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MASP-2, the effector enzyme of the lectin pathway of complement activation, modulates the immune response in models of intranasal *S. pneumoniae* infection and experimental poly-microbial peritonitis.

Thesis submitted for the degree of
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by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (MASP-2, the effector enzyme of the lectin pathway of complement activation modulates the immune response in models of intranasal *S. pneumoniae* infection and experimental poly-microbial peritonitis) is based on work conducted by the author at the university of Leicester mainly during the period between February 2006 and February 2009.

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: M. Dobó

Date 17/3/09

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Abstract

MASP-2, the effector enzyme of the lectin pathway of complement activation modulates the immune response in models of intranasal *S. pneumoniae* infection and experimental poly-microbial peritonitis.

Mohammed Youssif Ali

The aim of this project was to investigate the role of lectin pathway of complement activation in the innate immune defence against *S. pneumoniae* infection and poly-microbial septic peritonitis. In this study the only available model of total lectin pathway deficiency was used, a gene targeted mouse line deficient of the lectin pathway effector enzyme MASP-2.

MASP-2 deficiency increases the susceptibility of mice to *S. pneumoniae* infection and MASP-2 deficient mice showed a significantly higher bacterial load in blood and in lung tissues after intra nasal challenge with *S. pneumoniae* when compared to their wild type littermates. The MASP-2 deficient mice showed also a significantly higher rate of mortality when compared to the control wild type littermates after *S. pneumoniae* infection. The failure of the MASP-2 deficient mice to clear the infection is due to an impaired C3 deposition on the surface of *S. pneumoniae* and hence impaired opsonophagocytosis. The delayed inflammatory response of MASP-2 deficient mice may be also another factor that results in their inability to clear the infection.

In the CLP model of poly-microbial peritonitis, the MASP-2 deficient mice showed no significantly increased in mortality after CLP when compared to their MASP-2 sufficient littermates. Bacterial clearance however was significantly reduced in peritoneal lavage of MASP-2 deficient mice. Significant differences in the cytokine expression profiles between MASP-2 deficient and MASP-2 sufficient animals was also observed with TNF-α and IL-1β expression being significantly reduced in MASP-2 deficient animals following CLP. We conclude that MASP-2 deficiency compromises bacterial clearance, but limits the inflammatory response to septic peritonitis considerably, thus leading to a relative reduction of the inflammation driven mortality during septic shock.

The finding that the deficiency of MASP-2 may lead to a reduced inflammatory response during sepsis in CLP model and to the protection of mice from the lethal effect of a TNF-α driven severe inflammatory response has prompted the idea to generate antibodies against human and murine MASP-2 that could deplete MASP-2 and transiently inhibit lectin pathway functional activity. These antibodies could be used as therapeutic intervention during sepsis and septic shock. In addition, these antibodies may also serve as therapeutic agents to limit lectin pathway mediated ischaemia/reperfusion injury following myocardial infarction and other forms of ischemic diseases.

Abbreviations

APC	activated protein C
APS	Ammonium persulphate
AT	Anti-thrombin
BALF	Bronchoalveolar lavage
BBS	Barbital buffer saline
BSA	Bovine serum albumin
C1-INH	C1-inhibitor
C4bp	C4 binding protein
C5aR	C5a receptors
CCP	Complement control protein
cDNA	Complementary DNA
CFU	Colony forming unit
CHO	Chinese hamster ovary
CIAP	Calf intestinal alkaline phosphatase
CLP	Cecal ligation and puncture
CPS	Capsular polysaccharides
CR	Complement receptor
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
CVF	Cobra venom factor
CWPS	Cell wall peptidoglycans and cell wall polysaccharides
DAF	Decay-accelerating factor
DEPC	Diethyl pyrocarbonate
DIC	Disseminated intravascular coagulopathy

EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth faxtor
ELISA	Enzyme Linked Immunosorbent Assay
HAS	Human serum albumin
Hib	*Haemophilus influenzae* type b
HRP	horse raddish peroxidase
HSA	Human serum albumin
HUS	Hemolytic-uremic syndrome
IL	Interleukin
LPS	Lipopolysaccharides
mAbs	Monolconal antibodies
MAC	Membrane attack complex
MAp19	Mannan associated protein of 19 KD
MASP	Mannan associated serine protease
MBL	Mannan binding lectin
MCP	Membrane cofactor protein
MIP-2	Macrophage inflammatory protein 2
MLST	Multi-locus sequence typing
OD	Optical density
PCV	Pneumococcal conjugate vaccines
PEG1500	Poly ethylene glycol 1500
PPV	Pneumococcal polysaccharide vaccines
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
ROS	Reactive oxygen species
SAP	Serum amyloid protein

SDS-PAGE	SDS-poly-acrylamide gel electrophoresis
SLE	Systemic lupus erythematosis
TBS	Tris buffered saline
TD	T cell-dependant
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TFPI	Tissue factor pathway inhibitor
TNF	Tumor necrosis factor

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Chapter 1

Introduction

1. Introduction

1.1 Innate and adaptive immunity

The main role of the immune system is to protect the body against microbial infection to eliminate debris and to maintain the body's integrity. The immune system is divided into two major branches; the innate immune system and the adaptive immune system. The innate immune response comprises an array of nonspecific and specific defense mechanisms which can respond to all types of invading organisms and is readily available upon the first stages of infection. This system responds to antigens and microbial pathogens with fast kinetics, but lacks memory. Although the adaptive immune system launches attacks specific to an invading pathogen, it requires some time to elicit its custom-made response. The adaptive system "remembers" the encountered antigens and responds rapidly and efficiently the next time that antigen is presented, yet significantly slower than the innate system when confronted with pathogens against which no previous adaptive immune response was established (Borghesi and Milcarek, 2007). The adaptive immune system utilises antigen specific cellular receptors and humoral immunoglobulins that are generated by mechanisms relying on clonal expansion of subsets of T and B lymphocytes bearing these antigen specific receptors. In contrast, the innate immune response utilises a relatively small number of germ line–encoded receptors that detect a limited set of conserved antigens (Hoffmann *et al.*, 1999).

1.2 Complement system

The complement system is a major component of the innate immune defence and is composed of more than 32 components in plasma or on the cell surface

including positive and negative regulators (Makrides, 1998). Activation of the complement system encompasses a series of carefully regulated initiation, amplification and deactivation steps to adapt to specific physiological requirements (Parker, 1992; Liszewski *et al.*, 1996).

Complement activation acts through a cascade of sequential activation steps. Many complement components are zymogens that are present in plasma in their proenzymatic form and may be cleaved during the activation step and thereby converted into their enzymatically active state to form part of a multimolecular enzyme complex that cleaves and activates other complement components further downstream of the activation cascade.

Complement is activated by three pathways (Fig. 1.1) the classical pathway, the alternative pathway and the lectin pathway (Schwaeble *et al.*, 2002) all of which lead to the activation of C3, the key component of complement, and the subsequent formation of the cytolytic membrane attack complex (MAC). Following complement activation, the biologically active peptides C3a and C5a (released as a result of activation of C3 and C5) elicit a number of proinflammatory effects, such as chemotaxis of leukocytes, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and increase of vascular permeability (Köhl, 2001). In response to complement activation products, the inflammatory response is further amplified by subsequent generation of toxic oxygen radicals, induction of synthesis and release of arachidonic acid metabolites and cytokines (Kirschfink and Mollnes, 2003).

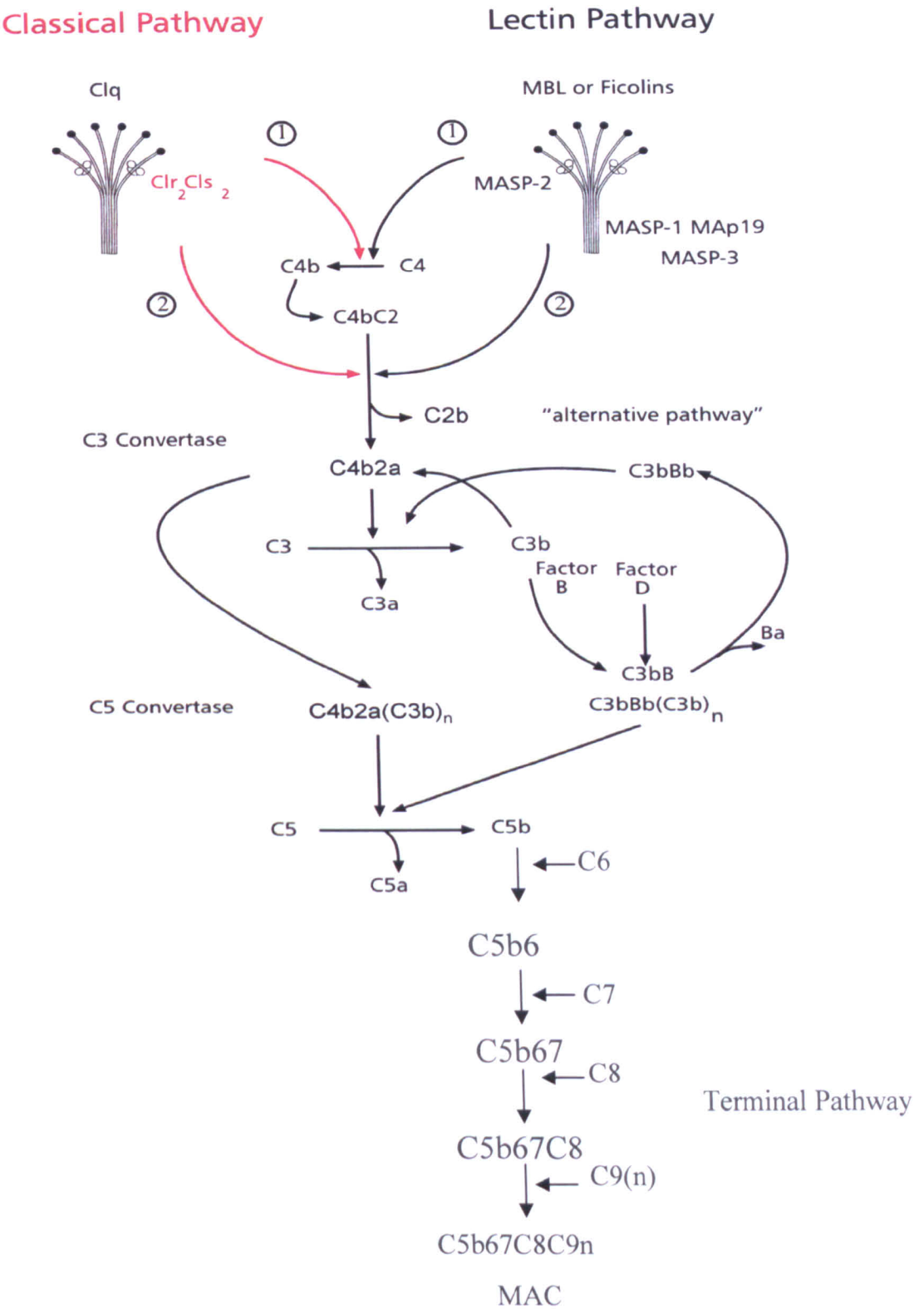


Figure 1.1: A simplified diagram of the three complement activation pathways, the classical, the lectin pathway and the alternative pathway (kindly provided by Professor W. Schwaeble).

