A^{-/-}$ mice showing a single band at 1300 bp. Lane 9 represents the DNA amplified from ficolin $A^{+/+}$ mice showing a single band at 935 bp. Lane 3 and 4 represent the DNA amplified from heterozygous mice showing two bands of 1300 and 935 bp.

3.1.2.2 Survival of ficolin A sufficient and ficolin A deficient mice following intranasal infection with *Streptococcus pneumoniae* D39

Ficolin A deficient mouse strain used in this study was provided by Prof. T. Fujita, Fukushima Medical University Japan. This mouse strain lacks the ficolin A mediated lectin pathway of complement activation where as MBL-A, MBL-C and CL11 mediated lectin pathway complement activation is completely functional. The potential role of ficolin A in activating lectin pathway of complement against *Streptococcus pneumoniae* was assessed using this mouse strain. 15 mice each from ficolin A sufficient and ficolin A deficient mice were challenged with $1x10^6$ CFU of *S. pneumoniae* D39 by intranasal infection and monitored for one week post infection. Animals showing severe symptoms were euthanised between 48 and 72 hours post

infection. The most critical time point during the course of infection was 72 hours when most of ficolin A deficient mice were euthanised on reaching severe signs of disease whereas only few ficolin A sufficient mice were euthanised until 72 hours. All the mice who survived 72 hour time point, survived until the end of experiment. After one week of infection, 66% of ficolin A sufficient mice and 13% of ficolin A deficient mice survived (Fig. 3.20). Scoring results of disease severity signs show that symptoms start to appear in both groups by 36 hours getting more severe in ficolin $A^{-/-}$ mice at 48 hours. Ficolin $A^{-/-}$ mice show significantly high scores of disease severity at time points 60 and 72 hours (Fig. 3.21).

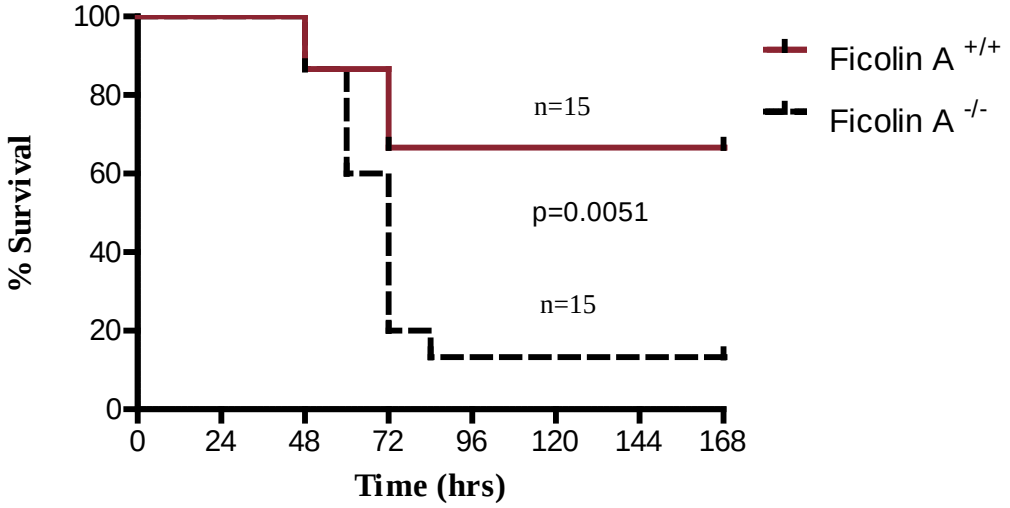


Figure 3.20: Survival of ficolin $A^{-/-}$ and ficolin $A^{+/+}$ mice after intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39. Ficolin A $^{-/-}$ mice show significantly higher mortality as compared to wild-type mice **p<0.01 (log-rank test).

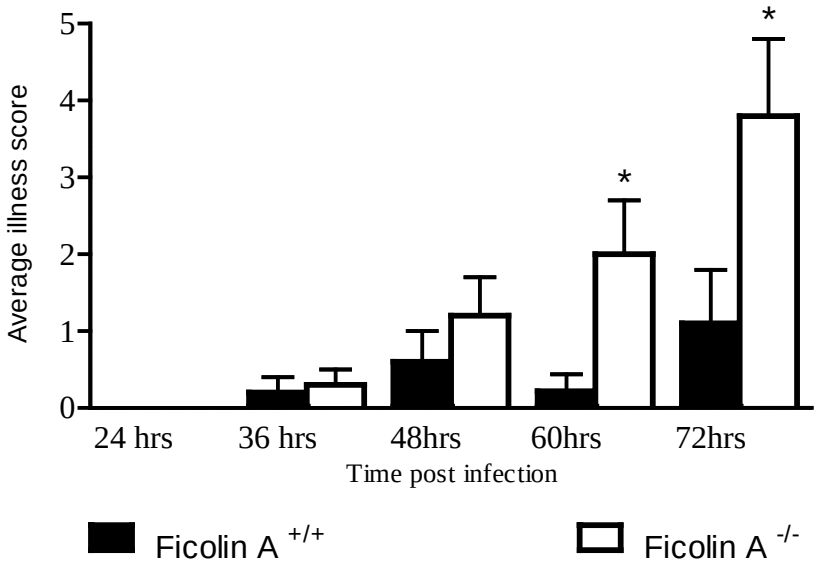


Figure 3.21: Average illness scores of ficolin A sufficient and ficolin A deficient mice over different time points after intranasal infection with *Streptococcus pneumoniae* D39. Results are means (±SEM). Scores were compared between both groups by Student's unpaired t test. *$p<0.05$

Score=0 "normal", Score=1 "hunched +", Score=2 "hunched ++", Score=3 "Starry coat +", Score=4 "Starry coat ++", Score=5 "Lethargic +", Score=6 "Lethargic ++"

3.1.2.3 Viable count of *Streptococcus pneumoniae* D39 in peripheral blood and lung homogenates

After intranasal infection with *Streptococcus pneumoniae* D39, blood was collected from both ficolin A deficient and ficolin A sufficient mice after 12, 24, 36, 48 and 72 hours post infection by tail bleed. Viable counts of bacteria at selected time points were determined by plating on blood-agar plates. Bacteria appeared in blood after 24 hours of infection and continued to increase in ficolin A deficient mice at 36 and 48 hours time points compared to ficolin A sufficient mice. After 48 hours ficolin A sufficient mice start clearing the infection and bacterial count drops. In ficolin A deficient mice, bacterial count keeps increasing after 48 hours of infection and reaches the maximum at 72 hours time point which was the peak time of infection, when most of the animals

showed severe symptoms of disease and were euthanised. Ficolin A deficient mice showed significantly higher bacterial burdens in blood at 60 and 72 hours time points as compared to ficolin A sufficient mice (Fig 3.22).

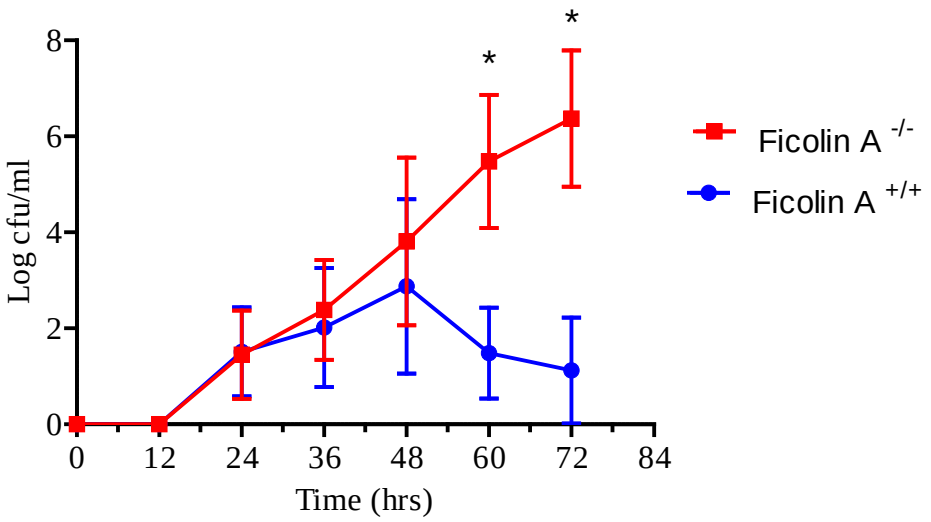


Figure 3.22: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Results are means (±SEM) of five animals at each time point. Bacterial loads were compared between both groups by Student's unpaired t test. *$p<0.05$

In another experiment, five mice from each group were sacrificed at 0, 12, 24 and 48 hour time points and lungs were homogenised to determine the viable count in lungs. Bacterial-counts remain same in lung homogenate at 0 and 12 hours time points in both groups. At 24 hours, count drops in ficolin A sufficient mice and remains same in ficolin A deficient mice. At time points 48 and 60 hours, bacterial count drops further in ficolin A sufficient mice and increased in ficolin A deficient mice showing significant difference (Fig 3.23).

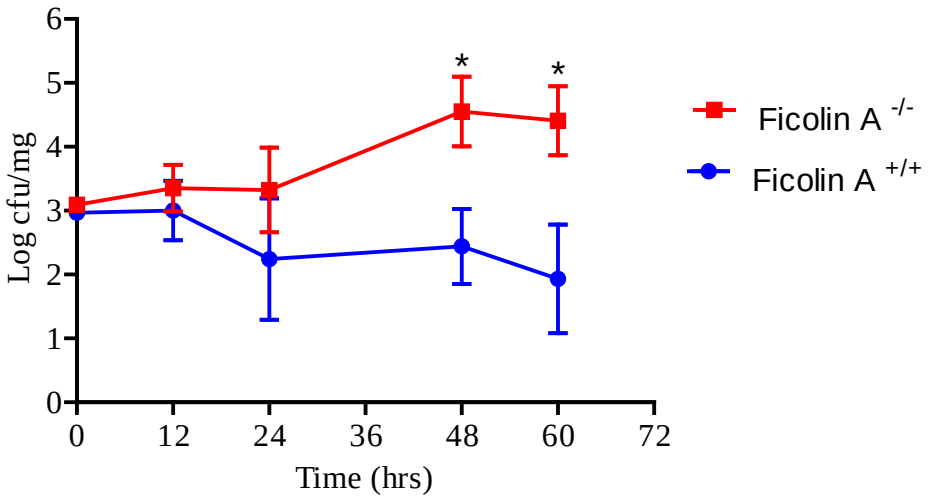


Figure 3.23: Viable counts of *Streptococcus pneumoniae* D39 in lung homogenates after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Results are means (±SEM) of five animals sacrificed at each time point. Bacterial loads were compared between both groups by Student's unpaired t test. *p<0.05

3.1.2.4 Survival and blood bacterial burden of MBL-A/C sufficient and MBL-A/C deficient mice after intranasal infection with *Streptococcus pneumoniae* D39

MBL-A/C deficient mice were purchased from MMRRC, Bar Harbor, Maine. This mouse strain lacks the MBL-A and MBL-C mediated lectin pathway of complement activation whereas ficolin A and CL11 mediated lectin pathway complement activation is completely functional. Five mice each from MBL-A/C sufficient and MBL-A/C deficient mice were challenged with $1x10^6$ CFU of *Streptococcus pneumoniae* D39 by intranasal infection and monitored for one week post infection. MBL-A/C deficient mice were resistant against pneumococcal infection. After one week of infection, all MBL-A/C deficient mice survived the infection and 60% of MBL-A/C sufficient mice survived (Fig. 3.24).

After intranasal infection with *S. pneumoniae* D39, blood was collected from both MBL-A/C deficient and MBL-A/C sufficient mice after 12, 24, 48 and 72 hours post infection by tail bleed. MBL-A/C deficient mice cleared the bacteria before bacteremia. No CFU was detected in MBL-A/C deficient mice throughout the course of infection. MBL-A/C sufficient mice exhibited very low bacterial levels at 24 hrs and 48 hrs post infection which were subsequently cleared after 72 hours of infection (Fig. 3.25).

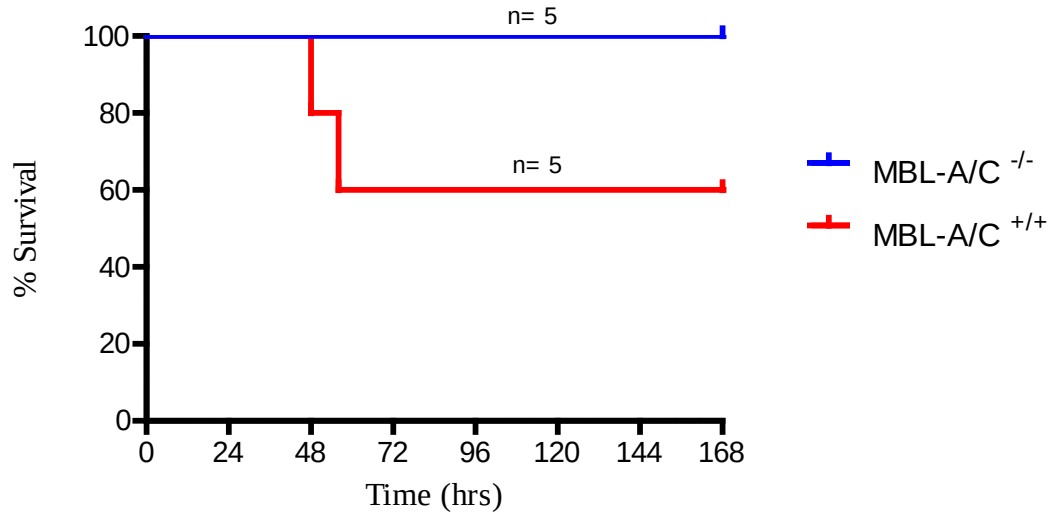


Figure 3.24: Survival of MBL-A/C $^{-/-}$ and MBL-A/C $^{+/+}$ mice after intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39. Both the groups were resistant to pneumococcal infection.

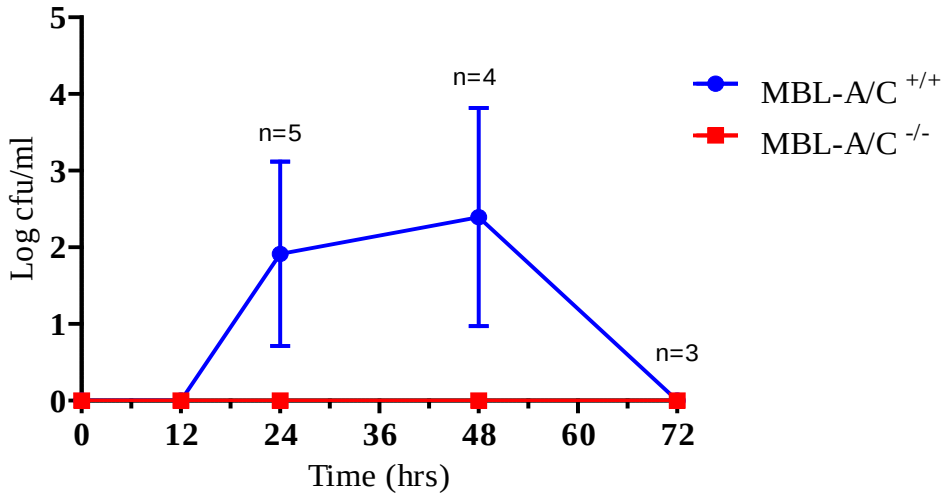


Figure 3.25: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Results are means (±SEM) of animals at each time point.

3.1.2.5: mRNA expression profile of inflammatory cytokines in mouse lung tissues at different time points post infection with *Streptococcus pneumoniae* D39

Expression levels of inflammatory cytokines in lungs of ficolin A deficient and ficolin A sufficient mice were determined by quantitative RT-PCR over different time points post infection using Roche Light-Cycler. Inflammatory response starts increasing in both groups after 6 hours and the level of expression of all the cytokines is roughly similar in both groups until 24 hours. TNFα expression is significantly higher at 24 and 48 hours in ficolin A deficient mice than in wild-type mice. IL6 expression is slightly higher in wild-type mice than ficolin A deficient mice at 24 hours but it is significantly increased in ficolin A deficient mice at time points 36 and 48 hours. MIP-2 expression is slightly higher in wild-type mice than ficolin A deficient mice at 24 hours and starts increasing in ficolin A deficient mice after 36 hours showing significantly higher expression at 48 hours in ficolin A deficient mice as compared to wild-type mice. IL-17

response is similar in both groups until 24 hours but significantly increases in ficolin A deficient mice at 36 and 48 hours. Onset of IL-10 response is very late as compared to other cytokines starting expression after 24 and 36 hours having a significantly greater response at 48 hours in ficolin A deficient mice. IL1β response is similar in both groups with no significant difference at all time points. However, it is slightly higher in wild-type mice at 12 hours and in ficolin A deficient mice at 24, 36 and 48 hours. INFγ response suddenly increases in both groups at 24 hours and then decreases at 48 hours with no significant difference between both groups (Fig3.26).

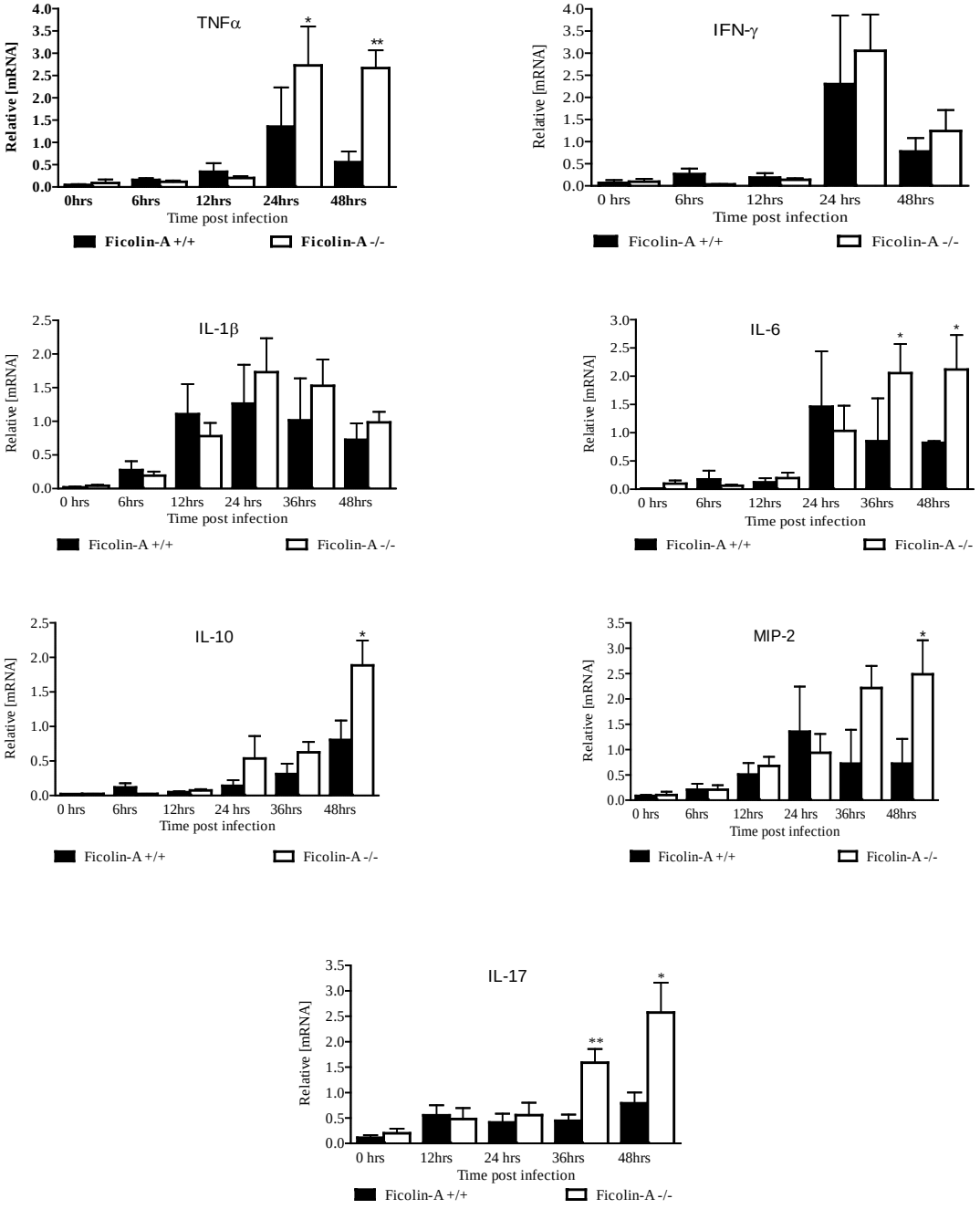


Figure 3.26: Quantitative RT-PCR analysis of mRNA expression of TNF-α, IFN-γ, IL-1β, IL-6, IL-10, MIP-2 & IL-17 in the lung following intra nasal infection with *Streptococcus pneumoniae* D39. Lungs were removed 0, 6, 12, 24, 36 and 48 hours after infection. Results are means (±SEM) of five different samples per time point. Relative expression was compared between both groups by students unpaired t test. *p<0.05 **p<0.01.