1.2.1 The classical pathway

The first component of the complement C1 is composed of a multimolecular initiation complex that triggers the classical pathway of complement activation. There is strong evidence that C1 is a major player in both the innate and the adaptive antimicrobial host defence and for the role of C1 in the maintenance of immune tolerance (Arlaud *et al.*, 2002).

The approximately 790 kDa C1 complex is consists of a recognition protein, C1q and a homodimers of C1r and C1s zymogens to form C1q:$C1r_2$:$C1s_2$ complex (Fig. 1.2). C1q is composed of six identical subunits joined together through their collagen like stalks that end in globular heads. Each subunit is consists of three identical polypeptide chains (Arlaud *et al.*, 2002). The classical pathway activation is initiated either by binding of C1q to a bacterial surface component or indirectly by binding to immune complexes (Boes *et al.*, 1998). Binding of C1q to complement activators leads to a conformational change in the collagenous region of C1q which in turn leads to the autoactivation of C1r, which subsequently activates C1s that translates C1q activation into cleavage of C4 and C2, followed by the formation of the C3 convertase leading to C3 activation (Arlaud *et al.*, 2002).

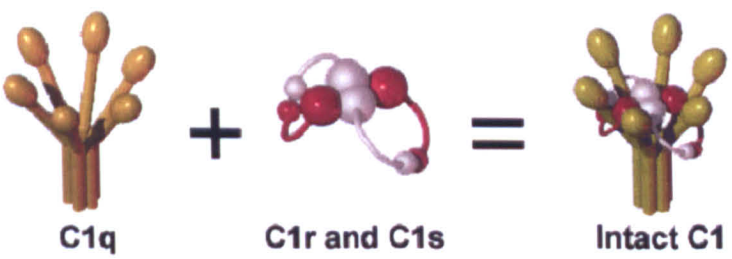


Figure 1.2: Structure of the first component of the classical pathway of complement showing binding homodimers of C1r and C1s with C1q molecule (modified from Arlaud *et al.*, 2002)

1.2.2 The lectin Pathway

The lectin pathway of complement activation is initiated by carbohydrate recognition molecules i.e. mannose binding lectin (MBL) and ficolins.

A single MBL monomer is composed of three identical polypeptide chains, each consisting of a short N-terminal cysteine-rich region, a collagen-like region, a neck region and carbohydrate recognition domain (CRD) (Fig. 1.3). MBL is present in serum as a mixture of higher grade oligomers (trimers, tetramers and hexamers) and the biological activity of MBL increases with the number of oligomers (Wallis, 2005).

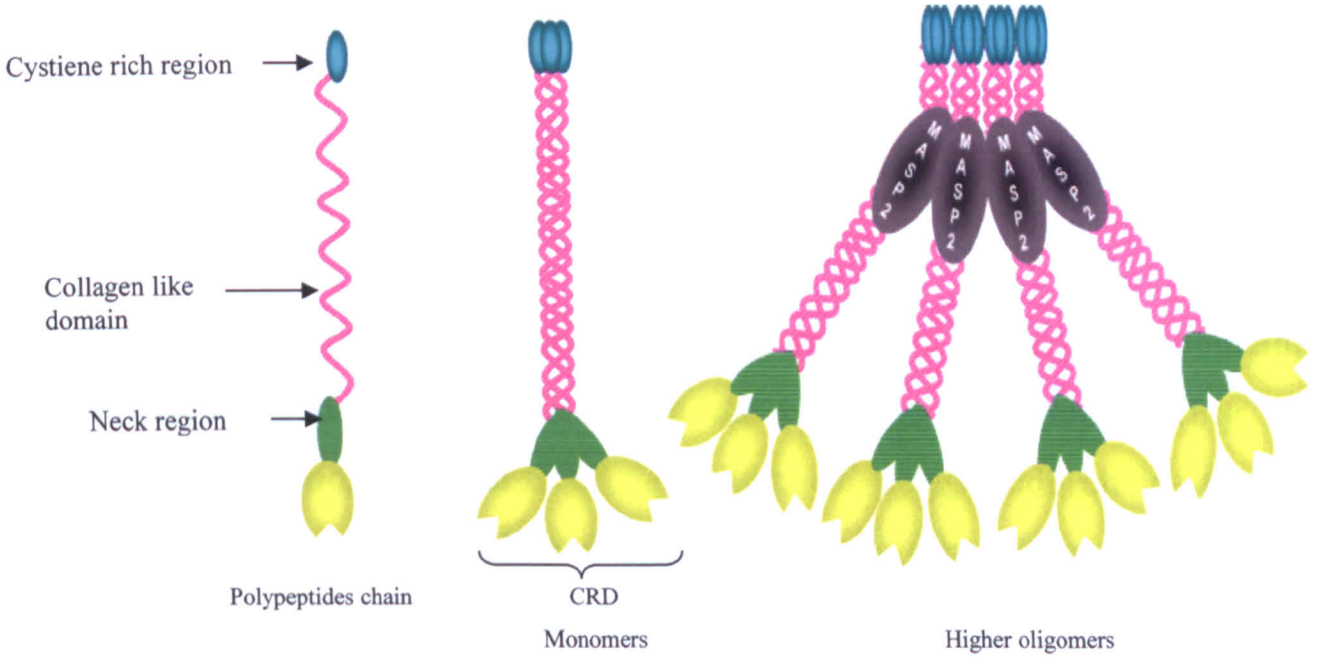


Figure 1.3: Structure of MBL. Each MBL monomer is composed of three identical polypeptide chains which binds together and give the high oligomer structure (Dommett *et al.*, 2006).

Ficolins are another group of carbohydrate recognition molecules which activate the lectin pathway and structurally resemble MBL. Two of the human ficolins (L-ficolin and H-ficolin) are serum proteins that activate the lectin pathway by direct recognition of the acetylated sugar moties in the surface of pathogens. The third type of human ficolins that may activate the lectin pathway is M-ficolin which is found on the surface of neutrophil and monocyte where it may act as a phagocytic receptor (Matsushita and Fujita, 2001). In addition, M-ficolin forms complexes with MASP-2 and MASP-1 and activates the lectin pathway of complement on N-acetyl glucosamine coated microtitre plates. M-ficolin was also shown to bind to certain types of bacteria such as *S. aureus* (Liu *et al.*, 2005). Unlike in man, the mouse has only two types of ficolins; ficolin A (which is found in the serum and resembles L-ficolin in humans) and ficolin B (which is found in bone marrow cells) (Runza *et al.*, 2008).

Ficolin polypeptides are composed of cysteine rich N-terminal domain, a collagen like domain, a neck region and fibrinogen like domain (Fig. 1.4). A ficolin monomer consists of three identical polypeptide chains that join together in the collagen like domain. The homotrimeric monomers bind to each other through the N-terminal domain to form oligomers with a bouquet like structure. Like MBL, ficolins binds to MASP-2 and catalyse the activation of the lectin pathway of complement via cleavage of C4 and C4b bound C2 with subsequent formation of C3 convertase (Runza *et al.*, 2008).