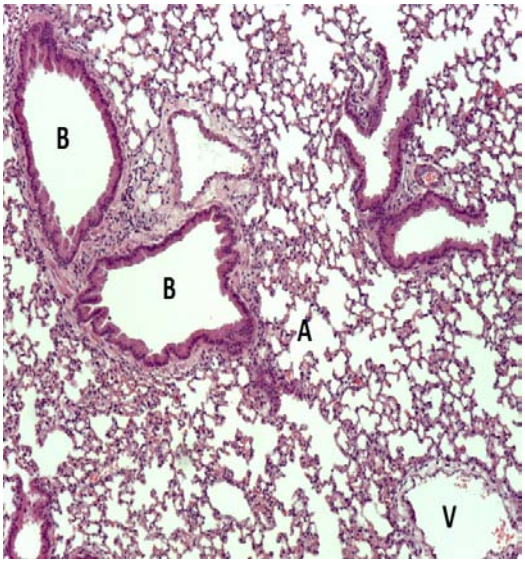
3.1.2.6 Histopathological examination of ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice lung tissues following infection with *Streptococcus pneumoniae* D39

Lung tissue sections from both groups of mice infected with *Streptococcus pneumoniae* D39 were histologically examined at time 0, 24, 48 and 60 hours post infection. Histopathological changes in ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice lungs were observed by light microscopy using 40x magnification. Each image shown in fig. 3.27 and fig. 3.28 best represents the general histopathological state of each mouse lung and is representative of four sections from two mice sacrificed from each group at prechosen time points post infection. Additional images from ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice lungs at 48 hours time point post infection are shown in addendum 1 as example (fig 7.6 & fig 7.7).

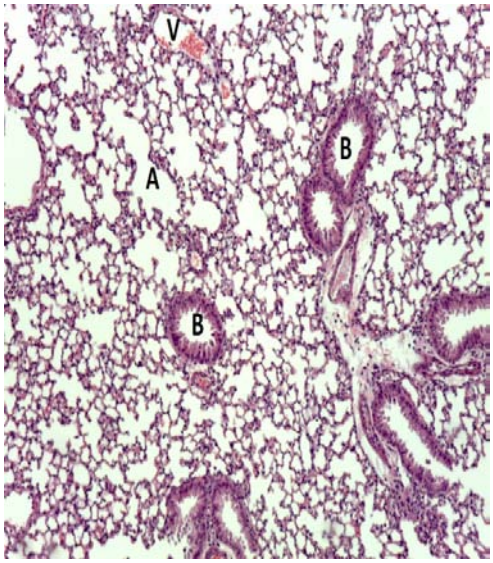
The histology in both groups of mice was similar at time 0 hours (Fig 3.27 A&B). After 24 hours of infection, cellular infiltration was observed in lungs of both groups of mice. However, level of infiltration seemed much higher in lungs of ficolin $A^{+/+}$ mice as compared to ficolin $A^{-/-}$ mice with more leukocyte infiltrating the alveolar space and the interstitium of alveolar septa. More leukocytes can be found around the vascular areas in ficolin $A^{+/+}$ mice as compared to ficolin $A^{-/-}$ mice (Fig 3.27 C&D). At 48 hours, infiltration of lungs of ficolin $A^{+/+}$ mice is slightly increased showing mild interstitial alveolitis and minor infiltration in the peribronchial and perivascular region. However, this infiltration of leukocytes seemed to be controlled (Fig 3.28 A). In contrast, lungs of ficolin $A^{-/-}$ mice showed signs of severe broncho-pneumonia with extremely high peribronchial and perivascular inflammatory cellular infiltration and severe interstitial alveolitis. There was massive tissue damage causing the collapse of alveolar spaces and

edema around blood vessels (Fig. 3.28 B). At 60 hours, perivascular and peribronchial cellular infiltration in ficolin $A^{+/+}$ mice lungs is decreased as compared to 48 hours (Fig 3.28 C) whereas ficolin $A^{-/-}$ mice lungs at 60 hours show similar picture as that of 48 hours with severe tissue damage (3.28 D).

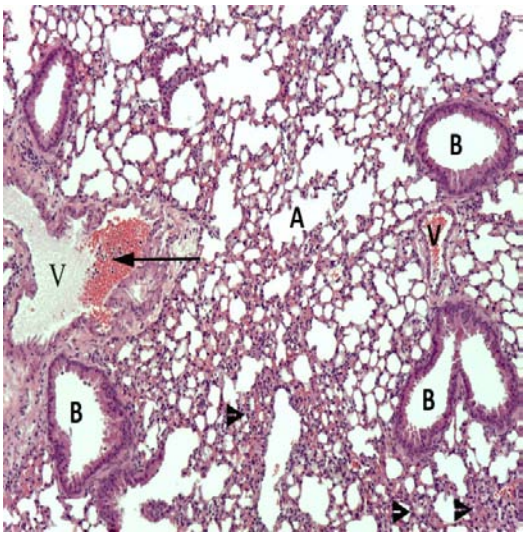
A 0hrs



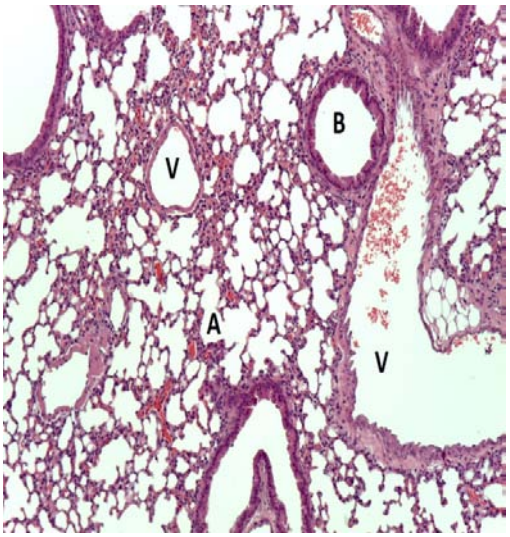
B 0hrs



C 24 hrs



D 24 hrs

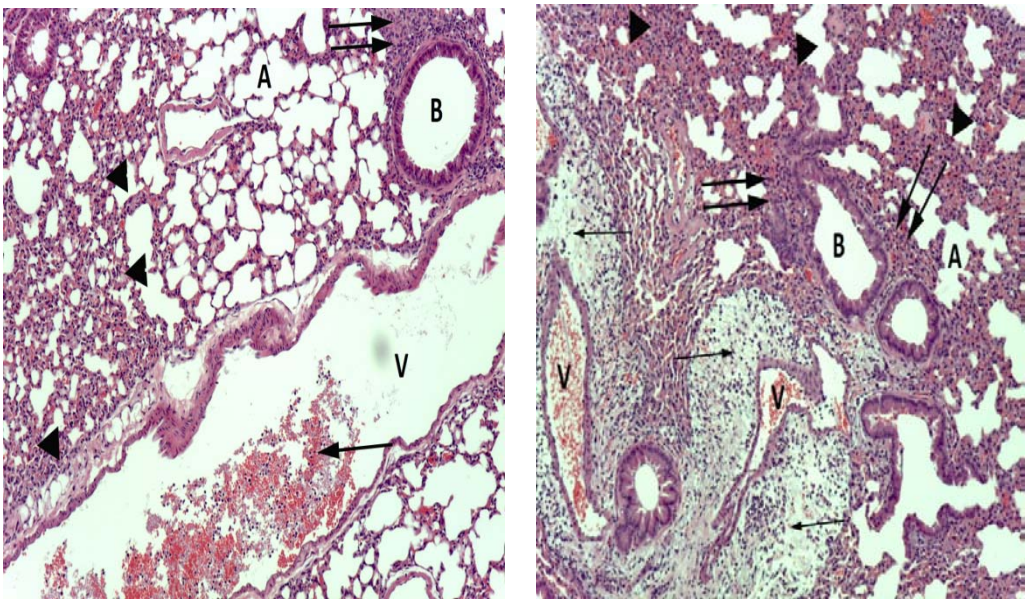


Ficolin A $^{+/+}$ Ficolin A $^{-/-}$

Figure 3.27: Histopathology of lung section of ficolin A$^{+/+}$ and ficolin A$^{-/-}$ mice stained with hematoxylin and eosin at time point zero (A&B), and 24 hours (C&D), after intranasal infection with *Streptococcus pneumoniae* D39. Each slide is representative of 2 mice. A, alveolar region; B, bronchioles; V, blood vessel. Arrow head indicates interstitial alveolitis; large thick arrow indicates the leukocyte increase in the blood stream.

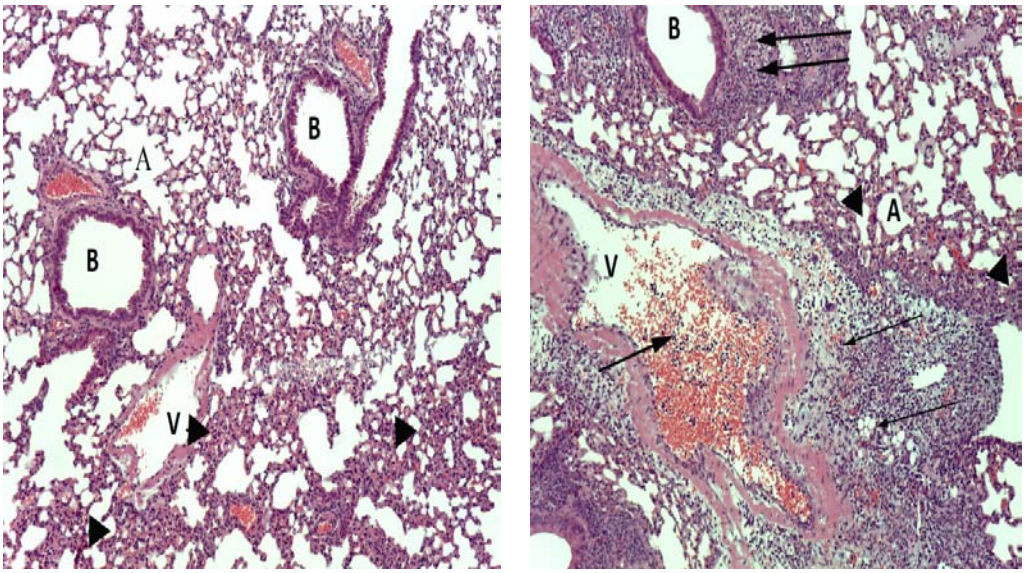
A 48 hrs

B 48 hrs



C 60 hrs

D 60 hrs



Ficolin A $^{+/+}$

Ficolin A $^{-/-}$

Figure 3.28: Histopathology of lung section of ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice stained with hematoxylin and eosin at time point 48hours (A&B), and 60 hours (C&D), after intranasal infection with *Streptococcus pneumoniae* D39. Each slide is representative of lung sections from 2 mice. A, alveolar region; B, bronchioles; V, blood vessel. Arrow head indicates interstitial alveolitis; large thick arrow indicates leukocyte increase in blood stream; large double arrow shows peribronchitis and small thin arrow indicates edema.

3.2 Discussion

Even after the introduction of antibiotics to treat bacterial infections, *Streptococcus pneumoniae* remains to be a major pathogen causing infections, including pneumonia, septicemia, and meningitis and continueous to be associated with high morbidity and mortality in children as well as adults (Varon *et al.*, 2010). The innate immune response plays a major role in providing protection against *S. pneumoniae*; a key feature of this is complement-dependent opsonophagocytosis (Yuste *et al.*, 2008).

Several studies investigated the protective role of the classical and the alternative pathways against *S. pneumoniae* using experimental models of pneumonia, systemic infection, meningitis and acute otitis in mice with engineered deficiencies of C1q, C4, C3 and factor B (Brown *et al.*, 2002; Rupprecht *et al.*, 2007; Tong *et al.*, 2010; Li *et al.*, 2011). These studies demonstrated the importance of both the classical and the alternative pathways in providing protection against *S. pneumoniae* with the classical pathway being the predominant mechanism for complement-mediated opsonisation and phagocytosis of *S. pneumoniae.* The role of the lectin pathway has not been investigated due to lack of available models of lectin pathway deficiency.

Recently, our group demonstrated the significant role of the lectin pathway in providing the protection against *S. pneumoniae* by using MASP-2 deficient mice, the first available model of total lectin pathway deficiency. These mice maintain fully functional classical and alterative activation pathways. MASP-2 deficient mice were severely compromised in their ability to survive *S. pneumoniae* infection concluding that the lectin pathway has an essential role in providing defence against *S. pneumoniae* in the non-immunised host (Ali, 2009).

My study was aimed to characterise the role of ficolin A in activating the lectin pathway against *S. pneumoniae.* The murine lectin pathway is activated via four carbohydrate recognition molecules MBL-A, MBL-C, ficolin A and CL-11 in mice (Schwaeble *et al.,* 2011). CL-11 is the most recently described lectin pathway recognition molecule (Keshi *et al.,* 2006). Its association with MASPs and its ability to activate complement was reported very recently (Hansen *et al.,* 2010). During the start of this project, I analysed the binding of MBL-A, MBL-C and ficolin A to *S. pneumoniae* D39. Ficolin A was the only recognition molecule that showed strong binding to *S. pneumoniae.* Since the role of the lectin pathway in protection against *S. pneumoniae* had been described (Ali, 2009), the role of ficolin A in activating MASP-2 and triggering the lectin pathway against *S. pneumoniae* was further investigated.

Binding of murine lectin pathway carbohydrate recognition molecules to *S. pneumoniae* has not been tested before. *S. pneumoniae* D39 was the strain used in this study. Results from binding assays indicated that MBL-C showed very weak binding, whereas MBL-A did not bind at all to *S. pneumoniae* D39 (Fig. 3.3). Ficolin A and CL-11 predominantly bound to *S. pneumoniae* D39 using murine serum, this was further confirmed using recombinant ficolin A and CL-11 by replacing murine serum with recombinant ficolin A and CL-11 (Fig. 3.2&3.5). Recombinant ficolin A showed very strong binding to *S. pneumoniae* D39 which was even higher than the binding to N-acetyl BSA used as positive control. Recombinant CL-11 also showed very strong binding to *S. pneumoniae* D39 and binding levels were similar to zymosan (which was used as positive control for CL-11 binding).

Binding of murine lectin pathway carbohydrate recognition molecules was further examined using other serotypes of *S. pneumoniae*. Ficolin A binding was further assessed in six serotypes of *S. pneumoniae,* i.e. serotype 6B, 18C, 3, 9V, 8 and 19A. Ficolin A binding significantly varied amongst the six serotypes tested (Fig. 3.6), initially giving the impression that this variation is dependent on variation in capsular polysaccharide. To further assess if capsular polysaccharide is responsible for variation of binding between different serotypes, ficolin A binding was further tested in nine clinical isolates, three each from serotype 6B, 18 C and 3. Three of these strains from each serotype had the same capsular polysaccharides but were genetically different. There was considerable variation in ficolin binding between different strains, indicating that genetic or strain variations but not the serotype variations affect ficolin A binding to *S. pneumoniae* (Fig. 3.7). Unlike ficolin, less variation was detected in CL-11, MBL-A and MBL-C binding to *S. pneumoniae*. CL-11 showed strong binding to all clinical isolates tested. MBL-A showed no or only weak binding whereas MBL-C showed weak binding to all the strains of serotype 6B, 18C and 3 (Fig. 3.8 & 3.9).

C3b deposition on *S. pneumoniae* varied between serotype 6B, 18C, 3, 9V, 8 and 19A (Fig 3.15). As evident from binding studies of lectin pathway recognition molecules to *S. pneumoniae,* ficolin A and CL-11 can recognise *S. pneumoniae*. Although CL-11 binds to *S. pneumoniae* and contributes towards the lectin pathway mediated C3b deposition, the binding of CL-11 to all the serotypes does not vary significantly, hence CL-11 binding cannot be responsible for the variation in C3b deposition seen on *S. pneumoniae*. Interestingly, levels of C3 deposition correlated with the binding of ficolin A to each strain. Strains showing strong binding to ficolin A showed higher C3b deposition indicating that ficolin A predominantly controls the C3 deposition on the

surface of *S. pneumoniae*. Variation of C3b deposition on the surface of *S. pneumoniae* was identical to the varied degree of C3b deposition between different serotypes reported previously. Some studies have linked these variations to capsular types concluding that differences in the structure of capsular polysaccharides are responsible for this variation, while ignoring genetic variations between strains of same types (Abeyta *et al.,* 2003; Melin *et al.,* 2009; Hyams *et al.,* 2010). Other studies reported that C3b deposition varies not only between the serotypes but also genetically variant strains of same type concluding that serotype-independent factors like variations in the genetic background are responsible for variation in C3b/iC3b deposition on *S. pneumoniae* strains while are independent of capsular type (Melin *et al.,* 2010a&b; Sabharwal *et al.,* 2009; Hyams *et al.,* 2011). However, all these studies were not able to answer precisely the mechanism behind this variation. Hyams *et al.,* correlated the differences in C1q binding with variation in C3 deposition.

In serum dilutions containing less than 5 % serum, the C3b deposition on *S. pneumoniae* is totally lectin pathway dependent (Ali 2009; this study Fig 3.16). My data shows that C1q does not have a role in mediating C3b deposition. The reason for that is that the classical pathway cannot be activated on the surface of *S. pneumoniae.* It decays all C4b deposited on its surface. Mechanism resulting in the loss of detection of C4b has been discussed in chapter 5. *S. pneumoniae* decays the larger haemolytically active fragment of C4 i.e. C4b, required for formation of the classical and the lectin pathway specific C3 covertases (C4b2a). The classical pathway is blocked in the absence of C3 covertases whereas the lectin pathway is still functional in the absence of C4 via a unique MASP-2-dependant C4-bypass mechanism (Schwaeble *et al.,* 2011), which was further confirmed by complete loss of C3b deposition on the surface of *S.*

pneumoniae using serum from MASP-2$^{-/-}$ mice (Fig. 3.16). Hence, the lectin pathway recognition molecules ficolin A and CL-11 are the only molecules that initiate C3 deposition directly by on the surface of *S. pneumoniae* by activating MASP-2. This study indicates that ficolin A binding is the critical step in activating complement on *S. pneumoniae* leading to C3b opsonisation on the bacterial surface. Several mechanisms like thickness of the capsule may affect C3b opsonisation on *S. pneumoniae* directly; differences in ficolin A binding between the serotypes and genetically variant strains of same type are the key factors in causing the variations in C3b deposition on the surface of *S. pneumoniae*. The essential role of ficolins in mediating C3b deposition on *S. pneumoniae* was also confirmed by showing a significant correlation between serum L-ficolin concentration and level of C3b deposition on *S. pneumoniae* D39 (see Ali *et al.*, 2012, addendum 2 Fig 1h).