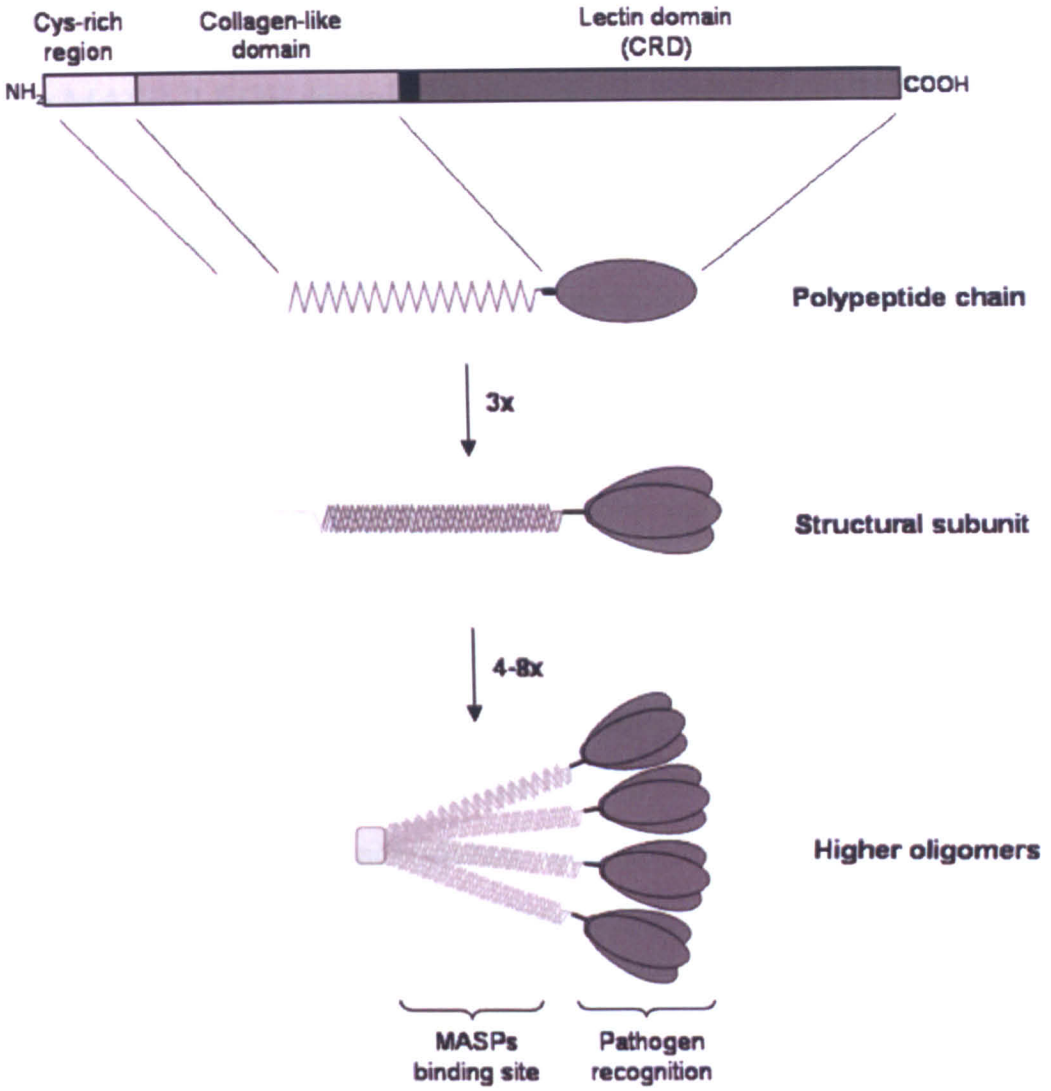


Figure 1.4: Schematic representation of ficolin structure, showing the polypeptide chain subunit and the higher oligomeric forms composed of multimers of the homotrimers subunit (Runza *et al.*, 2008).

Both MBL and ficolins are bound to MBL associated serine proteases (MASP-1, MASP-2 and MASP-3) and an enzymatically inactive protein, the 19 kDa MBL-associated protein MAp19 (Schwaeble *et al.*, 2002). MASPs molecules consist of two CUB domains (CUBI-CUBII), an epidermal growth factor (EGF) like domain, two complement control protein domains (CCPI-CCPII) and a C-terminal serine protease domain while MAp19 is composed of

the two N- terminal domains of MASP-2 i.e. the MASP-2 CUBI domain and the MASP-2 EGF domain (Stover *et al.*, 1999).

Previous studies aimed to determine the enzymatic activities of MASP-1, MASP-2, and MASP-3 and demonstrated convincingly that MASP-2 can cleave C4 and C4b bound C2 (Thiel *et al.*, 1997) whereas, MASP-1 can cleave C4b bound C2 but has no enzymatic activity towards C4 (Chen and Wallis, 2004). MASP-3 appears to be neither involved in C4 nor in C2 cleavage, indicating that MASP-2 is the key enzyme to initiate activation of the lectin pathway of complement. Unlike the classical pathway, the lectin pathway can be activated in the absence of immune complexes where the lectin pathway pattern recognition molecules MBL and/or ficolins can recognise the carbohydrate or N-acetylated carbohydrate structures on the surface of pathogens leading to the activation MASP-2 which in turn cleaves C4 and C4b bound C2 to generate the C3 convertase C4b2a (Wallis *et al.*, 2007).

1.2.3 The alternative pathway

Factor B, factor D and Properdin (factor P) are specific components of the alternative pathway of complement activation. Unlike the classical and the lectin pathway (which are initiated via specific recognition subcomponents such as C1q or MBL/ficolins respectively) the alternative pathway is initiated through a spontaneous steady state of hydrolysis of C3 to form $C3(H_2O)$ which in turn binds to factor B to form a $C3(H_2O)B$ zymogen complex. In this complex, factor B is cleaved by factor D releasing a Ba fragment while Bb remains attached to the complex formed with $C3(H_2O)$. The newly formed complex $C3(H_2O)$ Bb forms a C3 convertase enzyme and cleaves C3 into C3a and C3b. Once C3b is generated, it will bind to the surface of pathogens where

it can bind to another molecule of factor B and form a new alternative pathway C3 convertase C3bBb (Thurman and Holers, 2006). The new view point is that properdin can bind to the surface of pathogens and initiate the alternative pathway activation by stabilisation of C3bBb convertase (Kemper and Hourcard, 2008). The alternative pathway can also act as an amplification loop where C3b generated by either the classical or the lectin pathway binds to factor B, which is subsequently cleaved by factor D (Schwaeble and Reid, 1999).

1.2.4 Membrane attack complex (MAC)

C3 convertases produced either of the three complement activation pathways convert C3 into C3b and C3a. Accumulation of C3b in close proximity of either C4b2a or C3bBb complexes switches the substrate specificity of these C3 convertases from C3 to C5 forming the C5 convertase complexes C4b2a(C3b)n or C3bBb(C3b)n respectively which cleaves C5 into C5b and C5a. C5b then binds to cell surface and reacts with C6, C7 and C8 to form a C5b-8 complex that leads to polymerisation of C9 which inserts into the lipid bilayer of the bacterial cell membrane and initiate the membrane attack complex (MAC) formation leading to cell lysis (Podack *et al.*, 1982).

1.3 The biological effects of complement activation

Complement activation leads to a multitude of biological activities including opsonisation, initiation of a proinflammatory response, immune complex clearance and direct killing of cells via the membrane attack complex. Opsonisation of pathogens is mediated by the major opsonin C3b or iC3b (the haemolytically inactive cleavage product of C3b) and C4b to less extent. C3b coats the surface of microorganisms and enhances their phagocytosis by

leukocyte via binding to complement receptor type 1(CR1) and type 3 (CR3) (Aoyagi *et al.*, 2005). L-ficolin and MBL has been reported to initiate phagocytosis directly by binding to pathogens and enhances phagocytosis by binding to collectin receptors on the surface of the phagocytes (Jack *et al.*, 2001; Aoyagi *et al.*, 2005). In other instances complement can mediate direct killing of bacteria, especially Gram negative bacteria via the formation of the membrane attack complex which form pores in the cell wall leading to destruction of cytoplasmic membrane and cell lysis (Nauta *et al.*, 2004). During complement activation, proinflammatory cleavage products anaphylatoxins such as C5a and C3a are released. The release of anaphylatoxins increases the vascular permeability and formation of inflammatory exudates. These inflammatory exudates enhance the recruitment of inflammatory mediators and inflammatory cells to the site of injury and efficient elimination of invading pathogens or other inflammatory causing factors. Increased vascular permeability leads to extravasation of leukocyte to the site of inflammation, which helps in clearing invading pathogens. Anaphylatoxins, especially C5a, act as potent chemotactic factors that stimulate leukocyte migration. In addition, C5a was found to increase the synthesis of other chemotactic agents like echosanoides and chemokines. Schindler *et al.* reported that C5a stimulates the expression of interleukin-1 (IL-1) and tumor necrosis factor (TNF) genes (Schindler *et al.*, 1990). In addition, both C5a and C3a stimulate the production of monocyte chemotactant protein-1 and macrophage inflammatory protein-2 from mouse endothelial cells (Laudes *et al.*, 2002). C5a can also play a role in stimulation and release of IL-8 from human bronchial epithelial cells which enhance leukocyte infiltration to the site of infection (Hsu *et al.*, 1999).

Complement also plays a major role in the clearance of apoptotic and necrotic cells in addition to immune complexes. The globular head of C1q binds to the surface of apoptotic cells and facilitates the uptake of this complex by macrophages (Taylor *et al.*, 2000). In addition, opsonisation of apoptotic cells with iC3b leads to recognition of these cells by CR3 and CR4 receptors on the surface of phagocytes with subsequent engulfment of these cells (Mevorach *et al.*, 1998).

Complement also inhibits the precipitation of immune complexes and enhances its solubility by binding with C1q, C3b and C4b. This binding inhibits further increase in the size of the immune complex. These complexes bind to CR1 on the surface of erythrocytes which transfer them into the liver and the spleen where they are cleared from the circulation by the resident macrophages (Manderson *et al.*, 2004).

1.4 Control of Complement system activation

Excessive complement activation leads to damage of host tissues, so there are several complement regulatory proteins that restrict the spontaneous activation of complement. These regulators include fluid phase regulators and membrane bound regulators (Kirschfink and Mollens, 2003).

1.4.1 Fluid phase regulators

Complement C1 inhibitor (C1-INH) is a serine protease inhibitor that binds to C1 complex and prevents spontaneous activation of the proenzymes C1r and C1s via the formation of C1-INH-C1r-C1s-C1-INH complex (Harpel and Cooper, 1975). Also C1-INH was found to remove activated C1qrs complexes from surfaces and block further activation (Chen and Boackle, 1998).

C4 binding protein (C4bp) is a major regulator of the complement system. C4bp binds with C4b and inhibits its binding with C2 which prevents the formation of C3 convertase. Also C4bp acts as a cofactor in the factor I mediated conversion of C4b to iC4b, C4e and C4d (Jurianz *et al.*, 1999).

Factor H is the main fluid phase regulator of the alternative pathway. It accelerates the decay of the alternative pathway C3 convertase (C3bBb) by binding to C3b and removing it from the complex (C3bBb). Factor H acts as a cofactor in the factor I mediated conversion of C3b to iC3b, C3c and C3dg (Turnberg and Botto, 2003).

Clusterin and S protein are regulators for the terminal activation cascade of complement. They bind to C5b-7 complex and prevent the insertion of C8 and C9 leading to the inhibition of the MAC formation (Jenne and Tschopp, 1989).

1.4.2 Membrane bound regulators

Complement activation is also controlled by at least four characterised membrane bound proteins or receptors; complement receptor 1 (CR1), protectin (CD59), membrane cofactor protein (MCP) and decay accelerating factor (DAF).

CR1 is expressed by many cells including erythrocytes and lymphocytes. CR1 has a decay accelerating activity towards C3 and C5 convertases and serves as a cofactor for factor I which degrades C3b and C4b. MCP binds to C3b and acts as a cofactor for factor I and facilitates the inactivation of C3b (Whaley and Schwaeble, 1997). CD59 is another regulator of the MAC that binds to

C8 and C9, and subsequently inhibits binding to C5b-7 and formation of MAC (Rollins *et al.*, 1991).

DAF is attached to cell membranes and dissociates C3 and C5 convertases (Medof *et al.*, 1984).

1.5 Complement deficiencies

An intact complement system plays a major role in protection against infectious and non infectious diseases. Complement deficiencies are associated with recurrent invasive bacterial infections and development of autoimmune diseases (Mollnes *et al.*, 2007).

1.5.1 Classical and alternative pathway deficiencies

Activation of the classical pathway via C1q leads to the release of C3b with subsequent opsonophagocytosis of invading pathogens and removal of immune complexes from the circulation. A deficiency in any component of the classical pathway is associated with high incidence of immunological diseases and recurrent bacterial infections. C1q and C1s deficiency is associated with a high risk of systemic lupus erythematosis (SLE) development (Tom *et al.*, 2007; Amano *et al.*, 2008). The reason for the development of SLE in patients with such deficiencies is mainly due to the impaired function of the complement system in clearing immune complexes and removing apoptotic cells from the circulation (Carroll, 2004).

C2 deficiency was found as a predisposing factor for atherosclerosis (Jönsson *et al.*, 2005).

It is well established that complement deficiencies are highly associated with a high susceptibility for recurrent bacterial infections. C1q deficiency increases the risk of recurrent invasive bacterial infections (Brown *et al.*, 2002) and polymicrobial peritonitis (Celik *et al.*, 2001).

Children deficient in C2 are susceptible to recurrent pneumococcal infections (Jönsson *et al.*, 2005). C3 and factor B deficiencies significantly increase the risk of *S. pneumoniae* and *Pseudomona aeruginosa* infection (Brown *et al.*, 2002; Muller-Ortiz *et al.*, 2004). Deficiencies of factor H, Factor I (inherited or acquired) are associated with a typical hemolytic-uremic syndrome (HUS) which results in thrombotic microangiopathy associated with diarrhea (Thurman and Holers, 2006).

In a mouse model of properdin deficiency, it was found that the severity of polymicrobial peritonitis was significantly increased in deficient mice when compared to their wild type littermates (Stover *et al.*, 2008).

1.5.2 Lectin pathway deficiency

Lectin pathway activation is mediated by mannan binding lectin (MBL) and ficolins (L-Ficolin, H-ficolin and M-ficolin). Deficiency in any of these mediators is associated with high risk of recurrent bacterial infections and non bacterial disorders.

MBL deficiencies are due to three different mutations in exon 1 of the *mbl2* gene located on human chromosome 10. These mutations cause single amino acid substitution at codons 52, 54 and 57. A single point mutation at codon 52 leads to the substitution of glycine with aspartic acid at position 34 of the mature protein. Another point mutation at codon 54 leads to substitution of glycine with glutamic acid at position 37 of the mature protein. Those two mutations disrupt the collagenous region and alter the interchain disulfide

bonds within the N-terminal cross-linking region (Wallis and Cheng, 1999). The third single point mutation at codon 57 is due to substitution of cysteine with argenine at position 32 of the mature protein. This mutation leads to the disruption of the oligomerisation of polypeptide chains of the MBL (Wallis *et al.*, 2004).

These inherited MBL deficiencies are common and most individuals with these deficiencies are healthy and show no increase or predispositions for recurrent infections or morbidity when compared with MBL sufficient control populations (Dahl *et al.*, 2004). Under certain conditions, MBL deficiencies were found to be associated with an increased susceptibility for severe bacterial infections in children and adults especially when associated with other immunodeficiency conditions such as HIV infection (Turner, 2003) or post chemotherapy (Peterslund *et al.*, 2001). MBL deficiency was also associated with high risk of developing arterial thrombosis and cardiovascular disorders (Ohlenschlaeger *et al.*, 2004). Patients with MBL deficiencies are more prone to develop rheumatoid arthritis and persistent inflammatory conditions (Garred *et al.*, 2000). MBL deficiency was also associated with high risk for development of sepsis in pediatric patient (Fidler *et al.*, 2004).

For L-ficolin, another recognition component of the lectin pathway, it was found that low plasma levels play a significant role in recurrent respiratory tract infections in children (Atkinson *et al.*, 2004).

Although MASP-2 is the key enzyme that drives the lectin pathway, its role in the protection against infection is still incompletely understood. My thesis aims to shed light into the role of MASP-2 in fighting microbial infections.

1.6 MASPs of the Lectin pathway

1.6.1 Functional activity of MASPs

The mannan binding lectin associated serine proteases (MASPs) of the lectin pathway activation are three different serine proteases i.e. MASP-1, MASP-2 and MASP-3 (Schwaeble *et al.*, 2002). In addition, an enzymatically inactive truncated form of MASP-2 of 19 kDa called MAp19 was also described (Stover *et al.*, 1999). MASP-1 was the first MASPs described in 1992 by the pioneering work of Matsushita and Fujita and it was described as a C1s like serine protease associated with MBL. In 1997, a second mannan associated serine protease therefore named MASP-2 was described by Thiel *et al.* It was anticipated that MASP-1 and MASP-2 are zymogens complexing with MBL that activate the lectin pathway in a similar way as C1r first activates C1s which then translates this activation to cleave C4 and C4b-bound C2 leading to subsequent formation of the classical pathway C3 convertase (C4b2a). However, experimental evidence demonstrated that recombinant MASP-2 complexed with recombinant MBL is sufficient to drive the lectin pathway activation and generate C3 convertase. This result indicated that MASP-2 is the major serine protease responsible for the activation of the lectin pathway (Vorup-Jensen *et al.*, 2000).

Finally, the third member of this lectin pathway specific serine proteases family is MASP-3. MASP-3 was first described by Dahl *et al* in 2001 and it was found that MASP-3 may down regulate the proteolytic activity of MASP-2 towards C4 and C4b bound C2 by competition of MASP-3 and MASP-2 for the binding sites of the lectin pathway recognition molecules.

MAp19 is a truncated gene product of the *MASP2* gene which is enzymatically inactive 19 kDa protein (Stover *et al.*, 1999). Recent work also indicated that MAp-19 may negatively regulate lectin pathway activation by

competing with MASP-2 for the binding sites on lectin pathway recognition molecules (Iwaki *et al.*, 2006).

1.6.2 Genomic localisation of MASPs

MASP-1 and MASP-3 are alternative splicing and polyadenylation products of a single structural gene (*MASP1/3*) (Dahl *et al.*, 2001). This gene was mapped to the region 3q27-28 in human chromosome 3 (Takada *et al.*, 1995). The (*MASP1/3*) gene consists of 17 exons. Exons 1-10 encode the N-terminal domains of MASP-1 and MASP-3. These 10 exons are followed by the exon encoding for the linker region and the serine protease domain of MASP-3. Further down stream there are another 6 exons that encodes the linker region and the serine protease domain of MASP-1 (Fig. 1.5). Northern blot analysis and in situ hybridisation studies indicated that MASP-1 is only expressed in liver (Schwaeble *et al.*, 2002) while, MASP-3 is expressed in liver and in other tissues including spleen, lung, small intestine, brain and thymus (Lynch *et al.*, 2005).

MASP-2 and MAp19 are also products of a single structural gene and generated by alternative splicing of a single primary RNA transcript (Stover *et al.*, 1999). The human *MASP2/MAp19* gene is located on chromosome 1p36.2–3 and consists of 12 exons (Fig. 1.5). MASP-2 mRNA is encoded by 11 of these exons. The N- terminal domains of MASP-2 are encoded by the first 10 exons while the linker region and the serine protease domain are encoded by one exon only. Alternative splicing of Exon 5 of *MASP2/MAp19* gene allows either the generation of MAp19 or MASP-2 (Stover *et al.*, 2001). MASP-2 and MAp19 are exclusively synthesised in liver (Schwaeble *et al.*, 2002).