To confirm if the binding of the lectin pathway recognition molecules corresponds with the level of activation of the lectin pathway against *S. pneumoniae,* C3b deposition on *S. pneumoniae* D39 was assessed using ficolin A$^{-/-}$, MBL-A/C$^{-/-}$, MBL-A/C/ficolin A$^{-/-}$ and MASP-2$^{-/-}$ serum. As reported previously (Ali, 2009) C3b deposition was completely inhibited in MASP-2$^{-/-}$ serum indicating that C3b deposition on *S. pneumoniae* is lectin pathway dependent. The carbohydrate recognition molecules of the lectin pathway are the only PRPs responsible for initiating C3b deposition on *S. pneumoniae*. To further investigate the recognition molecules responsible for initiating C3b deposition on *S. pneumoniae,* C3b deposition was performed in above mentioned sera (Fig 3.16). MBL-A and MBL-C deficiency together did not affect C3b deposition on the surface of bacteria, which underlined the conclusion that MBL plays no role in activating the lectin pathway on *S. pneumoniae*. C3b deposition was significantly

reduced in ficolin A deficient serum confirming the role of ficolin A in triggering lectin pathway activation against *S. pneumoniae*. Interestingly, the level of C3b deposition in MBL-A/C/ficolin A deficient serum was similar as that of ficolin A deficient serum. Until recently, it has been believed that MBL-A, MBL-C and ficolin A activate MASP-2 to initiate the lectin pathway in mice. If this was true, lectin pathway would be completely dysfunctional in MBL-A/C/ficolin A deficient serum like MASP-2 deficient serum, because of the absence of any recognition molecules to activate MASP-2. Since C3b deposition on *S. pneumoniae* is completely lost in MASP-2 deficient serum, same would be expected in MBL-A/C/ficolin A deficient serum, but this was not the case. Hansen *et al*. (2010) recently reported that a C- type lectin CL-11 can form complexes with MASP-1 and MASP-3. Our group demonstrated that CL-11 also forms complexes with MASP-2 (See Ali *et al.,* 2012, addendum 2 Fig. S1). As CL-11 shows strong binding to *S. pneumoniae* (this study), this implies that C3b deposition in MBL-A/C/ficolin $A^{-/-}$ serum on *S. pneumoniae* is CL-11 mediated.

To further confirm if CL-11 mediates C3b deposition in the absence of MBL-A, MBL-C and ficolin A, CL-11 binding to different sugars and the effect of this binding on C3b deposition on these sugars was assessed. CL-11 did not bind to mannan but showed strong binding to zymosan and N- acetyl BSA (Fig. 3.17). No C3b deposition was detected on mannan using MBL-A/C $^{-/-}$ and MBL-A/C/ficolin $A^{-/-}$ serum whereas C3b deposition was very high in ficolin A deficient serum indicating that C3b deposition on mannan is totally MBL-A and MBL-C dependent (Fig 3.18a). This implies that in the absence of MBL-A, MBL-C, ficolin A and lack of CL-11 binding, there is no other recognition molecule that could trigger MASP-2 and mediate lectin pathway on mannan. Interestingly C3b deposition was detected on zymosan and N-acetyl BSA

using MBL-A/C/ficolin $A^{-/-}$ serum (Fig. 3.18 b&c). As CL-11 binds to both these sugars, it can be described that C3b deposition on these sugars is mediated by CL-11. Hence it is concluded that CL-11 works synergistically along with ficolin A to mediate lectin pathway activation after binding to specific PAMPs on the surface of *S. pneumoniae*.

Results from infection experiments clearly demonstrate that ficolin A deficient mice are severely compromised with significantly higher mortality as compared to ficolin A sufficient mice after intranasal infection with *S. pneumoniae* D39. After one week of infection, only 13 % of ficolin A deficient mice survived the infection compared to 66 % of ficolin A sufficient mice (Fig. 3.20). Ficolin A deficient mice showed a significantly higher bacterial burden in blood and lungs as compared to ficolin A sufficient mice. Ficolin A sufficient mice start to clear the infection after 48 hours whereas in ficolin A deficient mice, bacterial load kept increasing throughout the course of infection reaching the peak at 72 hours when most of the mice showed severe symptoms of disease and had to be euthanised (Fig. 3.21, 3.22 and 3.23). Comparing the survival graph, blood and lung bacterial burdens with the phenotype of MASP-2 deficient mice (Ali *et al.*, 2012, see addendum 2 Fig 5), it can be observed that the progression of infectious disease is slightly delayed in ficolin A deficient mice. While most MASP-2 deficient mice collapse at 48 hours post infection ($p=0.0006$), ficolin A deficient mice collapsed after time point 72 hours post infection ($p=0.005$). Statistical differences in bacterial loads of MASP-2 deficient mice and wild-type controls are also dramatically high at peak times of infection ($p<0.01$) as compared to ficolin A deficient mice and wild-type controls ($p<0.05$). This can be explained by the presence of CL-11 in ficolin A deficient mice. CL-11 might delay the onset of infection by two

mechanisms. As CL-11 can directly complex with MASP-2 (Ali *et al.*, 2012, see addendum 2 Fig. S1), in the absence of ficolin A, it continues to opsonise *S. pneumoniae* by activating MASP-2 leading to C3b deposition on bacteria (Fig 3.16). Secondly, CL-11 can also form complexes with MASP-1 and MASP-3 (Hansen *et al.*, 2010). After activating lectin pathway MASP-1 and MASP-3 (fig. 1.1), CL-11 might contribute towards alternative pathway amplification by converting zymogen factor D into enzymatically active form (Takahashi *et al.*, 2008) or catalyse the activation of C3b-bound factor B (Iwaki *et al.*, 2011). The presence of CL-11 might delay the onset of infection in ficolin A deficient mice but it is not sufficient on its own to protect the mice from pneumococcal infection. In contrast to ficolin A deficient mice, MBL-A/C deficient mice were resistant to pneumococcal infection, with no difference to the wild-type controls. After intranasal challenge with *S. pneumoniae* D39, all the mice survived the infection and completely cleared the bacteria from blood (Fig. 24 & 25). No bacteremia was detected in the blood at selected time points post infection. These results from *in vivo* studies underline the *in vitro* studies indicating that ficolin A (but not MBL-A or MBL-C) is a key recognition component of the lectin activation pathway in the innate host defence against pneumococcal infection.

Histopathological analysis shows increased neutrophil infiltration in lungs of ficolin A sufficient mice at 24 hours time point as compared to ficolin A deficient mice that remain more or less similar at 48 and 60 hours post infection. The early recruitment of neutrophils helps in clearing bacteria before they cause any damage. In ficolin A deficient mice, neutrophil infiltration was not observed at 24 hours. At 48 and 60 hours, lung tissues showed signs of severe broncho-pneumonia with extremely high

peribronchial and perivascular inflammatory cellular infiltration and severe interstitial alveolitis that might be due to the sudden increase in bacterial loads in the lungs.

To further explain the histopathological studies, levels of different cytokines were checked at different time points post infection (Fig. 3.26). Expression and release of pro-inflammatory cytokines are important steps in controlling the infection. TNF-α, IL-6 and IL-1 are the early response cytokines produced after the pneumococcal invasion. The early onset of pro-inflammatory cytokines facilitates the recruitment of neutrophils from the blood stream to lung tissues and alveoli to clear the bacteria before the infection gets worse (van der Sluijs *et al.,* 2006; Li *et al.,* 2002). Deficiency of signalling receptors of IL-1 (α and β) and TNF-α alters the bacterial clearance in a mouse model of pneumococcal infection as it impairs neutrophil recruitment and function (Jones *et al.,* 2005). Similarly, IL-1β deficient mice were highly susceptible to pneumococcal infection due to impaired neutrophil recruitment leading to acute pneumococcal infection (Kafka *et al.,* 2008).

In this study, ficolin A sufficient mice show higher expression of IL-1β at time points 6 and 12 hours, and TNF-α at time point 12 hours as compared to ficolin A deficient mice. Early upregulation of these two cytokines facilitates the neutrophil influx at the site of infection before the infection gets worse as indicated the results of the histopathological analysis. Lower expression of IL-1β has been reported previously in C3 deficient mice that could not survive the infection in a model of pneumococcal meningitis (Rupprecht *et al.,* 2007).

mRNA expression of both IL-6 and MIP-2 starts early in ficolin A sufficient mice as compared to ficolin A deficient mice at the 24 hours time point. Early expressions of these two mediators stimulate the influx of neutrophils into lung tissues and promote the clearance of bacteria. IL-6 is another early response cytokine having pro-inflammatory as well as the anti-inflammatory function. Expression of IL-6 is upregulated during acute phase of several infections models (Gadient and Patterson, 1999). In ficolin A deficient mice, expression of IL-6 and MIP-2 is higher at peak stages of infection indicates continuous macrophage activation whereas expression of cytokines decline in ficolin A sufficient mice at 36 and 48 hours post infection indicates that the infection is subsiding.

The level of interferon was similar in both groups, shooting up at 24 hours time point and declining shortly thereafter. IL-17 expression remains similar in ficolin A sufficient mice throughout the course of infection whereas in ficolin A deficient mice, IL-17 expression is significantly increased at 36 hrs and 48 hrs.

IL-10 expression starts around 24 hrs in both groups and continues to increase, reaching a maximum at 48 hrs. IL-10 is an anti-inflammatory cytokine that induces a downregulation of the expression of pro-inflammatory cytokines and chemokines (Bogdan *et al.,* 1991). The primary function of IL-10 is to control the overexpression of pro-inflammatory cytokines and to prevent the septic shock. Mice lacking the IL-10 response show higher mortality due to septic shock symptoms caused by excessive pro-inflammatory reactions (Latifi *et al.,* 2002). However, over expression of IL-10 itself can increase mortality due to unresolved infection (van der Poll *et al.,* 1997). In this study, the effect of expression of IL-10 on regulating the expression of pro-

inflammatory cytokines in ficolin A sufficient mice is evident. Following the expression of IL-10, the pro-inflammatory response starts to abate at 36 hrs and 48 hrs preventing fatal septic shock. In ficolin A deficient mice IL-10 mediated regulation of pro-inflammatory cytokines is only seen on INF-γ and IL-1β (which decline after 24 hours). Although IL-10 levels reach maximum at 48 hours in ficolin A deficient mice, it is not enough to control expression of TNF-α, IL-6, MIP-2 and IL-17 which remain significantly higher at 36 and 48 hrs time points and lead to septic shock symptoms as evidenced from histopathological slides of lungs of ficolin A deficient mice (Fig 3.26 b & d). Increased levels of IL-10 in ficolin A deficient mice are in line with previously performed studies showing persistently higher IL-10 levels in patients who do not survive sepsis than in patients who survive where IL-10 levels decrease over time (van der Poll *et al.,* 1997).

Taking together all the *in vitro* complement activation assays along with *in vivo* survival, histopathological and cytokine analysis, it is clear that the lectin pathway mediated opsonisation is critical in providing protection against *S. pneumoniae*. Ficolin A sufficient mice with fully functional lectin pathway opsonisation efficiently interact with innate host defences, eliciting early onset of pro-inflammatory cytokines enhancing neutrophil influx at the site of infection to phagocytose invading bacteria before these bacteria overpower the host immune defences. As ficolin A is the predominant lectin pathway recognition molecule that recognises *S. pneumoniae* in mice, ficolin A deficient mice show a significantly decreased degree of C3b deposition on *S. pneumoniae* affecting the opsonisation and subsequent clearing of these bacteria. The loss of lectin pathway specific responses correlates with reduced co-stimulatory

signals that booster the antimicrobial defences of the host. This delays the pro-inflammatory responses to *S. pneumoniae* invasion in ficolin A deficient mice. Neutrophil influx is significantly delayed which in turn leads to increased bacterial burden in the lungs and blood. The high bacterial burden results in a secondary pro-inflammatory reaction which mediates septic shock symptoms. Severe bacteremia and severe septic shock symptoms are responsible for the significantly higher lethality of ficolin A deficient mice when compared to their wild-type controls.

Binding studies to identify the human lectin pathway recognition components that bind to *S. pneumoniae* revealed a similar situation than that observed for the murine pathway recognition molecules. Human L-ficolin and CL-11 recognise all the strains of *S. pneumoniae* covering all four serotypes (Fig. 3.9 and 3.12). L-ficolin is regarded as the human orthologue of murine ficolin A (Endo *et al.,* 2011). The CL-11 sequence is also highly conserved amongst different species (Hansen *et al.,* 2010; Keshi *et al.,* 2006). Hence, the strong binding of L-ficolin and CL-11 in human serum was not surprising. However, unlike ficolin A, the degree of variation of L-ficolin binding to different serotypes or strains of same serotypes was very negligible. Binding of L-ficolin confirmed the previously reported binding of L-ficolin to *S. pneumoniae* serotype strains 11F, 11A and 11D (Krarup *et al.,* 2005). In this study, L-ficolin binding was further established in ten strains covering four serotypes. MBL, H-ficolin and M-ficolin did not bind to any of the strains tested in this study (Fig 3.8, 3.10 and 3.11). My results also confirm the lack of binding activity of MBL and H-ficolin to *S. pneumoniae* serotype 11F, 11A and 11D reported previously (Krarup *et al.,* 2005). The absence of binding of MBL and the strong binding of L-ficolin to *S. pneumoniae* was confirmed by C3b deposition studies on *S. pneumoniae* using human sera. The absence of MBL

deficient sera did not affect C3b deposition on the surface of *S. pneumoniae*. L-ficolin serum concentrations correlated with the amount of C3b deposition on *S. pneumoniae*. Sera with high concentrations of L-ficolin also showed increased C3b deposition and vice versa (Ali *et al.,* 2012, see addendum 2 Fig 1 g&h).

In the normal population one out of ten people suffers from MBL deficiency (Wallis and Lynch 2007). Many studies have focused on a possible association between MBL deficiency and pneumococcal disease. The majority of studies reported that MBL deficiency is not associated with community-acquired pneumonia or invasive pneumococcal disease (Endeman *et al.,* 2009; Carcia-Laorden *et al.,* 2008; Moens *et al.,* 2006). Unlike MBL, no complete deficiency of L-ficolin has been reported to date. However, variations and polymorphisms of the L-ficolin gene exist. The polymorphism of the L-ficolin gene can lead to low plasma levels of L-ficolin, but are not associated with an increased predisposition of pneumococcal disease (Chapman *et al.*, 2007). This indicates that even low levels of L-ficolin and the presence of CL-11 may be sufficient to mount an effective lectin pathway mediated immune response against *S. pneumoniae.*

This study underlines that the lectin pathway has a crucial role in providing protection against *S. pneumoniae* and underlines the results of the previously reported study showing the essential protective role of the lectin pathway (Ali, 2009). This challenges the previously published conclusion by Brown *et al*. (2002) that the lectin pathway may not be essential in host response against pneumococcal infection. Their conclusion is based on their observation that C1q deficient mice show a similar degree of susceptibility to pneumococcal infection than C4 deficient mice. Since the textbook

states that C4 deficient mice are likely to be deficient of lectin and classical pathways, their conclusion is based on this text book assumption rather than on data analysing models of lectin pathway deficiency. They also used the findings of previously reported studies showing poor binding of MBL to *S. pneumoniae* and weak association of genetic polymorphism of MBL with *S. pneumoniae* infection (Neth *et al.*, 2000; Kronborg *et al.*, 2002; Roy *et al.*, 2002). They used the historical and now overcome terminology for the lectin pathway describing this pathway as MBL pathway, which ignores the possible contribution of ficolin A or CL-11 as carbohydrate recognition molecules of the lectin pathway. This study underlines the redundant role of MBL in driving lectin pathway activation on pneumococci by demonstrating that C3b deposition is not affected in MBL-A/C deficient serum and MBL-A/C deficient mice show no predisposition to pneumococcal infection. This study underlines the critical role of the lectin pathway against pneumococcal infection by showing that ficolin A and CL-11 (but not MBL-A or MBL-C) recognise *S. pneumoniae* and drive lectin pathway mediated complement activation to clear *S. pneumoniae* infections. A complete loss of C3b opsonisation on *S. pneumoniae* in MASP-2 deficient serum with the complete deficiency of forming lectin pathway specific C3 and C5 convertases demonstrate that C3b deposition on *S. pneumoniae* is lectin pathway dependent. Hence, the lectin pathway contributes to the host defences by mediating C3b opsonisation of *S. pneumoniae* in the non-immunised host to facilitate phagocytosis of these bacteria. The increased mortality of C1q deficient mice reported by Brown *et al*. (2002) may not be due to loss of the classical pathway functional activity since C1q does not contribute to C3b opsonisation of *S. pneumoniae* but may act as opsonins by its own. Since the role

of C1q in phagocytosis of *S. pneumoniae* is very clear, C1q mediated phagocytosis may be due to direct opsonisation of C1q binding C1q receptors bearing phagocytes.

In conclusion, ficolin A has a crucial role in providing protection against pneumococcal infection. It predominantly activates MASP-2 along with CL-11 to trigger lectin pathway against *S. pneumoniae*. Ficolin A mediated deficiency of the lectin pathway significantly increases the susceptibility to infection and impairs the bacterial clearance from lungs and blood due to a delayed onset of neutrophil recruitment in lungs. Similarly, L-ficolin and CL-11 recognise *S. pneumoniae* and contribute to the lectin pathway activation in humans. Since this study describes clear evidence of the lectin pathway activation without any contribution from MBL, the existing view that MBL as the major initiator of the lectin pathway needs to be revisited.

4. Effect of α-MASP-2 antibody treatment on mortality in a mouse model of *Streptococcus pneumoniae* infection

4.1 Background

The significant role of the lectin pathway of complement in *Streptococcus pneumoniae* infection has been characterised in my previous study of *S. pneumoniae* infection using mice defcient in ficolin A, the carbohydrate recognition molecule that predominantly activates the lectin pathway against *S. pneumoniae.* A similar experimental model of *S. pneumoniae* infection was used in previous studies using mice deficient in the lectin pathway specific serine protease MASP-2, the only available model with complete deficiency of the lectin pathway (Ali, 2009). Both these studies showed that the lectin pathway has a significant role in protection against *S. pneumoniae* infection and mice deficient in the lectin pathway show significantly higher mortality.

The aim of this study was to investigate whether a similar effect could be achieved using specific α-MASP-2 inhibitory antibodies in an experimental model of *S. pneumoniae* infection, and check if antibiotic treatment affects inhibitory antibody treatment. The second aim was to assess if the inhibitory antibody delays tissue injury due to excessive complement activation by delaying the onset of inflammatory response against *S. pneumoniae* in an experimental mouse model of *S. pneumoniae* with high dose. Antibodies used in this study have been described previously (Schwaeble *et al.*, 2011).

4.2 Results

4.2.1 Survival of C57BL/6 mice pre-treated with α-MASP-2 mAb 16 hours before intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39

This study was performed in 4 groups of 8 weeks old C57BL/6 (12 mice in each group). The first group received a single *i.p.* dose of 1mg/kg of murine α-MASP-2 mAb 16 hours before infection with *Streptococcus pneumoniae* D39. The second group received a single dose of 1mg/kg of control antibody that specifically inhibits human but not murine MASP-2 and was used as control. The third group received a single dose of 1mg/kg α-MASP-2 and 20mg/kg ceftriaxone 16 hours before infection and ceftriaxone administered after every 12 hours until the termination of experiment. The fourth group received a single dose of 1mg/kg of control antibody and 20mg/kg of ceftriaxone 16 hours before infection and antibiotic administered every 12 hours after infection. Mice from all the groups were infected with $1x10^6$ CFU of *S. pneumoniae* D39 and monitored for a period of one week. All the animals in both the groups treated with ceftriaxone survived the infection. Mice treated with control antibody alone showed 60 % survival whereas mice treated with α-MASP-2 showed 25% survival after 72 hours of infection. All the mice asymptomatic after 72 hours of infection survived until the end of experiment (Fig 4.1).

Blood was collected from all mice at time points 24 and 48 hours post infection by tail bleed and determined count. Mice treated with α-MASP-2 had a significantly higher bacterial burden at both time points. No bacteremia was detected in groups of mice treated with Ceftriaxone (Fig 4.2). Average illness scores of both groups show that α-

MASP-2 mAb treated mice show significantly higher scores at time points 36 and 48 hours post infection. Symptoms start to appear in both groups around 36 hours. 48 hours was the critical time point for α-MASP-2 treated mice when half of the mice showed severe symptoms and had to be euthanised (Fig 4.3). Neither of the groups receiving antibiotics showed signs of any disease throughout the course of infection (not mentioned in the average illness score graph).

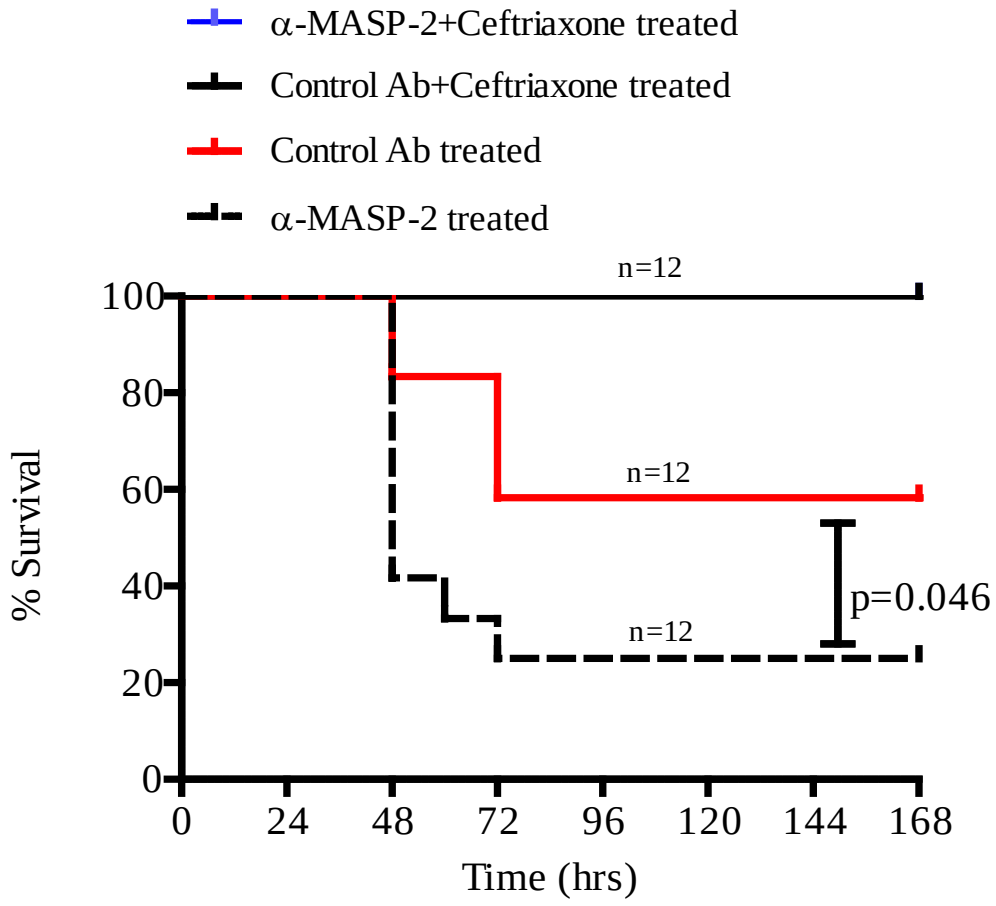


Figure 4.1: Kaplan Mayer Chart showing survival of four groups of mice after intra nasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39, 16 hours after *i.p.* treatment with antibodies. (I) C57BL/6 treated with α-MASP-2+Ceftrixone represented by blue line, (II) mice treated with control antibody+Ceftriaxone represented by black line, (III) mice treated only with control antibody represented by red line, (IV) mice treated only with α-MASP represented by black dotted line. $p<0.05$ (log-rank test).

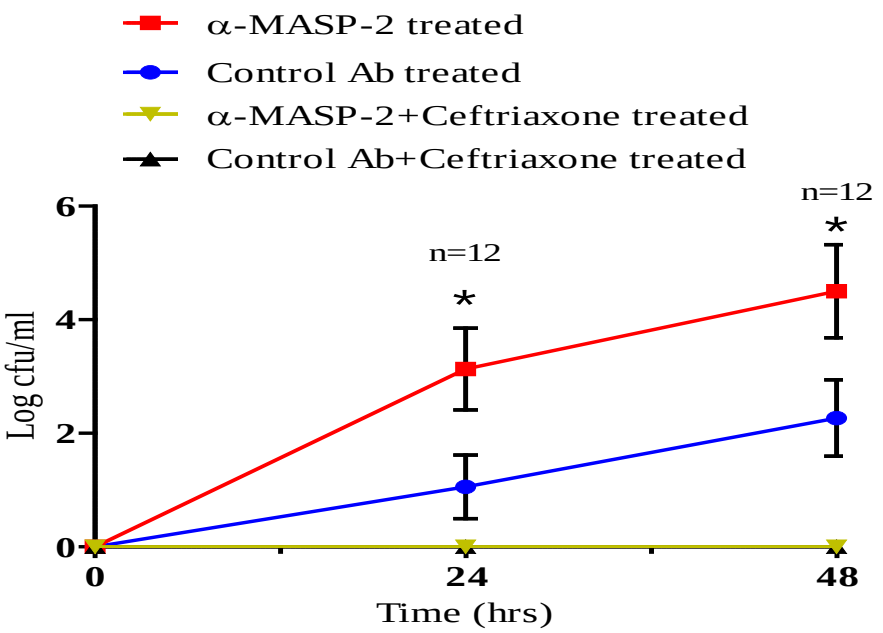


Figure 4.2: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood of four groups of C57BL/6 after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Results are means (±SEM) of 12 animals at each time point. Bacterial loads were compared between both groups by Student's unpaired t test. *p<0.05.

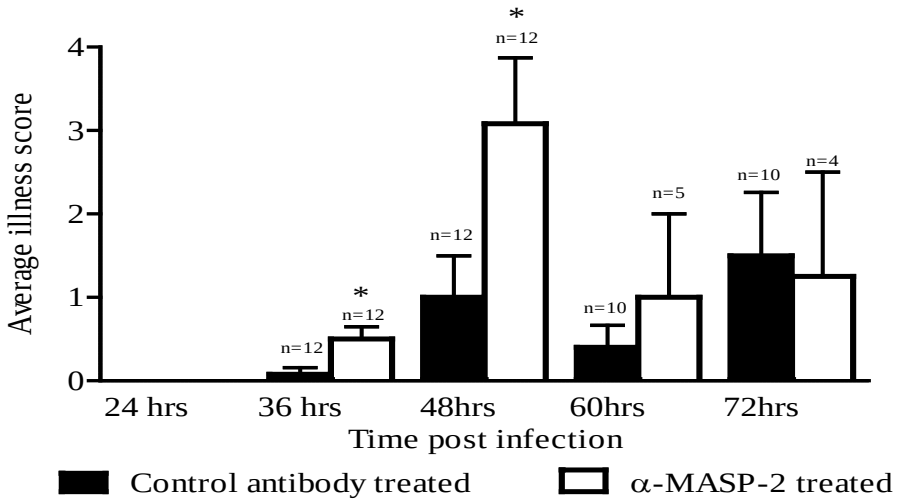


Figure 4.3: Average illness scores of α-MASP-2 mAb treated, and control antibody treated mice over different time points after intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39. Results are means (±SEM). Scores were compared between both groups by Student's unpaired t test. *p<0.05.

Score=0 "normal", Score=1 "hunched +", Score=2 "hunched ++", Score=3 "Starry coat +", Score=4 "Starry coat ++", Score=5 "Lethargic +", Score=6 "Lethargic ++".

4.2.2 Survival of C57BL/6 mice after intra nasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* treated with α-MASP-2 mAb six hours after infection

This infection study was performed to assess the effect on mortality of C57BL/6 when the lectin pathway is blocked after infection with *Streptococcus pneumoniae*, to determine the time point when MASP-2 becomes essential in fighting pneumococcal infection. Two groups of mice were infected with $1x10^6$ CFU *S. pneumoniae* D39 and treated with antibodies 6 hours post infection. The first group was treated with α-MASP mAb whereas second group was treated with control antibody. No significant difference was observed in survival of both groups. α-MASP-2 treated mice showed 80%, and control antibody treated mice showed 70 % survival after one week of infection. $1x10^6$ CFU is the dose targeted to achieve maximum survival in wild-type C57BL/6. Because of the low susceptibility of mice when the lectin pathway is blocked after infection with *S. pneumoniae*, survival was high in both groups (Fig 4.4). Bacterial counts remained very low throughout the course of infection and were efficiently cleared by both groups of mice (Fig. 4.5). Only few mice showed symptoms, i.e. 2 of the α-MASP-2 treated and 3 of the control antibody treated mice. The rest of the mice cleared the infection restricting the average illness scores to the lowest (Fig 4.6). This study indicates that treatment with α-MASP-2 mAb does not increase mortality in C57BL/6 when injected after infection with *S. pneumoniae.*

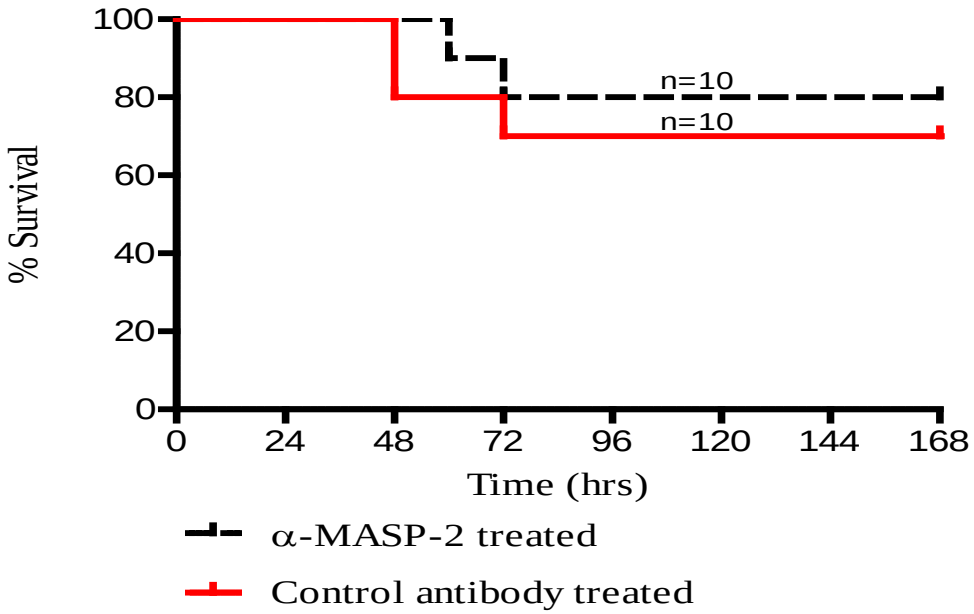


Figure 4.4: Kaplan Mayer Chart showing survival of α-MASP-2 treated C57BL/6 in black dotted line and control antibody treated C57BL/6 in red line after intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39 and *i.p.* injection of antibody six hours after infection (not significant).

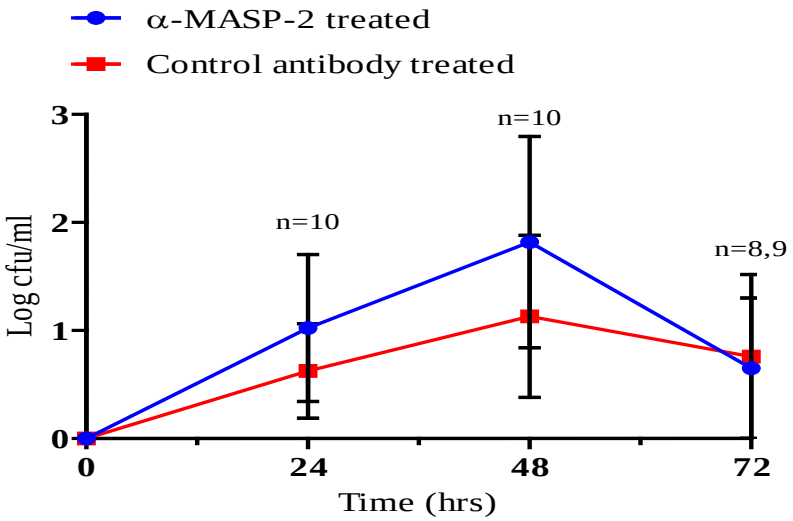


Figure 4.5: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood of α-MASP-2 treated, and control antibody treated C57BL/6 at different time points post infection with $1x10^6$ CFU *S. pneumoniae* D39 and antibodies administered after 6 hours of infection. Results are means (±SEM) of 10 animals at time points 24 hrs and 48 hrs and 8-9 animals at time point 72. Bacterial loads were compared between both groups by Student's unpaired t test (not significant).

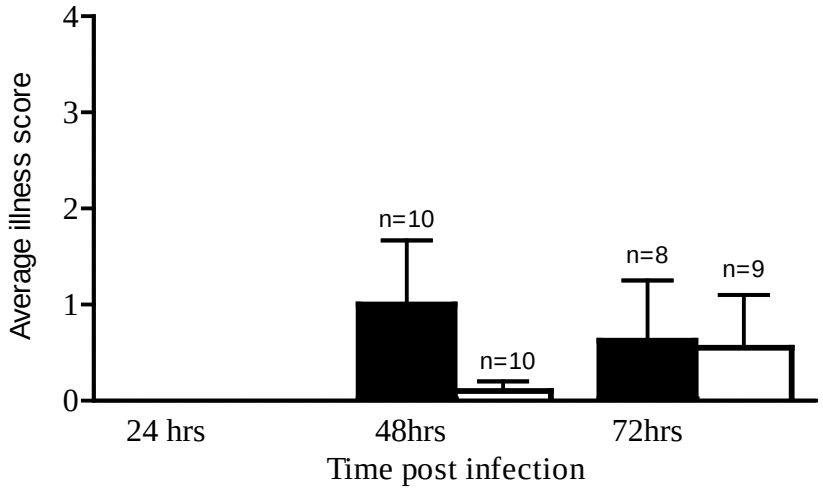


Figure 4.6: Average illness scores of α-MASP-2 mAb treated, and control antibody treated mice over different time points after intranasal infection with $1x10^6$ CFU *Streptococcus pneumoniae* D39 and antibodies injected after 12 hours of infection. Results are means (±SEM). Scores were compared between both groups by Student's unpaired t test (not significant).

Score=0 "normal", Score=1 "hunched +", Score=2 "hunched ++", Score=3 "Starry coat +", Score=4 "Starry coat ++", Score=5 "Lethargic +", Score=6 "Lethargic ++"

4.2.3 Survival of C57BL/6 mice after intra nasal infection with $2.5x10^6$ CFU *Streptococcus pneumoniae* and treated with α-MASP-2 mAb injected six hours after infection

This study aimed to investigate the effect of α-MASP-2 mAb in controlling septic shock. The infectious dose was designed to trigger severe sepsis by increasing the CFU of dose to $2.5x10^6$. Inhibitory antibody was administered six hours after infection to see if the antibody can delay the onset of severe inflammatory response against a very high dose of bacteria and can delay the tissue injury caused by excessive complement activation. Ten mice in two groups were infected with $2.5x10^6$ CFU *Streptococcus pneumoniae* D39 with one group receiving α-MASP-2 mAb and other group receiving control antibody after six hours of infection. Mortality in both the groups was almost

similar at the end of experiment. Only 20% mice treated with α-MASP-2 and 10 % mice treated with control antibody survived the infection. However, there was a significant overall delay in mortality of α-MASP-2 treated mice as compared to mice treated with control antibody with an average delay of 12 hours during the course of infection (Fig 4.7). The logrank test, which takes into account the survival as well as overall survival at the end of the experiment indicated a significantly better outcome in the α-MASP-2 treated group (p=0.042). Average survival time in mice receiving the isotype control was 48.44 hrs whereas mice receiving α-MASP-2 was 61.5 hrs. Bacterial counts were broadly similar in both groups at all time points (Fig 4.8). The average illness graph illustrates that symptoms in both groups started to show around 24 hours, unlike the mice infected with the normal dose of $1x10^6$ CFU in previous studies, which were normal after 24 hours of infection. At 36 and 48 hour points, control antibody treated mice showed significantly higher scores of disease severity than α-MASP-2 treated mice. The majority of control antibody treated mice were euthanised at these time points due to severe symptoms of the disease. α-MASP-2 treated mice showed severe signs of disease between 48 and 72 hours post infection (Fig. 4.9). The delayed onset of illness observed in α-MASP-2 treated mice is consistent with the hypothesis that lectin pathway inhibition suppresses the inflammatory response, reducing mortality due to septic shock and multiple organ failure.

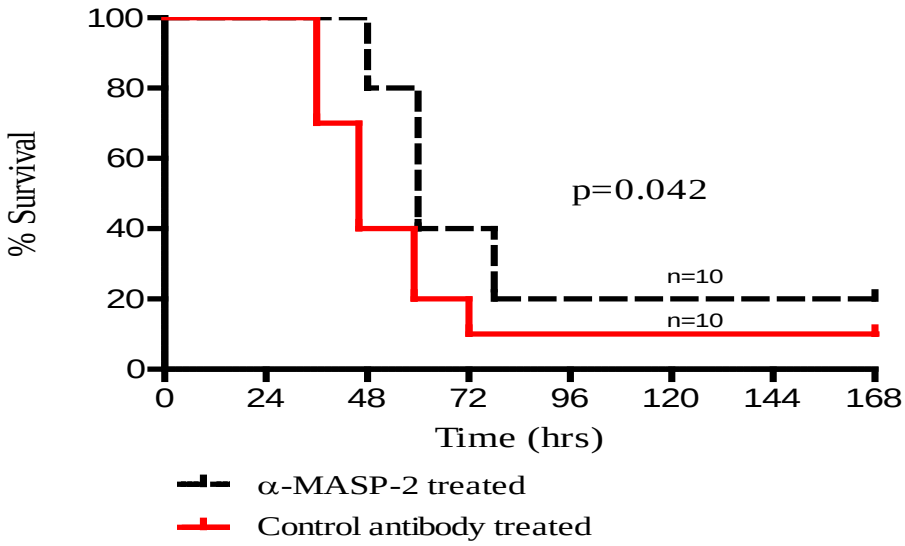


Figure 4.7: Kaplan Mayer Chart showing survival of α-MASP-2 treated C57BL/6 in black dotted line and control antibody treated C57BL/6 in red line after intranasal infection with a high dose of $2.5x10^6$ CFU *Streptococcus pneumoniae* D39 and *i.p.* injection of antibody after six hours of infection. *p<0.05 (log-rank test).