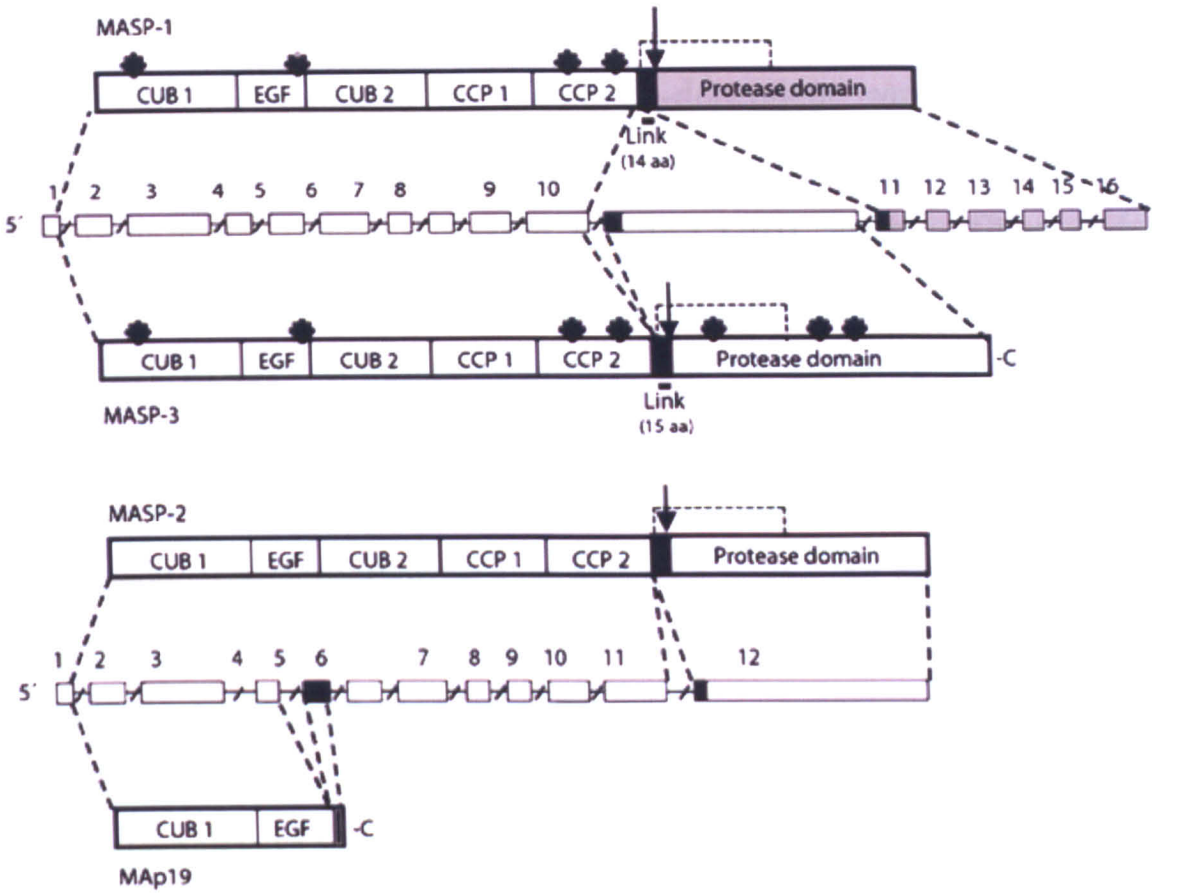


Figure 1.5: Genomic localisation and protein structure of MASPs (Sorensen *et al.*, 2005). Glycosilation sites are marked by a star (*)

1.6.3 MBL/MASPs complex

The binding between MBL and MASPs was described by several studies. Analysis of MBL/MASPs complex from human serum revealed that MASP-1 and MAp19 have high binding affinity with trimeric MBL oligomers. Whereas, MASP-2 and MASP-3 mainly associated with tetrameric MBL oligomers (Dahl *et al.*, 2001). In contrast to this finding, Teillet *et al* postulated in 2005 that MASPs and MAp19 have the same affinity towards trimeric and tetrameric MBL oligomers. This observation was confirmed by another study showed that MASPs can bind efficiently with a dimer form of rat MBL-A (Chen and Wallis, 2001).

MASP-1, MASP-2, MASP-3 and MAp19 form homodimers through binding via CUBI-EGF domains in a calcium-dependent way. The MASPs and MAp19 binding to MBL or ficolins occurs through a CUBI-EGF moiety and the strength of binding increased by the CUBII domain (Chen and Wallis, 2001).

The MBL/MASPs complex resembles the C1 complex in its structure but there are two major differences that discriminate the lectin pathway from the classical pathway. Firstly, activation of the classical pathway depends mainly upon sequential activation of C1r and C1s where the latter generates the C3 convertase (C4b2a). In contrast, the lectin pathway can be initiated by one enzyme only (MASP-2) to generate the C3 convertase. Secondly, the C1q complex is sensitive to high salt concentrations which dissociate the complex while the MBL/MASPs complex stays intact at high salt concentration such as 500 mM NaCl.

1.7 Overview of *S .pneumoniae* infection and Poly-microbial peritonitis:

1.7.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram-positive bacterium that grows as diplococci and forms short chains. *S. pneumoniae* infection is the major cause of pneumonia, otitis media, septicemia and meningitis in the UK and it causes substantial morbidity and mortality, especially in young children and elderly patients > 65 years old (Miller *et al.*, 2000; Kyaw *et al.*, 2003).

Historically, in 1900 *S. pneumoniae* was the major cause for children's death in the USA killing not less than 47 of every 1000 children every year. The increase in living standards and the improvement of nutrition limited the number of deaths even before the prevalence of effective antibiotic treatment (Mulholland, 2007). However, in developing countries in Africa and Asia, pneumonia is still a major cause of the high mortality seen in children and it accounts for approximately 25% of deaths in children under the age of five years and with more than 1.2 million infant deaths every year (Kadioglu *et al.*, 2008). This high rate of mortality is associated with very poor living standards, malnutrition and ineffective medical care. Old people are also subjected to the risk of *S. pneumoniae* infection with a mortality rate approximately 20% in patients older than 65 years increasing to 40% in patient older than 85 years (Butler and Schuchat, 1999).

Pneumococci colonise the nasopharynx of young children and the incidence of colonisation may reach up to 40% (Long, 2005). Several studies have reported a link between a high risk of acute and recurrent otitis media and the rate of colonisation in the nasopharynx (Dhooge *et al.*, 1999; Syrjanen *et al.*, 2001; Marchisio *et al.*, 2003). Certain diseases like diabetes, asplenia, chronic lung

diseases and AIDS can increase the risk of pneumococcal infections (Kyaw *et al.*, 2003, 2005).

The incidence of pneumococcal infection was significantly reduced in the UK following the introduction of pneumococcal vaccines (Kyaw *et al.*, 2003; McChlery *et al.*, 2005) but there is still a considerable number of cases of invasive pneumococcal disease (IPD), especially in winter months, even with the use of new generations of highly efficient antibiotics and the introduction of pneumococcal polysaccharides vaccines (Kyaw *et al.*, 2003).

The incidence of IPD in England and Wales is 8.6 cases per 100,000 of the population, but the incidence between children less than one year of age and the elderly (>65 years) approaches 30 per 100,000 (Miller *et al.*, 2000; Sleeman *et al.*, 2001). In Scotland, the incidence of IPD is around 11 cases per 100,000 of the population but the rate of incidence may reach 45 cases for children less than 1 year and 51 for people more than 65 years old (Kyaw *et al.*, 2003). Most IPD cases in the UK are associated with limited serogroups of *S. pneumoniae* (Table 1).

Country	**Serigraph/type**	**Reference**
England and Wales	14, 9, 6, 19, 23, 8, 1, 4, 18 and 7	George&Melegaro (2001)
Scotland	14, 8, 9V, 1, 3, 22F, 23F, 6B, 18C and 19F	McChlery *et al.* (2005)

Table 1.1: The most common serogroups/types of pneumococci causing invasive disease in the UK (Clarke, 2006).

1.7.1.1 Antibiotic resistance in *S. pneumoniae*

Penicillin treatment of *S. pneumoniae* was successfully introduced in 1960 but with time, penicillin resistant strains started to emerge causing life threatening invasive infectious diseases and more than this, some strains isolated from IPD were not only penicillin resistant but also macrolides and quinolones resistant (Baquero *et al.*, 1991). In the USA, 20% of the strains isolated from children were resistant to penicillin and 7% were resistant to ceftriaxone (Arditi *et al.*, 1998). In the UK, the situation is similar but the pneumococcus is still controlled by the use of antibiotics. However, more and more antibiotic resistant strains were recently isolated from IPD cases. In Scotland, the rate of penicillin resistant strains increased from 4.2% to 12% between 1992 and 1999 (Kyaw *et al.*, 2000). The rate of resistance to macrolides between different serotypes dramatically increased in the UK to reach 20% by 2001 (George and Melegaro, 2001). Worryingly, failure of vancomycin to treat some IPD cases is a clear indication for the emergence of vancomycin resistant strains. The emergence of a high rate of antibiotic resistance in *S. pneumoniae* may lead to an increase in the rate of IPD and so decrease the efficiency of the antibiotic treatment which may lead to an increase in the rate of mortality (Novak *et al.*, 1999).

1.7.1.2 Pneumococcal vaccines

Vaccination plays an important role in protection against *S. pneumoniae* infection and hence may increase survival, especially in pediatric cases. The vaccine strategy against two of the major causes of fatal infectious pneumonia i.e. *Haemophilus influenzae* type b (Hib) and *S. pneumoniae* has saved the life of up to 1 075 000 children every year (Madhi *et al.*, 2008).

1.7.1.2.1 Pneumococcal polysaccharides vaccines (PPV)

Pneumovax is currently the most available PPV. It is inexpensive and safe (Artz *et al.*, 2003). Pneumovax consists of polysaccharides of 23 different serotypes which cover up to 90% of the known different serotypes (George *et al.*, 1997; Kyaw *et al.*, 2000). Despite conflicting results concerning the efficacy of PPV in the prevention of *S. pneumoniae* infection, there is a solid evidence that PPV is effective in reducing IPD in adults and the elderly (Dear *et al.*, 2003) concluding that it gives 50-70 % protection in the elderly persons (Bulter and Schuchat, 1999). Unfortunately, the level of the protective antibodies produced by Pneumovax is decreasing over time leading to a decrease in the level of protection. In European studies, the levels of some specific antibodies have decreased to the pre-vaccination level after three years (Hedlund *et al.*, 2000). On the other hand, it was found that PPV is completely ineffective in children less than two years of age and at the same time it does not reduce the colonisation of the nasopharynx (Ledwith, 2001). One of the major drawbacks of the PPV vaccine is its T cell-independent behavior, where it activates mature B cells directly to produce antibodies in absence of T helper cells and so no cellular immunity is developed (Mond *et al.*, 1995).

1.7.1.2.2 Pneumococcal conjugate vaccines (PCV)

The capsular polysaccharides pneumococcal vaccines have poor antigenic characteristics and are not effective in protecting young children. The alternative vaccine for PPV is the pneumococcal conjugate vaccine (PCV), where the capsular polysaccharides are linked to a carrier protein. This type of vaccine is a T cell-dependent antigen (TD) that evokes an immune response

and results in immunological memory. TD antigens are recognised and internalised by B cells, phagocytic cells or dentritic cells where they are degraded into polypeptides that bind to class II MHC molecules and are then represented to helper T cells. Cytokines from T cells activate B cells to differentiate into antibody producing plasma cells and memory B cells that produce a strong immune response once re-exposed to the same antigen (Wood, 2001).

Prevenar is a PCV for seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) which are responsible for the majority of pneumococcal infections in young children. Prevenar vaccination significantly reduces the rate of IPD in children less than one year old (Black *et al.*, 2002). Infections with serotypes included in Prevenar were reduced by 90% in children less than 1 year (Clark *et al.*, 2006). In addition, a moderate protection against ear infection was also observed in children less than 3.5 years of age (Fireman *et al.*, 2003).

Prevenar vaccine reduces nasophyranyx colonisation and so decreases the rate of transmission of pneumococcus between individuals. In addition, it was found that Prevenar vaccination reduces the incidence of macrolides and penicillin resistance pneumococci as the majority of antibiotic-resistant pneumococci are covered by Prevenar (Whitney *et al.*, 2003). However, it is important to bear in mind that pneumococci are highly transformable. In a recent study (which looked at the genetic heterogeneity amongst 217 pneumococci isolates from invasive disease in children) 22 different serogroups/types were found (Clarke *et al.*, 2006b). These serotypes were further genotyped using multi-locus sequence typing (MLST) into 77 different sequence types. Although limited genetic heterogeneity was found amongst common serotypes, it highlights the possibility of an epidemiological shift in

serotypes distribution after the introduction of Prevenar has occurred (Meats *et al.*, 2003). The same phenomenon was observed for meningococci where the introduction of vaccines that contain only selected serotypes may have caused a capsule switch (Spratt and Greenwood, 2000; Jefferies *et al.*, 2004; Crook, 2006). Two different studies from the USA have already shown a shift in the epidemiology of pneumococcal disease and the emergence of new *S. pneumoniae* serotypes after the introduction of Prevenar (Cordeiro *et al.*, 2005; Pai *et al.*, 2005). Although the new emerging serotypes are not invasive and do not cause disease, they might select for a capsule switch and affect the efficacy of the present vaccines in the future (Clarke, 2006).

1.7.1.3 **Serotypes of *S. pneumoniae***

The serotyping technique (Quellung reaction) causes the *S. pneumoniae* capsule to swell when its exposed to the specific antiserum and so different serotypes can be identified once the specific antisera are present (Heineman, 1973). Serotypes which are antigenically close to each other are classified into serogroups e.g; 9A, 9L, 9N and 9V are belonging to serogroup 9, while strains which are antigenically not related are given other numbers such as; serotypes 1, 2, 3 and 4 (Heineman, 1973).

S. pneumoniae is classified into 46 serogroups which include more than 90 pneumococcal serotypes however; only limited numbers of serotypes are responsible for the majority of invasive and non invasive diseases (Henrichsen, 1995). *S. pneumoniae* strains are differentiated into different serotypes by their capsular polysaccharides which stimulate the production of serotype-specific antibodies. Each serotype can be identified by a unique

antigenic determinant on its capsular polysaccharides coat that stimulates the production of specific antibodies.

1.7.2 *S. pneumoniae* virulence factors

The pathogenicity of pneumococci is referred to its virulence factors. The high morbidity and mortality caused by *S. pneumoniae* is due to these virulence factors (Fig. 1.6).

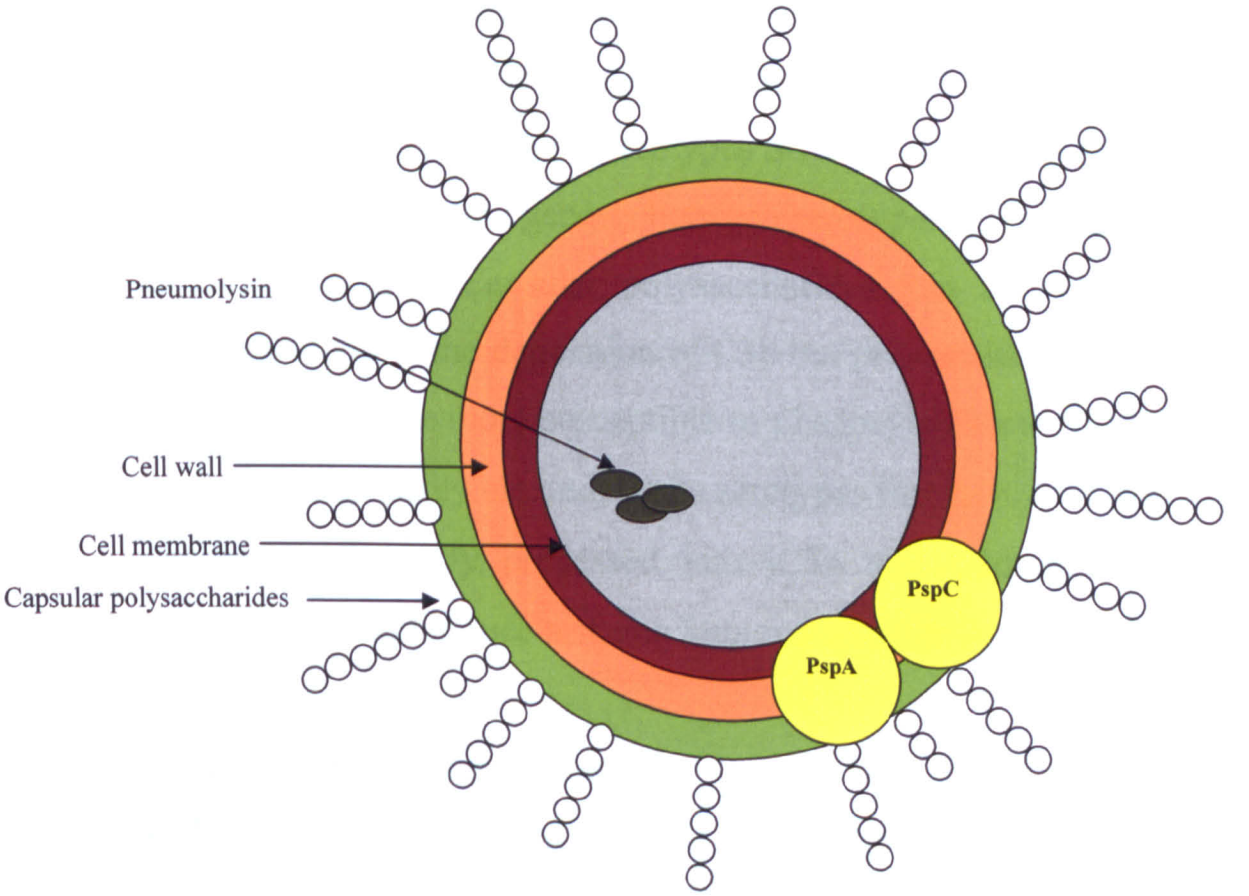


Figure 1.6: Major virulence factors of *S. pneumoniae* that modulate complement system activation during *S. pneumoniae* infection (modified from Kadioglu *et al.*, 2008).

1.7.2.1 Capsular polysaccharides (CPS)

The CPS forms the outermost layer surrounding the pneumococci. The thickness of the CPS is about 200-400 nm. Up to date, 91 different serological CPS serotypes have been identified (Kadioglu *et al.*, 2008). The capsule is the major virulence factor of *S. pneumoniae* where encapsulated strains were found to be more virulent than non-capsulated strains (Watson and Musher, 1990). Pneumococcal mutants that lack the polysaccharides capsule were found to be less virulent than the parent strains indicating that the virulence is determined by the capsule type (Kelly *et al.*, 1994).

The thickness and the chemical structure of the CPS determine the differential ability of various serotypes to survive in blood and to cause invasive diseases. Pneumococci from different serotypes differ in their ability to cause diseases. This difference in pathogenesis between serotypes is often due to the chemical structure of CPS. Capsular polysaccharides that activate the complement system and allow the deposition of C3b but prevent its further processing into iC3b and C3d, are more susceptible to phagocytosis and poorly immunogenic as they can be easily cleared, while serotypes that have C3b deposited on their capsules and quicly processed into iC3b and C3d are more resistant to phagocytosis and induce a strong humoral immune response (Hostetter, 1986).

1.7.2.2 Pneumococcal cell wall

Cell wall peptidoglycans and cell wall polysaccharides (CWPS) are potent inflammatory components and induce inflammatory reactions similar to that noticed after *S. pneumoniae* infection. Mice injected with purified peptidoglycans or CWPS showed an inflammatory response similar to that observed after infection with *S. pneumoniae* and typical pneumococcal disease in mice such as otitis media and pneumonia can be induced by injection of

these components (Tuomanen *et al.*, 1987; Carlsen *et al.*, 1992). These components activate the complement system and hence contribute to the generation of the complement anaphylatoxins C3a and C5a which enhance vascular permeability, induce mast cell degranulation and lead to neutrophil recruitment into the inflammatory site. The cell wall has also been found to be a potent inducer of IL-1 release by human monocytes (Alonso De Velasco *et al.*, 1995).

1.7.2.3 Pneumococcal proteins

Different proteins were suggested to play a role in the pathogenicity of *S. pneumoniae*. However, limited numbers of these virulent factors have been confirmed as virulence factors.

1.7.2.3.1 Pneumolysin

Pneumolysin is a potent virulence factor produced by all serotypes of *S. pneumoniae* (Kalin *et al.*, 1987). Pneumolysin is released as a 52 kDa soluble monomer. It binds cholesterol containing membranes and the monomer subunits oligomerise to form a pore in the target cell membrane and mediate cell death. Pneumolysin was also found to inhibit cilliary beating of respiratory epithelium, inhibit phagocytosis, induce cytokine synthesis and mediate $CD4^+$-T cell activation and chemotaxis (Kadioglu *et al.*, 2004; Tilley *et al.*, 2005).