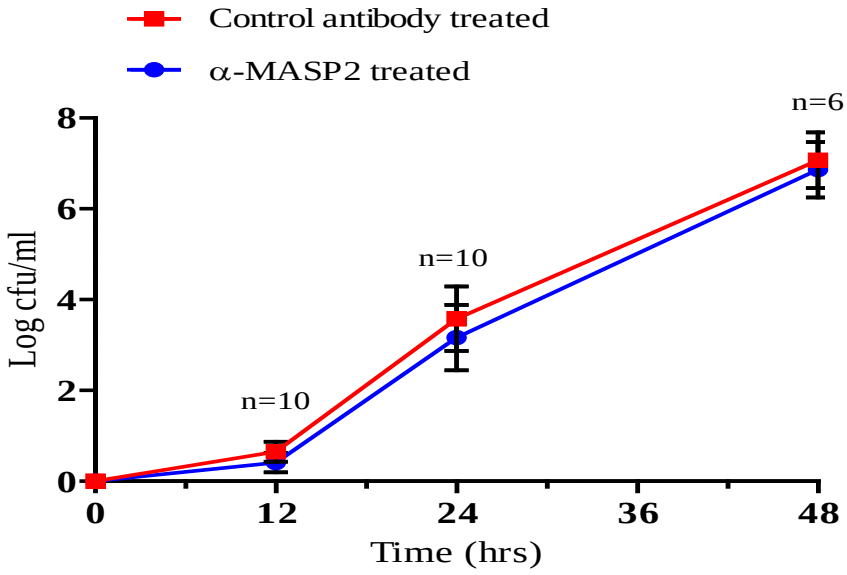


Figure 4.8: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood of α-MASP-2 treated, and control antibody treated C57BL/6 at different time points post infection with a high dose of $2.5x10^6$ CFU *S. pneumoniae* D39 and antibodies administered after 6 hours of infection. Results are means (±SEM) of 10 animals at time point 12 and 24hours and 6 animals at 48 hours. Bacterial loads were compared between both groups by Student's unpaired t test.

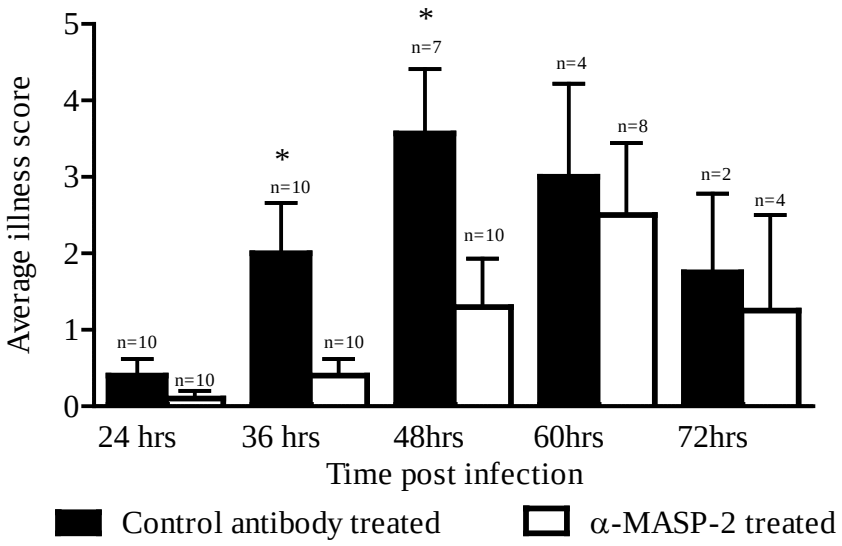


Figure 4.9: Average illness scores of α-MASP-2 mAb treated, and control antibody treated mice over different time points after intranasal infection with high dose of $2.5x10^6$ CFU *Streptococcus pneumoniae* D39 and antibodies injected after 6 hours of infection. Results are means (±SEM). Scores were compared between both groups by Student's unpaired t test. *$p<0.05$.

Score=0 "normal", Score=1 "hunched +", Score=2 "hunched ++", Score=3 "Starry coat +", Score=4 "Starry coat ++", Score=5 "Lethargic +", Score=6 "Lethargic ++".

4.3 Discussion

The lectin pathway plays a vital role in protection against *Streptococcus pneumoniae* by enhancing the lectin pathway mediated opsonisation and facilitating phagocytosis. The protective role of the lectin pathway was first described in MASP-2 deficient mice, the model with complete deficiency of lectin pathway activation (Ali, 2009). Results presented in chapter 3 further confirmed the essential role of the lectin pathway against *S. pneumoniae* and described that ficolin A (but not MBL-A and MBL-C) mediated activation of lectin pathway provides protection against *S. pneumoniae.* The aim of this study was to validate the *S. pneumoniae* infection model in MASP-2 deficient mice reported previously (Ali, 2009), and check if a similar effect could be achieved using MASP-2 inhibitory antibody by depleting the lectin pathway in normal C57BL/6 and assess whether susceptibility of MASP-2 deficient mice is directly due to loss of MASP-2 driven lectin pathway activity or indirect result of MASP-2 deficiency on the development of animal's immune response.

The α-MASP-2 mAb has been tested previously. A single *i.p.* dose of 0.6 mg/kg body weight stimulates MASP-2 deficiency resulting in loss of more than 90% of lectin pathway activity for up to seven days (Schwaeble *et al.,* 2011). The same antibody was used in this study to block the lectin pathway using a dose of 1mg/kg body weight, injected 16 hours before infection. Wild type C57BL/6 treated with α-MASP-2 mAb showed significantly greater mortality as compared to untreated control after intranasal infection of *S. pneumoniae* D39. At the end of the infection experiment, 75 % mortality was recorded in C57BL/6 treated with α-MASP-2 mAb whereas 40% mortality was recorded in the control group injected with the isotype control. Similarly α-MASP-2

mAb treated mice showed severe symptoms of disease and significantly higher bacterial loads throughout the course of infection. Hence treatment of wild-type C57BL/6 with α-MASP-2 mAb validated the studies reported previously in MASP-2 $^{-/-}$ mice showing a mortality of 85% (Ali, 2009; see Ali *et al.,* 2012, addendum 2 fig. 5), suggesting that increased susceptibility to *S. pneumoniae* in MASP-2 $^{-/-}$ is the direct result of the loss of MASP-2 driven lectin pathway activity rather than an indirect result of MASP-2 deficiency on the development of animal's immune response.

It has been demonstrated previously that α-MASP-2 mAb can limit tissue injury after ischaemia reperfusion by blocking the lectin pathway driven inflammation (Schwaeble *et al.,* 2011). This antibody is currently under consideration as a therapeutic. This study suggests that besides limiting the tissue injury in ischaemic pathologies, α-MASP-2 mAb treatment can also increase susceptibility to pneumococcal infection. Hence before initiating the antibody therapy, the immune status of individual should be considered. Another option to decrease the risk of pneumococcal infection after antibody treatment would be concurrent antibiotic treatment. To examine the effect of antibiotic treatment along α-MASP-2 mAb treatment, two additional mice groups receiving α-MASP-2 mAb and isotype control were treated with Ceftriaxone. Treatment with 20mg/kg body weight Ceftriaxone 12 hours before infection and every 12 hours thereafter showed complete protection in both the groups treated with α-MASP-2 mAb and isotype control. All the mice treated with Ceftriaxone survived the infection and did not show any bacteremia throughout the course of infection suggesting that prophylactic antibiotic treatment along with α-MASP-2 mAb will be safe to decrease the risk of pneumococcal infection in patients with a history of pneumococcal infection.

In previous experiments, the susceptibility of wild-type C57BL/6 was increased with treating with α-MASP-2 mAb prior to infection. To examine the effect of α-MASP-2 mAb treatment after infection, α-MASP-2 mAb was *i.p.* injected after 12 hours of infection with $1x10^6$ CFU *S. pneumoniae* D39. Antibody treatment after the infection did not increase the mortality in C57BL/6 mice. Eighty percent of mice treated with α-MASP-2 mAb survived the infection as compared to 70 % survival in mice treated with isotype control suggesting that mice are only susceptible when they are compromised in lectin pathway prior infection with *S. pneumoniae.* Blocking the lectin pathway post infection allows the mice to elicit early response against *S. pneumoniae.* As discussed chapter 3 (Fig. 3.26), response of early expression cytokines starts rapidly in wild-type mice as compared to ficolin A deficient mice. This was also noted in wild-type mice used as control for MASP-2 deficient mice (Ali *et al.,* 2012, see addendum 2 fig. 5). In both experiments, cytokine expression was higher in wild-type mice at 12 and 24 hours post infection and delayed in lectin pathway deficient mice. Blocking the lectin pathway 12 hours post infection is too late to enhance the infection in C57BL/6 mice. Thus, it would appear that the protective inflammatory response is so far advanced at 12 hrs that blocking the lectin pathway has no further detrimental effect. In mice group receiving α-MASP-2, 12 hrs post infection, carbohydrate recognition molecules of lectin activation pathway, ficolin A and CL-11 recognise *S. pneumoniae,* trigger the the lectin pathway activation and prevent the blood invasion before the lectin pathway is blocked.

Histopathological studies in ficolin A deficient mice showed that increased mortality in ficolin A deficient mice is due to higher bacterial loads after infection with *S. pneumoniae* as well as tissue injury caused by increased neutroutrphil infiltration in

lungs due to the severe inflammatory response at the peak stages of infection. MASP-2 deficient mice were similar (Ali, 2009). To investigate the effect of α-MASP-2 mAb in controlling the septic shock, a higher dose was used to induce septic shock (2.5×10^6 CFU). After intranasal infection, mice receiving isotype control showed severe symptoms at 36 and 48 hours, whereas, mice receiving α-MASP-2 did not show severe symptoms. This suggests that blockage of lectin pathway activation suppresses the inflammatory response, reducing septic shock. This allows the α-MASP-2 treated mice to survive longer as compared to mice treated with isotype control. Although overall percent mortality and bacterial burden at different time points are similar in both groups, number of mice alive at various time points was higher in α-MASP-2 treated mice as compared to isotype control treated mice. Also, the average overall survival time was higher in α-MASP-2 treated mice (61.5 hours for mice who couldn't survive the infection) as compared to isotype control mice (48.44 hours). In conclusion, early mortality and severe symptoms observed in mice treated with isotype control were probably due to septic shock as well as bacteremia. This study further confirms the role of α-MASP-2 mAb in controlling tissue loss in ischaemic pathologies by blocking the lectin pathway driven inflammation as reported previously (Schwaeble *et al.,* 2011).

5. C4 deposition on *Streptococcus pneumoniae*

It is well established that *Streptococcus pneumoniae* inhibits C4b deposition on its surface (Li *et al.,* 2007; Li *et al.,* 2009; Krarup *et al.,* 2005). Previous studies suggested that PspA and PspC are responsible for inhibition of C4b deposition on the pneumococcal surface (Li *et al.,* 2007). This study was performed to understand the mechanism of inhibition of C4 deposition on the surface of *S. pneumoniae* using several capsulated and non capsulated strains of different serotypes. Furthermore, *pspA* and *pspC* mutants of serotype 2, strain D39 were used to investigate the role of PspA and PspC in blocking C4 deposition on *S. pneumoniae*.

5.1 Results

5.1.1 C4 deposition on strains of different serotypes and non-capsulated strains of *Streptococcus pneumoniae*

C4 deposition was assayed on 11 different strains of *Streptococcus pneumoniae* covering six serotypes to check if C4 deposition is consistently inhibited on all the strains of *S. pneumoniae.* No C4 deposition was detected on any strain of these serotypes tested, showing that the differences in capsular polysaccharide do not affect C4 deposition or the absence of C4 deposition on the surface of *S. pneumoniae* (Fig.5.1). The effect of capsulation on C4 inhibition was further tested by performing C4 deposition assays on eight non-capsulated forms representing different serotypes of *S. pneumoniae*. No C4 deposition was detected on the surface of these strains further confirming the redundant role of the capsule in C4 inhibition on the surface of *S. pneumoniae*. (Fig.5.2). In this assay C4 deposition was detected using a specific rat anti-mouse C4 from Hycult, which recognises intact C4 and can also cross-react with the C4b fragment of C4. As a positive

control, *Escherichia coli* was included to show that this assay is capable of detecting C4b binding to the bacterial surface.

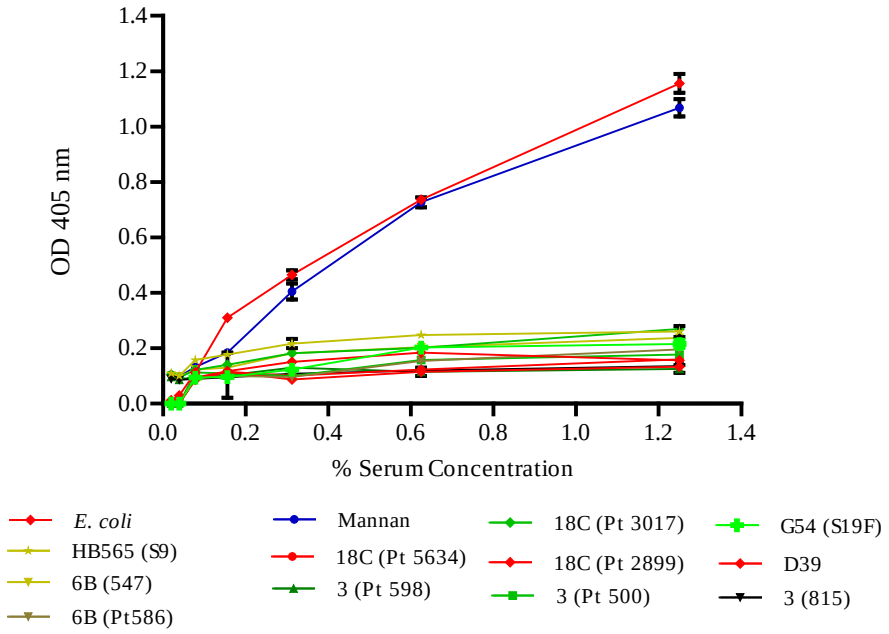


Figure 5.1: C4 deposition assay on strains of *Streptococcus pneumoniae* type 6B, 18C, 3, 19F, 9, 2 and *Escherichia coli*: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal mice serum, C4 deposition was detected using a specific rat anti-mouse C4 (Hycult). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

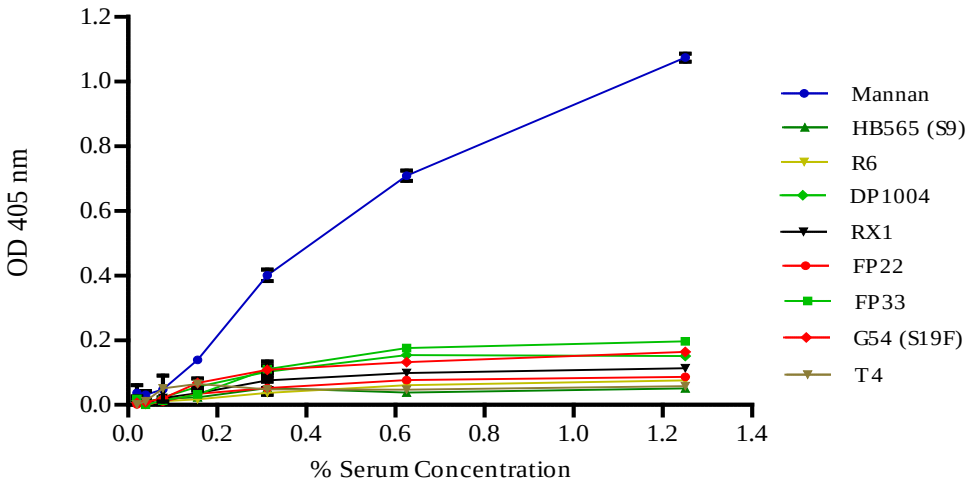


Figure 5.2: C4 deposition assay on non-capsulated strains of *Streptococcus pneumoniae*: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal mice serum, C4 deposition was detected using specific rat anti-mouse C4 (Hycult). Results are mean (±SEM) duplicates and are representative of three independent experiments.

5.1.2 C4 deposition on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39

C4 deposition was tested on three *pspA* mutants of *Streptococcus pneumoniae* D39, kindly provided by Dr. Hasan Yasilkaya and *pspC* mutant was kindly provided by Dr. Aras Kadioglu to assess the role of pneumococcal surface proteins, PspA and PspC inhibiting C4 deposition on the surface of *S. pneumoniae*. C4 deposition was investigated on these strains along with wild-type *S. pneumoniae* D39 using normal mice serum. No C4 deposition was detected on the surface of any of mutant tested using a specific rat anti-mouse C4 (Hycult) that recognises intact mouse C4 and can cross react with C4b (Fig 5.3). C4 deposition on *pspA* and *pspC* mutants was further checked by using human serum. Specific chicken anti-human C4 was used for detection of C4. This antibody recognises intact human C4 and can cross react with C4b fragment of C4. No C4 deposition was detected using human serum on any of mutant tested (Fig 5.4).

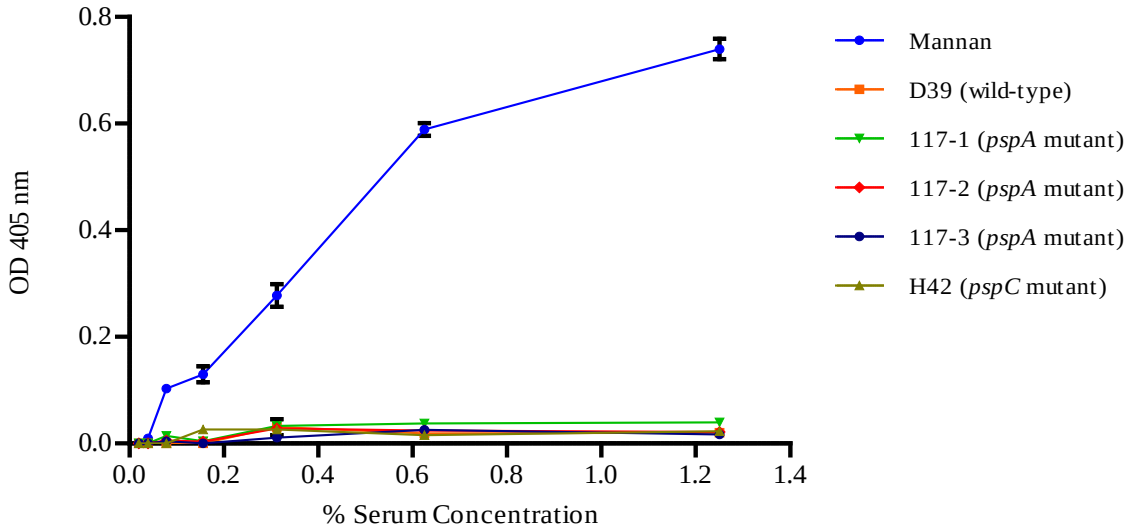


Figure 5.3: C4b deposition assay on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using mouse serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal mice serum, C4 deposition was detected using specific anti C4 (Hycult). Results are means of duplicate experimenst and are representative of three independent experiments.

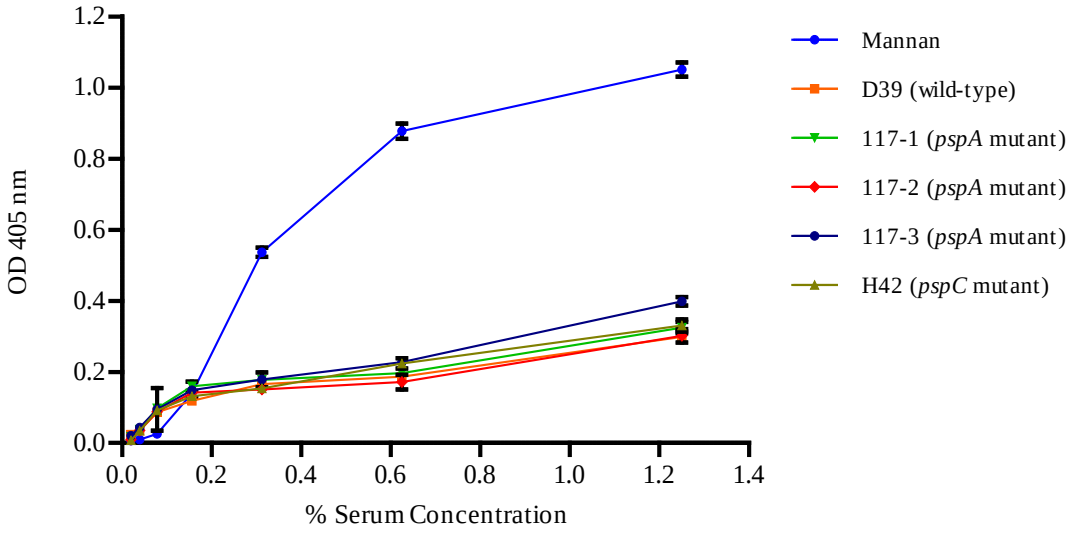


Figure 5.4: C4b deposition assay on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using human serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal human serum, C4 deposition was detected using specific anti C4 (Agrisera). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

C4 deposition was further checked by a different C4 activation assay in which exogenous human C4 was added to each well after incubation with normal mice serum. Binding of human C4 or its fragment C4b and C4c were detected by two different antibodies; chicken anti-human C4 which reacts with human C4 fragment and can cross react with C4b, and mouse anti-human C4 that reacts with intact human C4 and cross-reacts with fragment C4c. No binding of intact C4 or its fragments, C4b or C4c was detected on any mutant tested (Fig 5.5 A and B).

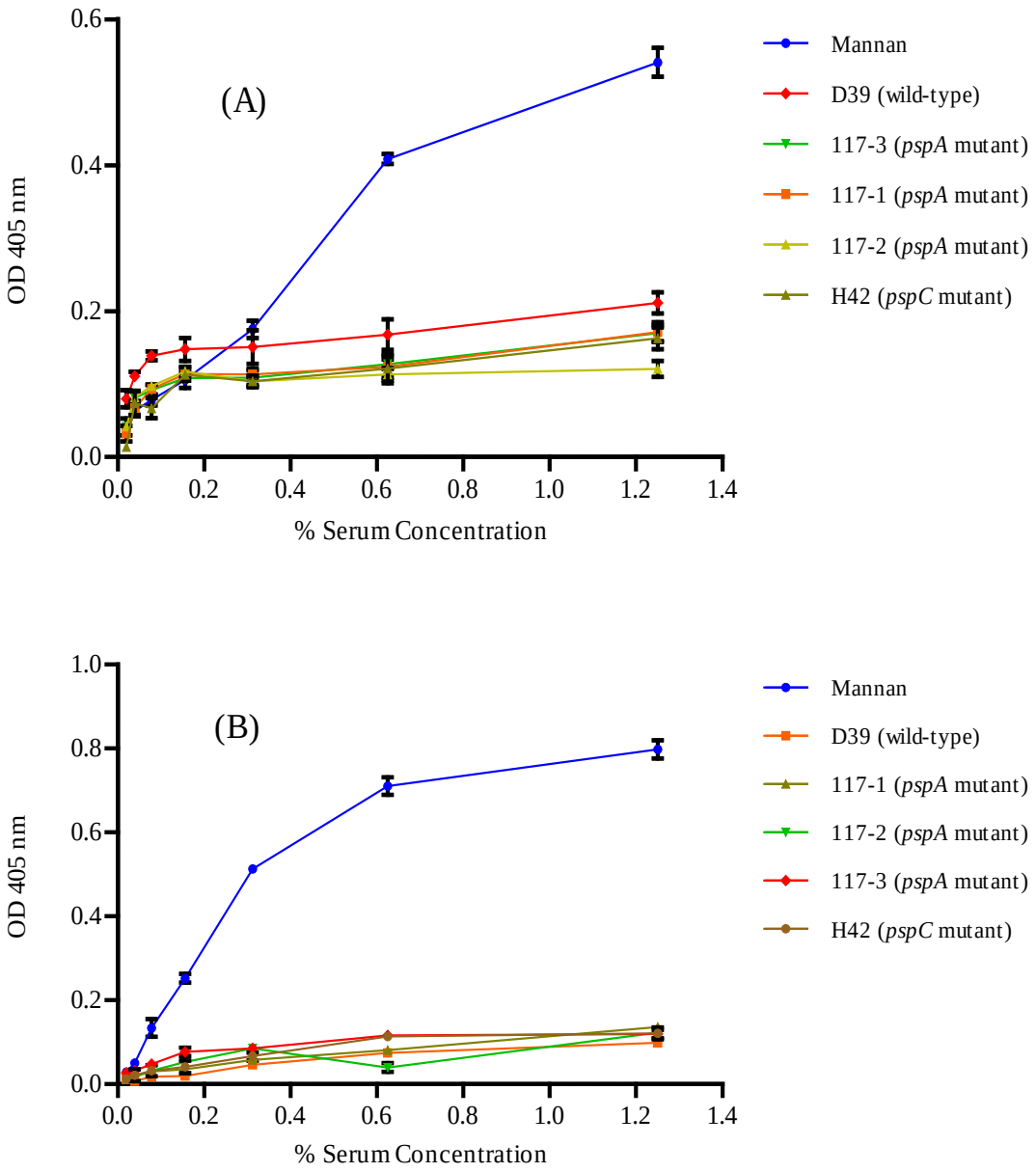


Figure 5.5: Exogenous C4b and C4c deposition assay on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using mice serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal mice serum first and exogenous human C4 later, C4 deposition was detected using specific Chicken anti-human C4 from Agisera that also cross reacts with C4b fragment (A), and mouse anti-human C4 from Santa Cruz that also cross reacts with C4c fragment (B). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

5.1.3 C3b deposition on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39

C3 deposition was tested on *pspA* and *pspC* mutants along with wild-type D39 to see if PspA or PspC deficiency in *Streptococcus pneumoniae* affects C3 deposition on its surface. No significant difference in level of C3b deposition was detected on wild-type D39 and its *pspA* mutants (Fig 5.6).

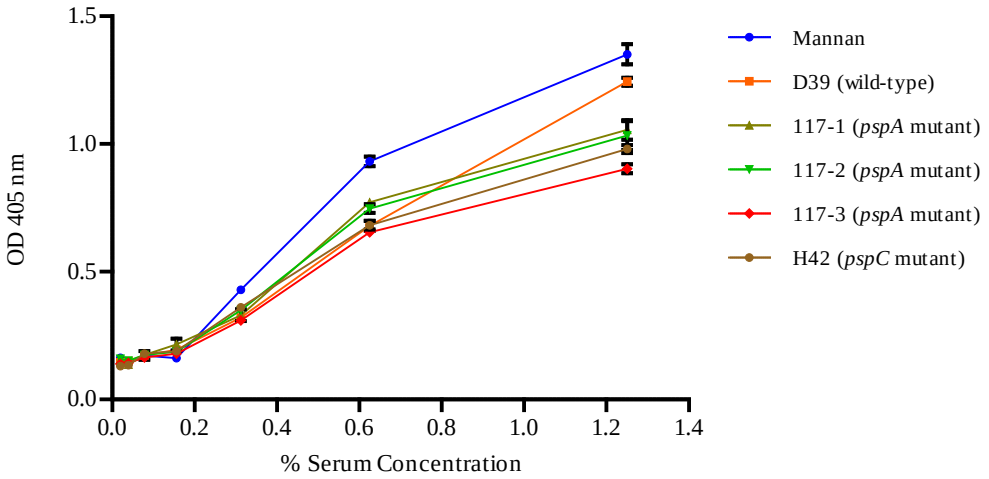


Figure 5.6: C3b deposition assay on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using mice serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal mice serum, C3b deposition was detected using specific rabbit anti-human C3c (Dako). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

5.1.4 C4dg deposition on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39

Deposition of C4dg, last factor I mediated degradation product of C4b was checked on wild-type D39 and its *pspA* and *pspC* mutants. C4dg was detected on all *pspA* and *pspC* mutants tested as well as wild-type D39. There was no difference in the level of C4dg deposition on all the bacteria tested. This result indicates that C4 is degraded into subcomponents after attaching to pneumococcal surface leaving behind the haemolytically inactive fragment C4dg on the surface. C4dg could not be detected on mannan indicating that for degradation of C4, bacterial surfaces are required that utilise the complement inhibitors of hosts to trigger factor I mediated cleavage of C4b. Due to the presence of

whole C4 fragment, C4dg binding sites are hidden that cannot be detected by anti-C4dg antibody.

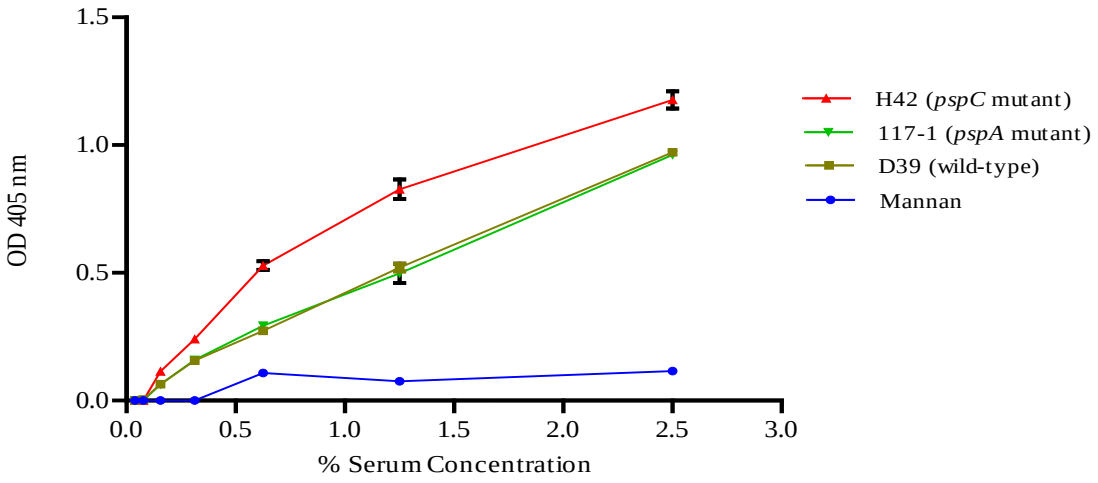


Figure 5.7: C4dg deposition on *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using human serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal human serum, C4dg deposition was detected using anti C4dg. Results are mean (±SEM) of duplicates and are representative of three independent experiments.

5.1.5 C4bp binding to *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39

Binding of C4bp, the fluid phase inhibitor of the classical and the lectin pathway was assessed on the surface of *Streptococcus pneumoniae* D39 and its *pspA* and *pspC* mutants to check if the absence of PspA or PspC affects the C4bp binding to *S. pneumoniae.* C4bp bound to all the mutants wild-type D39 supporting the factor I mediated cleavage of C4b on *pspA* and *pspC* mutants of *S. pneumoniae* D39. Mannan did not bind to C4bp, and this might be the reason of no C4dg detection on mannan in fig. 5.7. Due to lack of binding of C4bp, C4 was not degraded and due to the presence of full C4 fragment, C4dg could not be detected on mannan.

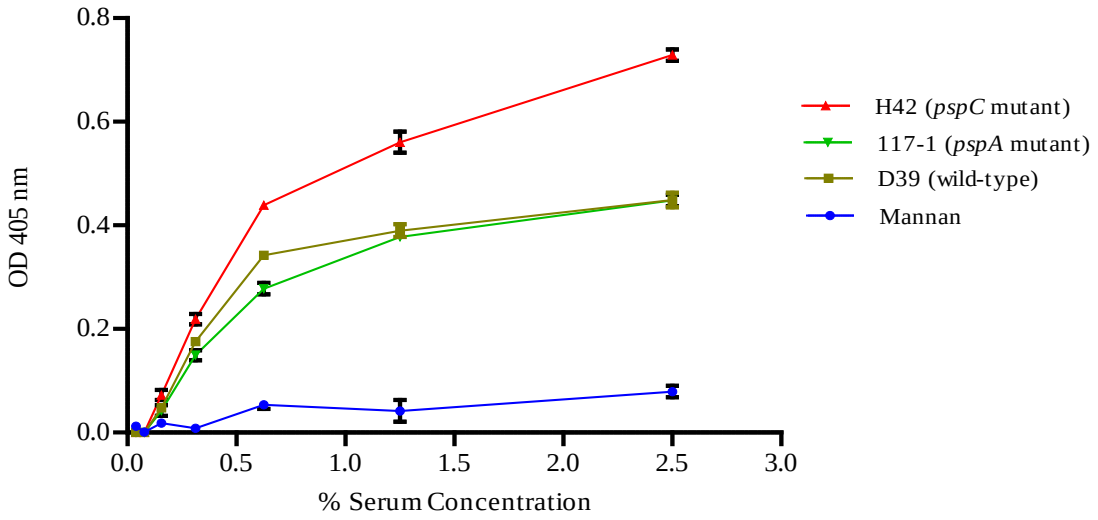


Figure 5.8: C4bp binding to *pspA* and *pspC* mutants of *Streptococcus pneumoniae* D39 using human serum: Following coating of microtitre plates with mannan and formalin fixed bacteria, incubation with normal human serum, C4bp binding was detected using anti C4bp (Quidal). Results are mean (±SEM) of duplicates and are representative of three independent experiments.

5.2 Discussion

Complement system is an integral part of innate host defences to eliminate the invading pathogens by a series of actions that kill the pathogens directly, mediate C3b opsonisation to facilitate phagocytic uptake or produce anaphylotoxins to attract phagocytes (Laarman *et al.,* 2010). On the other hand, pathogens adopt strategies to evade complement attack by expressing virulence factors interfering with complement activation on bacterial surfaces or by utilising the complement inhibitors of the host to inhibit the direct lysis or oposonophagocytic killing (Blom *et al.,* 2009). *Streptococcus pneumoniae* express an array of virulence factors that target specific complement components and reduce the complement mediated bacterial clearance. The most important virulence factors interfering with complement activation include capsular polysaccharides, PspA, PspC and pneumolysin (Kadioglu *et al.,* 2008). It is well established that *S. pneumoniae* inhibits C4 deposition on its surface (Krarup *et al.,* 2005; Li *et al.,* 2007). Recent studies demonstrated that PspA and/or PspC interfere with C4 deposition on the surface of *S. pneumoniae* (Li *et al.,* 2007). However, it is not clear if PspA and/or PspC directly inhibit C4 deposition on the surface of *S. pneumoniae* or protect the bacteria from C4 opsonisation by binding to negative host complement regulators. This study was performed to understand the mechanism of inhibition of C4b deposition on *S. pneumoniae* and bacterial virulence factors affecting it.

To investigate if serotype or strain variations are responsible for affecting C4b deposition on bacterial surface, C4b deposition was checked on 11 strains of *S. pneumoniae* covering six serotypes. C4b could not be detected on any of the strains tested indicating that capsular polysaccharides or genetic variations between the strains do not contribute in inhibition of C4b deposition on the pneumococcal surface. Efficient C4b deposition on mannan and *E. coli*

suggested that this assay was capable of detecting C4b not only on mannan but also on the bacterial surface. To further investigate if virulence factors related to capsule or non-capsular virulence factors inhibit C4b deposition on the surface of *S. pneumoniae*, C4b deposition was assessed on eight non-capsulated strains representing different serotypes. None of the non-capsulated types of these bacteria showed any C4b deposition suggesting that non-capsular virulence factors produced by *S. pneumoniae* interfere with C4b deposition on the pneumococcal surface.

The effect of non-capsular virulence factors on C4 deposition on *S. pneumoniae* surfaces was assessed using *pspA* and *pspC* mutants to assess their role in inhibiting C4 deposition. To find out if C4b is completely blocked on bacterial surface or degraded by these surface proteins, different fragments of C4 were explored on the surface of bacteria using specific antibodies against mice and human C4 fragments. Degradation of C4 results in formation smaller fragments C4b, C4c and C4dg. C4b or C4c, larger haemolitically active degradation fragments of C4 could not be detected on any of the *pspA* or *pspC* mutants using both mice and human sera. However smaller haemolytically inactive fragment C4dg was abundantly deposited on all the wild-type D39 along with *pspA* and *pspC* mutants of *S. pneumoniae* serotype 2. The presence of C4dg suggests that C4 deposition is not blocked by *S. pneumoniae*. The absence of active fragments of C4 on the surface of *S. pneumoniae* is due to rapid degradation of C4 on pneumococcal surface supporting the hypothesis that *S. pneumoniae* sequester the host complement protein to accelerate the factor I mediated breakdown of C4b (Li *et al.*, 2007; Dieudonne-Vatran *et al.*, 2009). C4dg deposition on both *pspA* and *pspC* mutants indicates that both PspA and PspC can mediate C4b breakdown on the surface of *S. pneumoniae* on their own by accumulating the negative regulators of complement activation to accelerate factor I mediated C4b breakdown. Absence of PspA or

PspC is not enough to rule out the factor I mediated breakdown of C4b. Hence this finding supports the resurgence of C4b deposition of *pspA* and *pspC* double mutant of *S. pneumoniae* reported previously (Li *et al.*, 2007) but does not agree with C4b deposition on *pspA* mutant reported in the same study.

To further investigate the factor I mediated cleavage of C4 fragments on pneumococcal surface, binding of C4bp was assessed. C4bp is the fluid phase inhibitor of the classical and the lectin pathways by acting as the cofactor of serine protease factor I which cleaves C4b and C3b (Meri *et al.*, 2004; Dahlback *et al.*, 1983). C4bp binding to *S. pneumoniae* has been reported previously through pneumococcal surface protein C (PspC) (Dieudonne-Vatran *et al.*, 2009). In this study, C4bp binding was detected on *S. pneumoniae* wild-type D39 strains as well as *pspA* and *pspC* mutants of *S. pneumoniae* D39 showing that C4bp binding is not through specific surface proteins. PspC contributes in C4bp binding to *S. pneumoniae* but in the absence of PspC, PspA retains C4bp binding to the pneumococcal surface hence mediating factor I dependant cleavage of C4b. Similarly, in the absence of PspA, PspC binds to C4bp to mediate factor I dependant cleavage of C4b. Hence PspA and PspC work synergistically contributing in cleavage of C4b on the pneumococcal surface.