Several studies described the effect of pneumolysin as a virulence factor in murine models of pneumonia. Infection of mice with mutant stains that lack pneumolysin are less virulent than wild type *S. pneumoniae* and produce less inflammation, lower numbers of lung neutrophil recruitment, limited multiplication of the pneumococci in lung and blood and significantly less T

cell infiltration (Kadioglu *et al.*, 2000). Immunisation of mice with pneumolysin protects them from pneumococcal infection indicating the crucial role of pneumolysin as a virulence factor during pneumonia (Alexander *et al.*, 1994). Also it has been reported that intranasal challenge of mice with *S. pneumoniae* deficient in pneumolysin showed a significantly lower number of bacteria in lung, trachea and nasopharynx. These data confirm the role of pneumolysin in nasopharyngeal colonisation by *S. pneumoniae* (Kadioglu *et al.*, 2002). On the other hand, Pneumolysin activates the classical pathway of complement in the lung after intranasal infection and induces an inflammatory response that delays the onset of bacteremia in mice. Intranasal infection of mice with *S. pneumoniae* D39 mutant strain deficient in pneumolysin production showed better clearance of the pneumolysin mutant strain after 24 hrs in comparison to wild type D39 (Jounblat *et al.*, 2003).

1.7.2.3.2 **Pneumococcal cell-surface proteins**

Cell-surface proteins are known as potent virulence factors that can stimulate the production of opsonic antibodies, thus a lot of attention has been directed towards these proteins as vaccine antigens (Kadioglu *et al.*, 2008).

S. pneumoniae produces several cell surface proteins, the most important ones are pneumococcal surface protein A (PspA) and PspC (Bergmann and Hammerschmidt, 2006).

A) Pneumococcal surface protein A (PspA)

PspA is expressed by all clinical isolates and it appears to play a major role in the pathogenesis of *S. pneumoniae.* Previous studies reported that PspA interferes with complement activation and C3 deposition on the pneumococcal surface and hence it protects the bacteria from complement mediated

phagocytosis (Tu *et al.*, 1999; Ren *et al.*, 2003). Infection of mice with PspA deficient *S. pneumoniae* showed a longer survival time when compared to mice infected with the wild type strain (Ren *et al.*, 2004). Pre-immunisation of mice with PspA prior to intravenous infection with *S. pneumonaie* protects the mice from the lethal sepsis and prolongs the survival time of the immunised mice in comparison to the non immunised controls (Roche *et al.*, 2003).

Using of PspA conjugated with capsular polysaccharides (CPS) from pneumococcus serotype 23F significantly increased the immunogenicity of the capsular polysaccharides. Mice immunised with CPS-PspA conjugate produced a high antibody titre response against both CPS and PspA. In addition, infection of the immunised mice with *S. pneumoniae* expressing PspA showed a significant improvement in the survival time in comparison to mice challenged with PspA alone or in combination with CPS. This increased protection after using this conjugate vaccine was due to the high capacity of the produced antibodies to bind *S. pneumoniae* and enhance complement deposition (Csordas *et al.*, 2008).

B) Pneumococcal surface protein C (PspC)

PspC is a major virulence factor of *S. pneumoniae* and contributes to many different biological functions. It binds to immunoglobulin A, complement component C3 and factor H. Binding of factor H (complement regulatory protein of the alternative pathway) is a defense mechanism that protects the bacterium form complement attack and complement mediated phagocytosis (Jarva *et al.*, 2002). It was also reported that PspC helps the adherence of pneumococcus into the lung tissues and enhances colonisation of the nasopharynx (Rosenow *et al.*, 1997). Previous studies reported that PspC is upregulated when pneumococcus adheres to the host epithelial cells in the

respiratory tract and its expression is required for subsequent tissue invasion (Orihuela *et al.*, 2004; Orihuela *et al.*, 2004b). Immunisation of mice with purified PspC protects the mice from bacterial colonisation and *S. pneumoniae* infection. Also it was found that mutants deficient in PspC have a reduced ability to colonise the nasopharynx and cause lung infection when compared to the wild type strains (Balachandran *et al.*, 2002). In a model of pneumococcal sepsis, mice infected with serotype 2 and serotype 3 mutant strains deficient in PspC showed a significant increase in the survival in comparison to the wild type strains (Iannelli *et al.*, 2004).

1.7.3 Colonisation and invasion of *S. pneumoniae*

S. pneumoniae colonise the upper respiratory tract of many healthy individuals (Fig. 1.7). Once entering the nasal cavity, mucous secretions act as a first line of defense against *S. pneumoniae* invasion. The expression of thick layers of negatively charged capsular polysaccharides increases the repulsion between pneumococcus and the sialic acid-rich mucopolysaccharides. By this simple mechanism, the pneumococcus can overcome the entrapment in the mucous secretion and reach the epithelial layer lining the nasopharynx (Kadioglu *et al.*, 2008). Adherence of *S. pneumoniae* to respiratory epithelial cells is enhanced by neuraminidase enzyme, which cleaves sialic acid from glycosphingolipids that present in human lung tissues. Removal of sialic acid exposes several receptors allowing adhesion of *S. pneumoniae* (Krivan *et al.*, 1988). At the epithelial surfaces the thick capsule is not an advantage because it is negatively charged and so the capsule will decrease the adherence. So during early stage of colonisation the pneumocccus express a thinner capsule that facilitates the adherence to the host tissues (Weiser *et al.*, 1994). Expression of hyaluronidase enzyme by *S. pneumoniae* facilitates the spread

through the connective tissues as it hydrolyses hyaluronan-containing polysaccharide components of connective tissues (Jedrzejas *et al.*, 2002).

Absence of specific IgA and loss of nonspecific reflexes (mucosal secretion and ciliary transport) in the respiratory tract (by the inhibitory effects of pneumolysin on the ciliary beating as well as by the effects of an IgA1 protease secreted by *S. pneumoniae*) can impair the immune defense and facilitate colonisation (Boulnois, 1992). The most dangerous event in *S. pneumoniae* infection is the invasion of the lower respiratory tract and blood stream infection where it causes pneumonia, septicemia and meningitis (Obara and Adegbola, 2002). The bacterial hyaluronidase enzyme can facilitate the invasion of *S. pneumoniae* due to its ability to degrade hyaluronic acid of the connective tissues. Previous studies reported the important role of hyaluronic acid during invasion of *S. pneumoniae* where strains expressing high level of hyaluronidase enzyme disseminate into blood more effectively than strains expressing lower level of hyaluronidase enzyme (Volkova *et al.*, 1994).

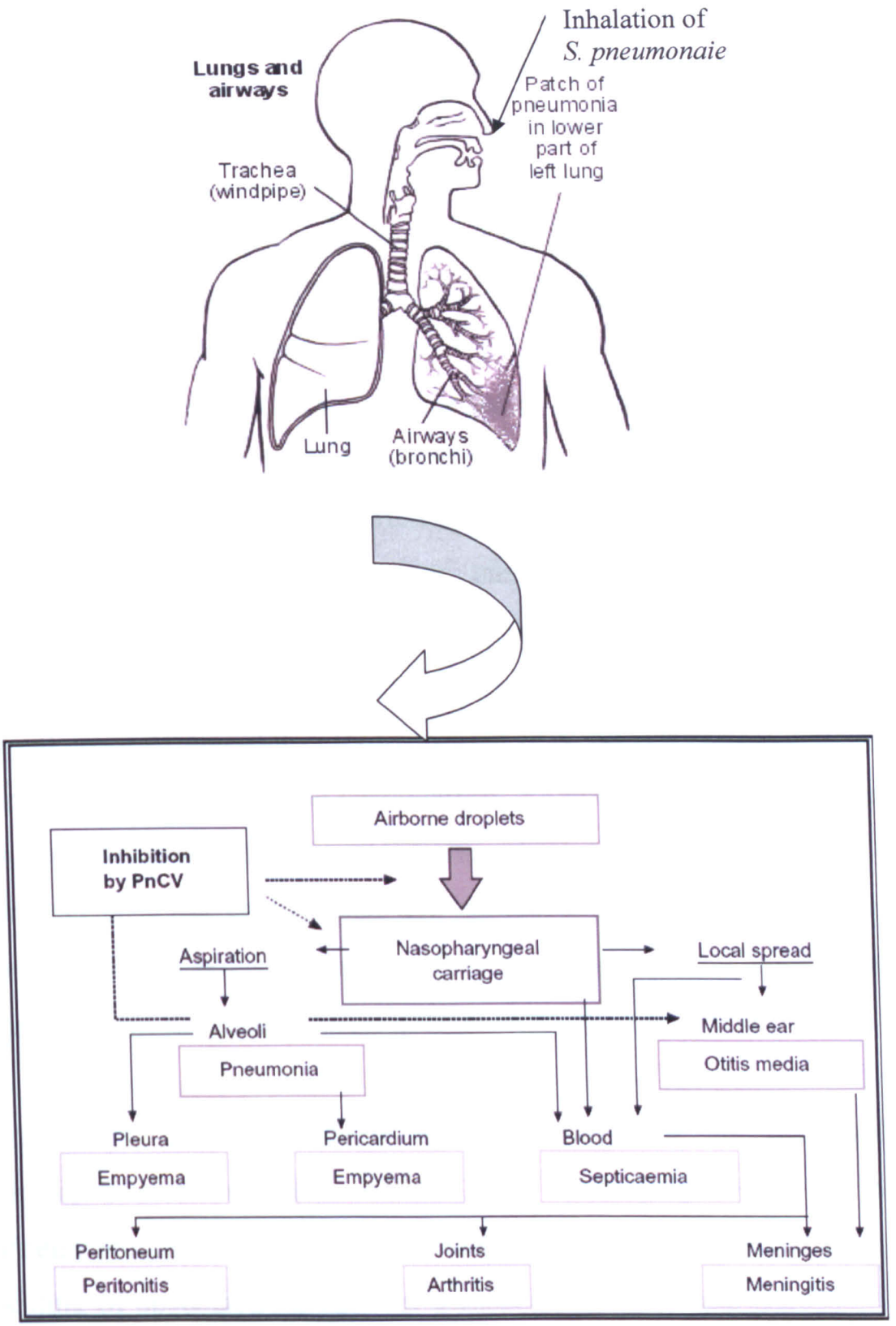


Figure 1.7: Schematic diagram showing the steps of spread of invasive *S. pneumoniae* (modified from Obara and Adegbola, 2002).

1.7.4 Host defense against *S. pneumoniae*

An effective immune defense against *S. pneumoniae* infection depends on the collaboration between humoral and cell mediated immunity in addition to non-immunological mechanisms. The main mechanism in protection against pneumococcal infection is opsonophagocytosis (Bruyn, 1992).