The identification of the mechanism responsible for the inhibition or degradation of C4b deposited on the surface of *S. pneumoniae* is essential to understand the role of different complement activation pathways in fighting pneumococcal infection. My results strongly support the view that *S. pneumoniae* decays the larger haemolytically active fragment of C4 i.e. C4b, which is required for formation of the classical and the lectin pathway specific C3 covertases (C4b2a) through a factor I mediated cleavage by binding to the negative complement regulators and factor I cofactors C4bp and factor H. Since the classical pathway can not proceed further in the absence of the C3 covertase C4b2a, absence of haemolytically

active fragments of C4 on the surface of *S. pneumoniae* rules out the possibility of a central protective role of the classical pathway in fighting pneumococcal infection. The essential role of lectin pathway activation is explained by the fact that MASP-2 can still activate complement C3 in the absence of C4 via a unique MASP-2 dependant C4-bypass mechanism as described previously (Schwaeble *et al.*, 2011). Since MASP-2 activates the lectin pathway in the absence of C4, ficolin A and CL-11 are the relevant carbohaydrate recognition molecules that initiate the lectin pathway mediated C3 deposition directly on the surface of *S. pneumoniae* through activation of MASP-2.

6. Summary

Complement system is part of the innate immune system and acts as a first line of defence against pathogens invading the body. The complement system is composed of plasma zymogens which upon activation trigger a reaction cascade resulting in the generation of activation products that either enhance phagocytosis or attract phagocytes or kill pathogen directly by the formation of membrane attack complex. Complement activation is mediated via three different pathways; the classical pathway, initiated by binding of the classical pathway recognition component C1q to antibodies bound to pathogens; the alternative pathway, which is in a constant low grade turnover state that is kept in check by activation inhibiting or activation promoting regulatory components; and the lectin pathway, initiated by carbohydrate recognition molecules binding to microbial carbohydrates on the surface of microorganisms. Recognition molecules that initiate the lectin pathway in mice are MBL-A, MBL-C, ficolin A and recently described CL-11.

Streptococcus pneumoniae is an important human pathogen causing a variety of diseases, including pneumonia, otitis media, septicemia and meningitides and remains the major cause of morbidity and mortality throughout the world. Innate immunity plays a critical role in providing protection against *S. pneumoniae*. The complement system provides the key component of the innate as well as the adaptive immune response against *S. pneumoniae.* In *S. pneumoniae* infections complement is essential in mediating opsono-phagocytosis.

Several studies have demonstrated the protective role of the classical and the alternative pathways of complement activation against pneumococcal infection using mice with genetically engineered deficiencies in classical and alternative pathways using different infection models of *S. pneumoniae*. Very recently, a critical pathophysiological role of the lectin pathway has been demonstrated using MASP-2 deficient mice who present with a complete deficiency of the lectin pathway functional activity through their inability to form the lectin pathway specific C3 or C5 convertases. In this study, the role of the lectin pathway recognition molecule ficolin A in fighting pneumococcal infection was demonstrated using a mouse strain with gene targeted deficiency of *Fcna* gene (provided by Prof. Teizo Fujita, Fukushima Medical University Japan). This strain is completely deficient in ficolin A mediated lectin pathway activation while having a perfectly functional MBL and CL-11 mediated lectin pathway activation.

The first part of this study assessed the binding of lectin pathway specific carbohydrate recognition molecules to *S. pneumoniae*. Binding results using mouse sera clearly demonstrated that ficolin A and CL-11 predominantly bind to the surface of *S. pneumoniae.* This was also confirmed by using recombinant mouse ficolin A. CL-11 binding was consistent among all the *S. pneumoniae* strains tested. However, ficolin A binding showed varying binding affinity with the 10 *S. pneumoniae* strains tested. Murine MBL-A did not bind to any of the strains tested, whereas murine MBL-C showed very weak to no binding to all the 10 strains tested. Similarly, in humans, L-ficolin and CL-11 showed strong binding to *S. pneumoniae* whereas MBL, M-ficolin and H-ficolin did not bind to *S. pneumoniae* at all. Hence, binding studies imply that murine ficolin A and CL-11 and human L-ficolin and CL-11 are the key recognition components capable of activating lectin pathway on *S. pneumoniae*.

Ficolin A binding and C3b deposition on different *S. pneumoniae* strains showed a high degree of correlation. The degree of C3 deposition was markedly higher on the serotypes showing a strong binding to ficolin A and vice versa. This indicates that the level of ficolin A binding affects C3b deposition on different serotypes of *S. pneumoniae*. Hence, ficolin A binding is critical for the activation of the lectin pathway on *S. pneumoniae* and critical in mediating lectin pathway dependent C3b opsonisation on *S. pneumoniae.*

To further investigate the contribution of ficolin A, CL-11, MBL-A and MBL-C in activating the lectin pathway on the surface of *S. pneumoniae*, C3b deposition was measured by using sera from different gene targeted mouse strains with selective complement deficiencies including deficiencies of either ficolin A, MBL-A/C or MBL-A/C/ficolin A. MASP-2 deficient serum did not show any C3 deposition on *S. pneumoniae* indicating that C3b deposition on *S. pneumoniae* is totally MASP-2 dependent. C3b deposition was not affected in MBL-A/C deficient serum when compared to wild-type serum implying that neither MBL-A nor MBL-C is critical in the activation of the lectin pathway on *S. pneumoniae.* This further confirms and underlines the outcome of the binding studies for MBL-A and MBL-C binding to *S. pneumoniae*. Ficolin $A^{-/-}$ serum showed significantly reduced C3b deposition on *S. pneumoniae* confirming the critical role of ficolin A in lectin pathway activation. Interestingly, MBL-A/C/ficolin $A^{-/-}$ serum, deficient in all lectin pathway recognition components except CL-11, deposited C3b on the surface of *S. pneumoniae*. Since mouse CL-11 shows strong binding to *S. pneumoniae* (this work), and since CL-11 forms complexes with MASP-2 (Ali *et al.*, 2012, see addendum 2), we concluded that CL-11 works

synergistically with ficolin A to contribute in the activation of the lectin pathway of complement against *S. pneumoniae*.

C3b deposition on zymosan, mannan and N-acetyl BSA using sera from mice deficient in various lectin pathway recognition molecules further underlines the conclusion that CL-11 activates MASP-2 and initiates the lectin pathway. CL-11 does not bind to mannan and as a result no C3b deposition is detectable on mannan using MBL-A/C/ficolin $A^{-/-}$ serum sufficient (which is sufficient in CL-11). On the other hand, CL-11 shows strong binding to zymosan and N-acetyl BSA resulting in C3b deposition in MBL-A/C/ficolin $A^{-/-}$ serum.

In vivo studies demonstrated that ficolin A deficient mice are severely compromised in their ability to fight pneumococcal infection showing significantly higher mortality (87%) as compared to ficolin A sufficient mice (13%) after intranasal challenge with *S. pneumoniae*. In addition, ficolin A deficient mice showed a higher bacterial burden in blood and lungs as compared to ficolin A sufficient mice at different time points post infection. MBL-A/C deficient mice were highly resistant to pneumococcal infection. All MBL-A/C deficient mice survived the infection and did not show any bacteremia after intranasal challenge with *S. pneumoniae*, validating the *in vitro* results that ficolin A (but not MBL-A and/or MBL-C) is the key recognition component of the lectin pathway activation against *S. pneumoniae*.

The histopathological analysis of lung section from ficolin A sufficient mice showed increased neutrophils infiltration at 24 hours time point as compared to ficolin A deficient mice. Neutrophil infiltration was dramatically high in ficolin A deficient mice at time points 48 and 60 hours post infection. Early recruitment of neutrophils in lungs

tissues of wild-type mice helps the mice in clearing bacteria before bacteria cause any damage, whereas delayed onset of neutrophil recruitment in ficolin A deficient mice results in a sudden increase in the bacterial burden in lungs at 48 and 60 hours post infection as a result of which there is a huge influx of neutrophils at the site of infection causing tissue damage.

The results of the histopathological analysis were underlined by the mRNA expression profiles, established for pro-inflammatory mediators and revealed that ficolin A sufficient mice show relatively higher expression levels of proinflammatory cytokines such as IL1-β, IL-6, MIP-2 and TNF-α as compared to ficolin A deficient mice at the early stages of infection. Higher levels of these inflammatory mediators at early stages of infection lend to early immune response against *S. pneumoniae*, which enhance the recruitment of leukocytes to the site of infection, providing protection against *S. pneumoniae*. Lower levels of these inflammatory mediators in ficolin A deficient mice delay the immune response and allow the bacteria to multiply. Due to the higher bacterial burden, expression levels of IL1-β, IL-6, MIP-2, TNF-α, IL-17 and IL-10 are significantly increased in ficolin A deficient mice at the peak stages of infection. Overexpression of these inflammatory mediators results in excessive neutrophil activation, a process confirmed by the histopthological analysis of lung sections from ficolin A deficient mice at time point 48 and 60 hours post infection.

Both the *in vitro* and *in vivo* studies demonstrate that the lectin pathway has a crucial role in providing protection against *S. pneumoniae* supporting the previously reported study showing the protective role of the the lectin pathway. My results obtained by analysing a ficolin A deficient mouse strain underlines the previously obtained results when analysing MASP-2 deficient mice with complete deficiency of lectin pathway

functional activity (Ali, 2009). This study also highlights the importance of the MBL independent activation of the lectin pathway in *S. pneumoniae* infections and shows that ficolin A and CL-11 (but not MBL-A or MBL-C) bind to *S. pneumoniae* and drive the lectin pathway on the surface of *S. pneumoniae*. My *in vivo* studies focused on the identification of the role of ficolin A in fighting pneumococcal infection and demonstrate that ficolin A deficient mice are highly susceptible to pneumococcal infection with significantly higher mortality and higher blood and lung bacterial burden.

The critical role of the lectin pathway in providing protection against *S. pneumoniae* was further assessed using specific α-MASP-2 mAb, which deplete MASP-2 from plasma. Previous studies showed that a single *i.p.* dose of α-MASP-2 mAb administered as 0.6mg/kg body weight results in the loss of lectin pathway functional activity for up to seven days (Schwaeble *et al.,* 2011). Lectin pathway functional activity was blocked in C57BL/6 using the mouse specific α-MASP-2 antibody, AbD04211 administered *i.p.* administered 16 hours prior to *S. pneumoniae* infection. C57BL/6 mice treated with α-MASP-2 mAb showed significantly greater mortality (75%) as compared to untreated control group (40%). Antibiotic treatment (Ceftriaxone, 20mg per kg body weight *i.p.* 12 hours before infection and every 12 hours thereafter) resulted in complete protection against pneumococcal infection both in antibody treated and non treated groups. Schwaeble *et al.,* 2011 suggested the therapeutic utility of α-MASP-2 mAb to limit tissue injury through ischaemia reperfusion injury by blocking lectin pathway driven ischaemia reperfusion injury. This study implies that this approach to limit tissue injury in ischaemic pathologies, using α-MASP-2 mAbs may also increase the susceptibility to pneumococcal infection. Prophylactic antibiotic

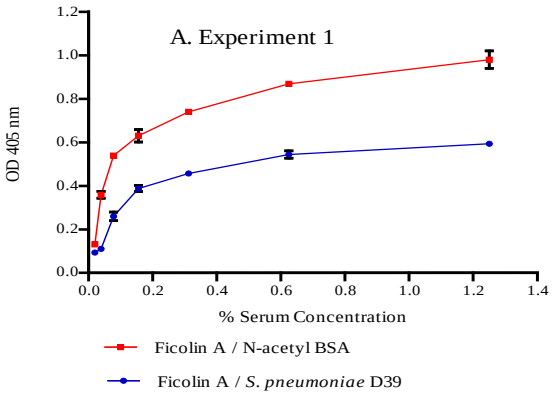
treatment prior to administration of MASP-2 antagonists may be required to reduce the risk of pneumococcal infection.

Previous studies have reported that PspA and PspC inhibit C4 deposition on the surface of *S. pneumoniae* (Li *et al.,* 2007; Li *et al.,* 2009). This study indicates that deletion of *pspA* or *pspC* alone is not enough to recover C4b/C4c deposition on the surface of these bacteria as no C4b/C4c was detected on *pspA* or *pspC* mutants of *S. pneumoniae*. However, C4dg, the final product of C4 decay was abundantly deposited on the surface of *S. pneumoniae* suggesting that all the wild-type, as well as *pspA* and *pspC* mutants of *S. pneumoniae* sequester complement inhibitory proteins from host serum to accelerate the breakdown of C4b on the bacterial surface. This was further confirmed by the detection of abundant C4bp deposition on the surface of wild-type, *pspA* and *pspC* mutants of *S. pneumoniae*. Inhibition of haemolytically active C4b as well as C4c was also investigated using various serotypes and non capsulated strains of *S. pneumoniae*. No C4b or C4c was detected on the surface of these strains ruling out the effect of capsule and serotype differences in inhibiting C4b and C4c deposition on the surface of *S. pneumoniae*.

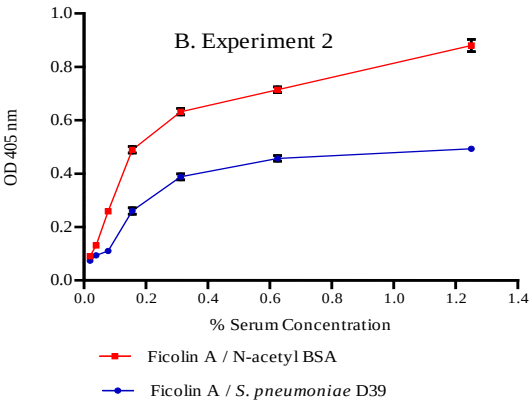
7. Addendum 1

7.1. Presentation of ELISA graphs

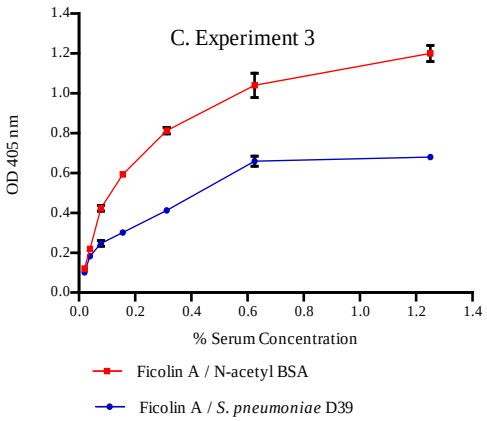
Each ELISA result shown in this thesis is from a single experiment representative of three seperate experiments, where the replicates of each experiment showed similar results with high degree of reproducibility. Fig. 7.1, 7.2 and 7.3 show three replicate experiments each representing Fig. 3.1, 3.2 and 3.3 respectively in chapter 3. Corresponding tables show the final $OD405_{nm}$ readings recorded at the end of each experiment. In all the assays, negative control wells of coating substance were used, where serum was replaced by buffer used for serum dilution. Negative control reading was subtracted from each sample of corresponding column to obtain the actual reading by eliminating the background value. Error bars indicate ±SEM of duplicates.



A. Experiment 1				
% serum conc.	D39		N-acetyl BSA	
1.25	0.616	0.672	1.013	1.089
.625	0.578	0.610	0.936	0.944
.312	0.484	0.530	0.792	0.830
.156	0.425	0.451	0.674	0.728
.078	0.291	0.329	0.606	0.614
.039	0.156	0.164	0.416	0.444
.019	0.140	0.148	0.194	0.210
-ve	0.051	0.049	0.073	0.069

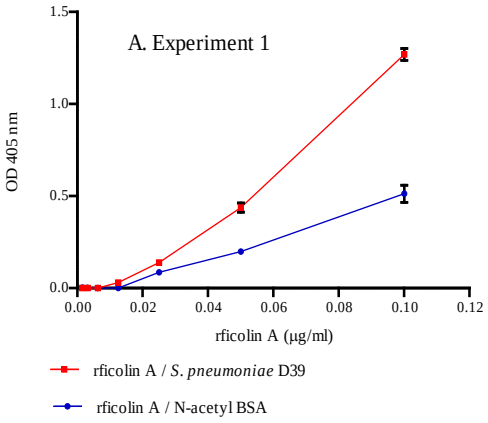


B. Experiment 2				
% serum conc.	D39		N-acetyl BSA	
1.25	0.519	0.540	0.920	0.969
.625	0.481	0.506	0.766	0.791
.312	0.411	0.438	0.683	0.710
.156	0.282	0.311	0.540	0.567
.078	0.139	0.154	0.313	0.334
.039	0.124	0.137	0.186	0.205
.019	0.103	0.118	0.144	0.165
-ve	0.034	0.039	0.063	0.066

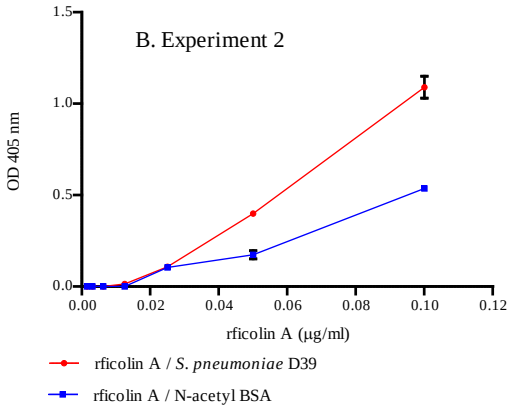


C. Experiment 3				
% serum conc.	D39		N-acetyl BSA	
1.25	0.740	0.766	1.196	1.380
.625	0.705	0.759	1.066	1.190
.312	0.474	0.496	0.884	0.918
.156	0.360	0.388	0.669	0.693
.078	0.304	0.336	0.496	0.526
.039	0.248	0.264	0.301	0.313
.019	0.170	0.178	0.203	0.215
-ve	0.071	0.075	0.087	0.089

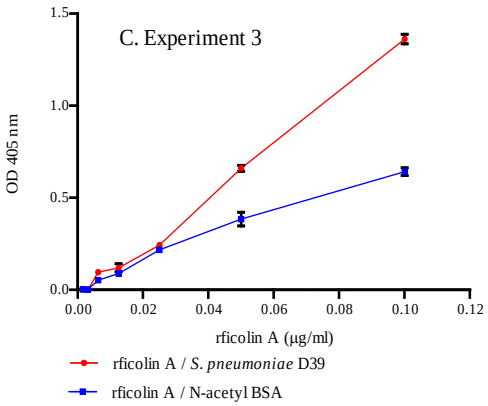
Figure 7.1: Ficolin A binding assay to determine binding of ficolin A with *Streptococcus pneumoniae* D39 by ELISA: Fig 3.1 in chapter 3 represents above three experiments performed separately. Graph A was chosen to represent the binding of ficolin A to *S. pneumoniae* D39.



A. Experiment 1				
% serum conc.	N-acetyl BSA		D39	
1.25	0.512	0.608	1.316	1.382
.625	0.241	0.252	0.492	0.543
.312	0.127	0.1393	0.211	0.227
.156	0.046	0.049	0.108	0.111
.078	0.046	0.049	0.095	0.086
.039	0.047	0.0506	0.084	0.082
.019	0.049	0.0529	0.081	0.085
-ve	0.046	0.049	0.079	0.081

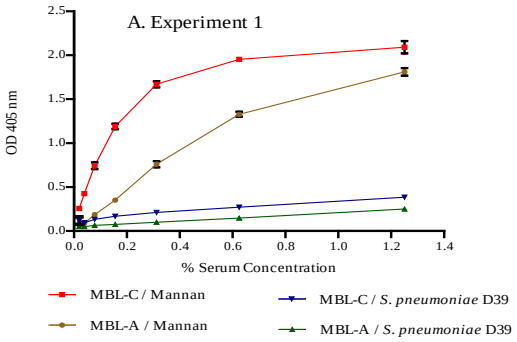


B. Experiment 2				
% serum conc.	N-acetyl BSA		D39	
1.25	0.593	0.611	1.082	1.205
.625	0.215	0.263	0.446	0.459
.312	0.159	0.179	0.157	0.166
.156	0.062	0.063	0.061	0.074
.078	0.059	0.061	0.050	0.045
.039	0.063	0.066	0.051	0.049
.019	0.064	0.07	0.048	0.053
-ve	0.063	0.067	0.052	0.055



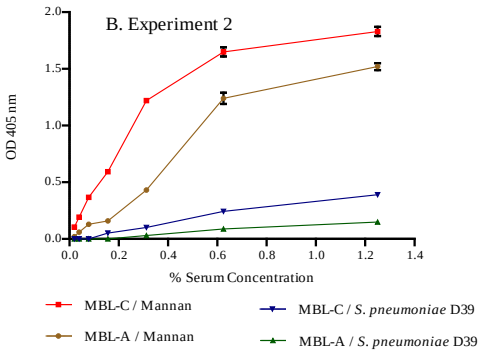
C.. Experiment 3				
% serum conc.	N-acetyl BSA		D39	
1.25	0.694	0.739	1.415	1.470
.625	0.420	0.497	0.723	0.758
.312	0.281	0.302	0.314	0.333
.156	0.158	0.169	0.177	0.224
.078	0.118	0.135	0.170	0.183
.039	0.0736	0.078	0.083	0.093
.019	0.0761	0.08	0.081	0.089
-ve	0.073	0.076	0.079	0.082

Figure 7.2: Ficolin A binding assay to determine binding of recombinant ficolin A with *Streptococcus pneumoniae* D39 by ELISA: Fig 3.2 in chapter 3 represents above three experiments performed separately. Graph A was chosen to represent binding of ficolin A to *S. pneumoniae* D39.



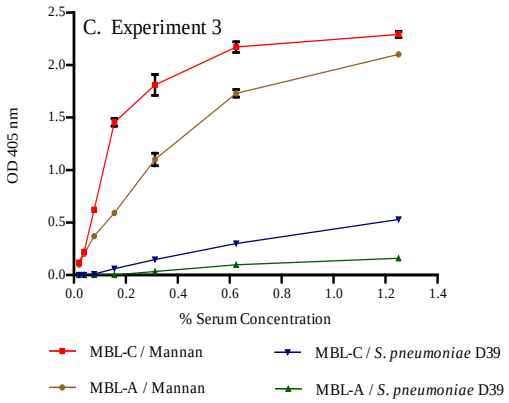
A. Experiment 1

% serum conc.	MBLA/Mannan		MBL-C/Mannan		MBL-A / D39		MBL-C / D39	
1.25	1.946	1.862	2.262	2.124	0.308	0.312	0.454	0.457
.625	1.448	1.396	2.054	2.055	0.207	0.203	0.337	0.345
.312	0.888	0.821	1.805	1.739	0.155	0.161	0.28	0.281
.156	0.453	0.439	1.322	1.262	0.135	0.134	0.235	0.241
.078	0.285	0.277	0.882	0.808	0.122	0.123	0.200	0.205
.039	0.179	0.189	0.547	0.51	0.109	0.111	0.162	0.164
.019	0.164	0.165	0.363	0.358	0.108	0.114	0.144	0.233
-ve	0.092	0.096	0.102	0.104	0.056	0.061	0.069	0.071



B. Experiment 2

% serum conc.	MBLA/Mannan		MBLC/Mannan		MBL-A / D39		MBL-C / D39	
1.25	1.854	1.766	2.16	2.02	0.252	0.251	0.385	0.386
.625	1.356	1.300	1.952	1.951	0.151	0.142	0.268	0.274
.312	0.796	0.725	1.703	1.635	0.099	0.1	0.211	0.21
.156	0.361	0.343	1.22	1.158	0.041	0.066	0.076	0.17
.078	0.193	0.181	0.78	0.704	0.048	0.064	0.066	0.079
.039	0.087	0.093	0.445	0.406	0.052	0.065	0.068	0.074
.019	0.072	0.069	0.261	0.254	0.053	0.053	0.065	0.075
-ve	0.092	0.096	0.102	0.104	0.056	0.061	0.069	0.071



C. Experiment 3

% serum conc.	MBLA/Mannan		MBLC/Mannan		MBL-A / D39		MBL-C / D39	
1.25	2.186	2.164	2.303	2.445	0.218	0.232	0.603	0.587
.625	1.842	1.768	2.203	2.305	0.160	0.17	0.373	0.357
.312	1.236	1.114	1.793	1.995	0.0924	0.109	0.219	0.205
.156	0.674	0.656	1.501	1.575	0.054	0.073	0.132	0.118
.078	0.447	0.443	0.693	0.717	0.053	0.072	0.075	0.075
.039	0.2822	0.272	0.296	0.314	0.059	0.071	0.069	0.068
.019	0.183	0.165	0.197	0.209	0.061	0.075	0.067	0.071
-ve	0.076	0.074	0.083	0.085	0.063	0.069	0.064	0.066

Figure 7.3: MBL-A and MBL-C binding to *Streptococcus pneumoniae* D39: Fig 3.2 in chapter 3 represents above three experiments performed separately. Graph A was chosen to represent binding of ficolin A to *S. pneumoniae* D39.

7.2. Survival and blood bacterial burden of MBL-A/C sufficient and MBL-A/C deficient mice after intranasal infection with *Streptococcus pneumoniae* D39

Figure 3.24 and 3.25 in chapter 3 show results from the preliminary infection experiments performed with 5 MBL-A/C$^{+/+}$ and 5 MBL-A/C$^{-/-}$ mice, intra-nasally infected with 1x10^{6} CFU *Streptococcus pneumoniae* D39. To improve statistical power, the, infection study was repeated with 10 mice in each group. The results were similar the preliminary infection study. After one week of infection, all MBL-A/C$^{-/-}$ mice survived the infection whereas 70% of MBL-A/C$^{+/+}$ mice survived (Fig. 7.4).

Blood bacterial burden was also similar to that observed in the preliminary experiment. No bacteria were detected in the blood of MBL-A/C$^{-/-}$ mice throughout the course of infection. MBL-A/C$^{+/+}$ mice showed very low bacterial loads in blood at 24 hrs and 48 hrs post infection, which was subsequently cleared after 72 hours of infection (Fig. 7.5).

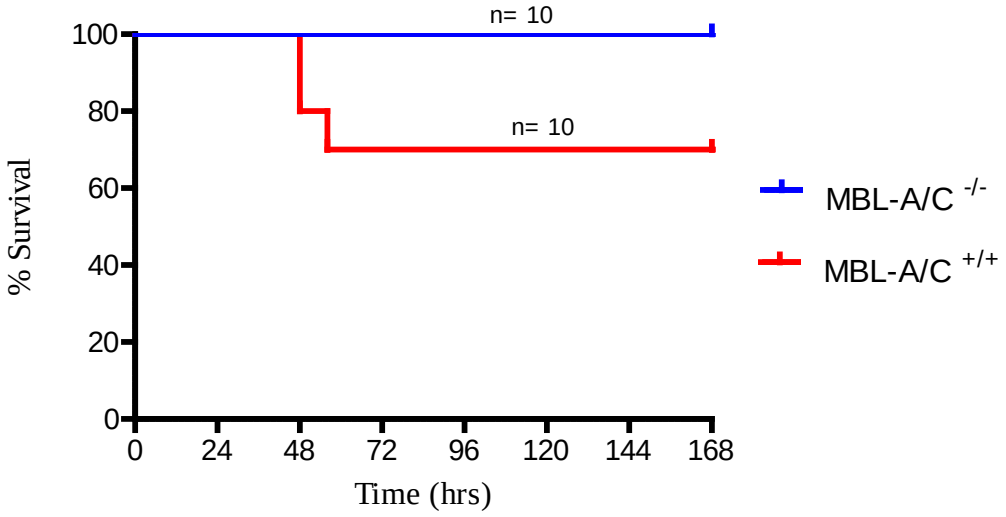


Figure 7.4: Survival of MBL-A/C$^{-/-}$ and MBL-A/C$^{+/+}$ mice after intranasal infection with 1x10^{6} CFU *Streptococcus pneumoniae* D39.

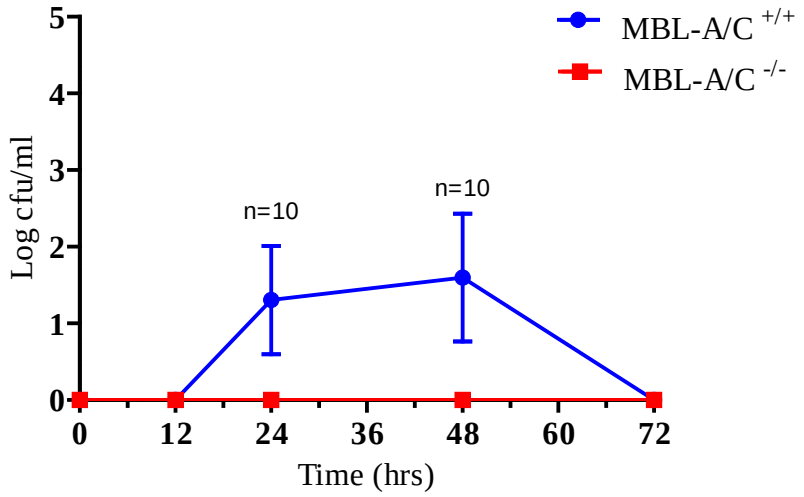
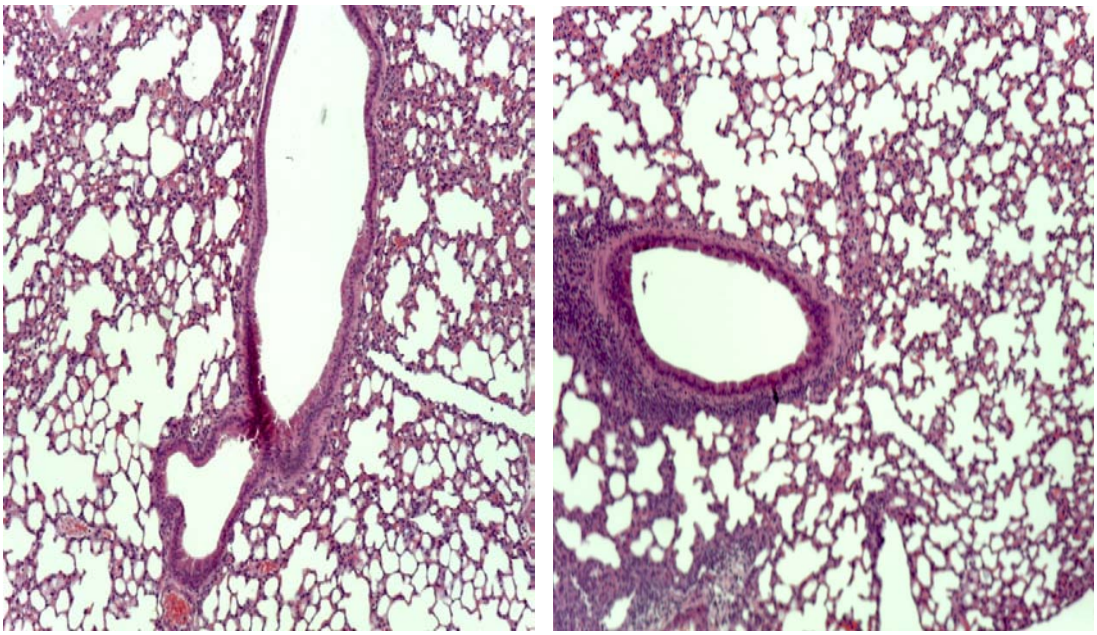


Figure 7.5: Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Results are means (±SEM) of 10 mice at each time point.

7.2. Histopathological slides of ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice lungs

Each image shown from histopathological slides in fig. 3.27 and 3.28 is representative of 4 sections from two different mice in each group sacrificed at pre-chosen time point post infection with *Streptococcus pneumoniae* D39. Additional representative images of histopathological slides from lung sections of both ficolin $A^{+/+}$ and ficolin $A^{-/-}$ mice after 48 hours of infection with *S. pneumoniae* D39 are shown here as examples (Fig 7.6 & 7.7).

A. B.



C. D.

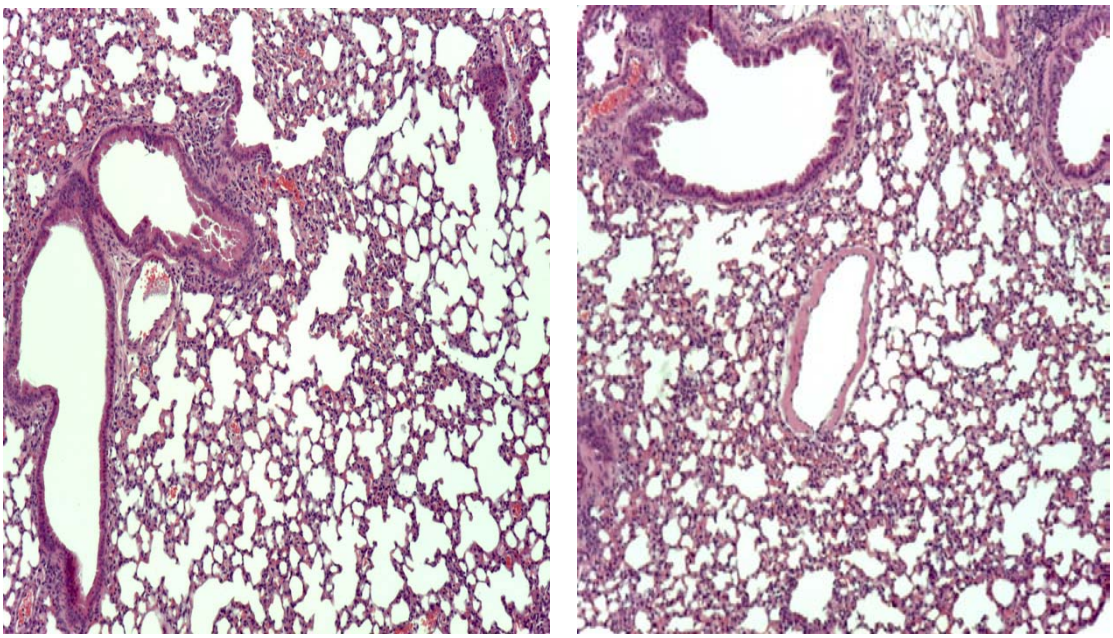
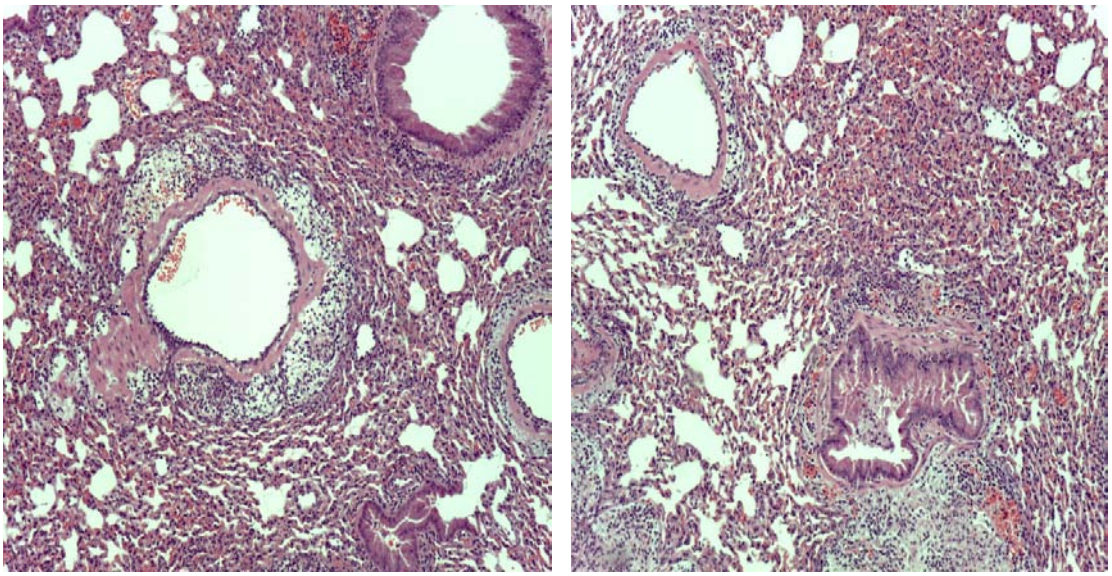


Figure 7.6: Additional histopathological slides from each section of ficolin A $^{+/+}$ mice lungs sacrificed after 48 hours of infection with *Streptococcus pneumoniae* D39.

A. B.



C. D.

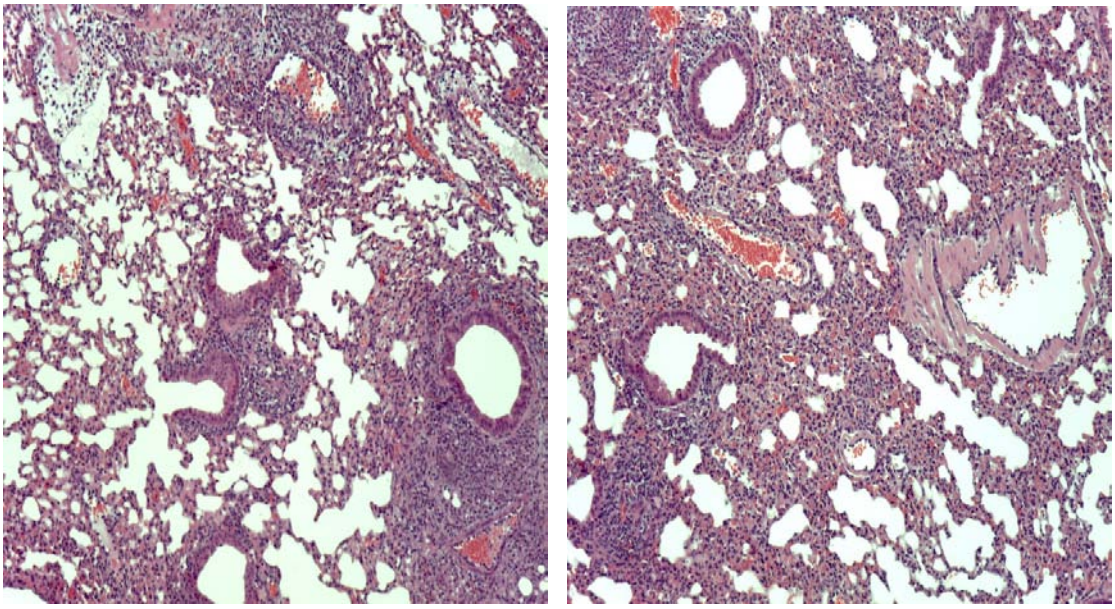


Figure 7.7: Additional histopathological slides from each section of ficolin $A^{-/-}$ mice lungs sacrificed after 48 hours of infection with *Streptococcus pneumoniae* D39.

8. Addendum 2

Manuscript draft submitted to PLoS Pathogens.

Manuscript in addendum 2(P. 181-199) is not available in electronic version of this thesis due to third party copyright restrictions. The full version can be consulted at the University of Leicester Library.

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