Mucosal immunity plays the key role in the protection against the respiratory pathogens, where the mucous secretion covering the epithelial cells prevents adhesion of these microbes to the epithelial cells and facilitates its removal by the ciliary movement (Lamblin and Roussel, 1993). Extra-cellular killing of inhaled bacteria has been reported by several antimicrobial factors in the lung lavage including; lysosymes, iron binding proteins and fibronectin with subsequent clearance of these bacteria by phagocytosis (Coonrod, 1986).

Breakdown of mucosal defenses results in *S. pneumoniae* colonisation of the upper respiratory tract and nasopharynx. Immunoglobulin A (IgA) participates in defense against the pneumococcus where IgA provides a local defense to prevent bacterial colonisation and spread of infection as it mainly interacts with capsular polysaccharides of *S. pneumoniae* and appears to activate complement-dependent opsonophagocytosis (Fasching *et al.*, 2007).

Limitation of the mucosal defense results in an increased risk of pneumococcal infection which may eventually lead to bacteremia. Pneumococcal capsular polysaccharides specific IgA provides dose dependent killing of opsonised bacteria by human phagocytic cells (Janoff *et al.*, 1999). The ability of IgA to control bacterial colonisation was confirmed by infection of mice deficient in polymeric IgA receptors, where the wild type mice

showed a significant protection against pneumococcal colonisation (Sun *et al.*, 2004).

Complement proteins have been reported to be synthesised by alveolar macrophages and lung epithelial cells (Ackerman *et al.*, 1978; Cole *et al.*, 1983; Strunk *et al.*, 1988). Complement activation following *S. pneumoniae* infection in the lung plays a major role in the host defense against further bacterial invasion. Activation of complement C3 and its deposition on the surface of *S. pneumoniae* significantly facilitates phagocytosis by alveolar leukocytes. Opsonophagocytosis and killing of pathogens by resident alveolar macrophages are the major mechanisms leading to the clearance of invading bacteria from the lung. Because resident macrophages cannot phagocytose most strains of *S. pneumoniae*, recruited PMNs play the main role in clearance of all strains of *S. pneumonaie* from the alveolar area (Coonrod *et al.*, 1987). Opsonisation of bacteria is considered to be initiated by antibodies against pneumococcal capsular polysaccharides, complement deposition or soluble lung surfactant proteins such as surfactant protein A (SP-A) which enhance phagocytosis of *S. pneumoniae* in non immunised subjects (Tino and Wright, 1996; Kuronuma *et al.*, 2004). The complement system is well-characterised in serum and can be either activated via the classical, the alternative or the lectin pathway. Complement activation enhances bacterial clearance through opsonisation and phagocytosis.

C-reactive protein (CRP) is a human acute phase protein that is mainly produced in the liver. Its levels are dramatically increased in response to infection or tissue damage. CRP binds to phosphocholine residues on *S. pneumoniae* capsular polysaccharides in Ca^{+2} dependent manner and appears to activate the classical pathway of complement in human serum

independently of the presence of specific antibody (Kaplan and Volanakis, 1974).

In contrast to human CRP, murine CRP is not an acute phase protein and this is the reason to use transgenic mice expressing human CRP as a model of testing the role of the human CRP in protection against infection. The use of human CRP transgenic mice in a mouse model of pneumococcal infection showed that transgenic mice have a significant lower mortality than non transgenic mice. The high resistance of the transgenic mice to infection was also associated with a significant reduction in bacteremia (Szalai *et al.*, 1995).

In another mouse model of *S. pneumoniae* infection, passive administration of human CRP had shown a significant protective role against *S. pneumoniae* infection and the results showed improvement in the survival time and a significantly lower bacteraemia (Mold *et al.*, 1981). Interestingly, the protective role of CRP appears to be complement independent. This finding was confirmed by using mutant form of CRP that does not bind with C1q and so does not activate the classical pathway of mouse complement. Passive administration of the mutant form and recombinant wild type CRP (that binds C1q and activate the classical pathway more efficiently than the wild type form) showed the same level of protection in mice following experimental *S. pneumoniae* infection (Suresh *et al.*, 2006).

Serum amyloid protein (SAP) is another acute phase protein that plays an important role in protection against *S. pneumonia* infection. SAP binds to pneumococcus, increases complement deposition via the classical pathway and thus facilitates phagocytosis. Mice deficient in SAP showed impaired clearance of *S. pneumoniae* and a significantly higher rate of mortality.

Reconstitution of these mice with human SAP reduced the disease severity during the course of infection and mice showed a clear improvement in controlling the infection with a significant increase in survival (Yuste *et al.*, 2007).

1.7.5 Role of complement activation in the innate immune defense against *S. pneumoniae*

Once the pneumococcus invades the host tissues, the innate immune response is immediately activated leading to deposition of opsonins on the surface of *S. pneumoniae* inducing phagocytosis. Complement activation is one of the first host defense mechanisms that play a major role in fighting bacterial infection. It was perceived that complement plays a pivotal role in defense against *S. pneumoniae* infection for nearly 100 years. Since then, a lot of studies have been done to characterise the exact role of complement system in pneumococcal infection.

Recently, several gene-deficient mice with deficiencies in one or more complement components have been used to assess which pathway of the complement system is most important for protection against *S. pneumoniae*.

C1q deficient mice were found to be more susceptible to infection with *S. pneumoniae* after intranasal infection than the wild type mice (Brown *et al.*, 2002). This finding indicates that the classical pathway has a protective role against *S. pneumonaie* infection. Natural IgM antibodies against capsular polysaccharides can activate the classical pathway. μ-/- mice (which are deficient in IgM) are more susceptible to infection with a higher bacterial load in blood and in lung tissues in comparison to the wild type controls. The alternative pathway was also found to have a protective role against *S.*

pneumoniae, but to a lesser extend than the classical pathway. Mice deficient in factor B showed a significant higher level of bacteria in lung and in blood in comparison to the wild type (Brown *et al.*, 2002).

Complement component C3 appears to be the central mediator for the activation of the three pathways of the complement to system. Mice lacking C3 are deficient in all of the three pathways of complement activation. This deficiency affects the innate as well as the adaptive immune response to the *S. pneumoniae* through the course of *S. pneumoniae* infection (Brown *et al.*, 2002).

Regardless which pathway mediates the activation of the complement system; deposition of C3b on the surface of bacteria is a key step in the downstream complement activation cascade leading to C3b mediated phagocytosis and clearance of pathogens as well as the formation of MAC leading to lysis of bacteria (Paterson and Mitchell, 2006). As a consequence, the pneumococcus developed several mechanisms to resist complement attack. The capsular polysaccharides are a major factor to protect pneumococci from complement attack and opsonophagocytosis (Jarva *et al.*, 2002).

The pneumococcus evades the alternative pathway activation by expression of factor H binding protein on its surface preventing complement attack through the recruitment of functionally active host factor H on the bacterial surface which shifts alternative pathway activation on the surface in the favor of the inhibitory regulatory mechanisms as surface bound factor H dissociates C3b complexes formed and serves as a cofactor in the factor I mediated conversion of C3b to iC3b (Jedrzejas, 2001).

The pneumococcal surface protein A (PspA) was recently found to be involved in inhibition of complement deposition on the surface of *S.*

pneumoniae. This protein is highly electronegative and has an inhibitory effect on the C4 or C4b deposition on the bacterial surface (Li *et al.*, 2007).

Pneumolysin was also found to have a protective role against complement mediated clearance. Mutagenic strains deficient in pneumolysin showed increased C3 deposition on the bacterial cell surface, indicating the important role of pneumolysin in the pathogenesis of *S. pneumoniae* infection (Yuste *et al.*, 2005).

Pneumolysin and capsular polysaccharides may activate the complement system leading to the release of C3a and C5a (which is potent chemotactic agents for neutrophil recruitment). In parallel to complement activation, extracellular bacteria are potent inducers of the proinflammatory mediators, IL-1, TNF-α, IL-6 and MIP-2. The proinflammatory cytokines trigger a cascade of inflammatory responses that help in the immune defense against *S. pneumoniae* infection (Henderson and Wilson., 1996).

1.8.1 Poly-microbial peritonitis

Intra-abdominal infection is a severe medical complication associated with a high level of morbidity and mortality. A breakdown of the barriers between the gut lumen and the peritoneum subsequently may lead to poly-microbial infections of the peritoneum with mixed aerobic and anaerobic comensal bacterial flora in the intestine. Poly-microbial peritonitis is a life-threatening condition frequently occurring after ruptured appendicitis or after rupture of colonic diverticulum (Stover *et al.*, 2008). Poly-microbial peritonitis is also one of the major complications of continuous peritoneal dialysis (Kim and Korbet, 2000). Sepsis is characterised by a massive infiltration of neutrophils into the peritoneum where neutrophils help in the clearance of the invading pathogens. When the innate immune defence fails to overcome the bacterial infection, the pathogens find their way into the blood stream and disseminate through all host organs, inducing an exaggerated inflammatory response (Riedemann *et al.*, 2003). During sepsis, macrophages, neutrophils, lymphocytes and endothelial cells produce powerful inflammatory mediators including TNF-α, IL-6, IL-1 and IL-8 in addition to acute phase proteins such as C-reactive protein. At the same time, the complement system is shifted towards activation and produces potent proinflammatory mediators such as C5a which stimulate the production of the cytokines and chemokines. The proinflammatory mediators produced in the early stages of sepsis recruit and activate the phagocytic cells (neutrophils and macrophages) which become hyperactive and produce reactive oxygen species as (H_2O_2) which is effective in killing bacteria, but at the same time may also cause tissue injury. The next step of sepsis is characterised by production of anti-inflammatory mediators such as IL-10, transforming growth factor-β and IL-13 which down-regulate

the production of the proinflammatory mediators and so the innate immune response becomes hypoactive and finally the immune defence is suppressed leading to the final stage septic shock (Fig. 1.8) (Riedemann *et al.*, 2003b).

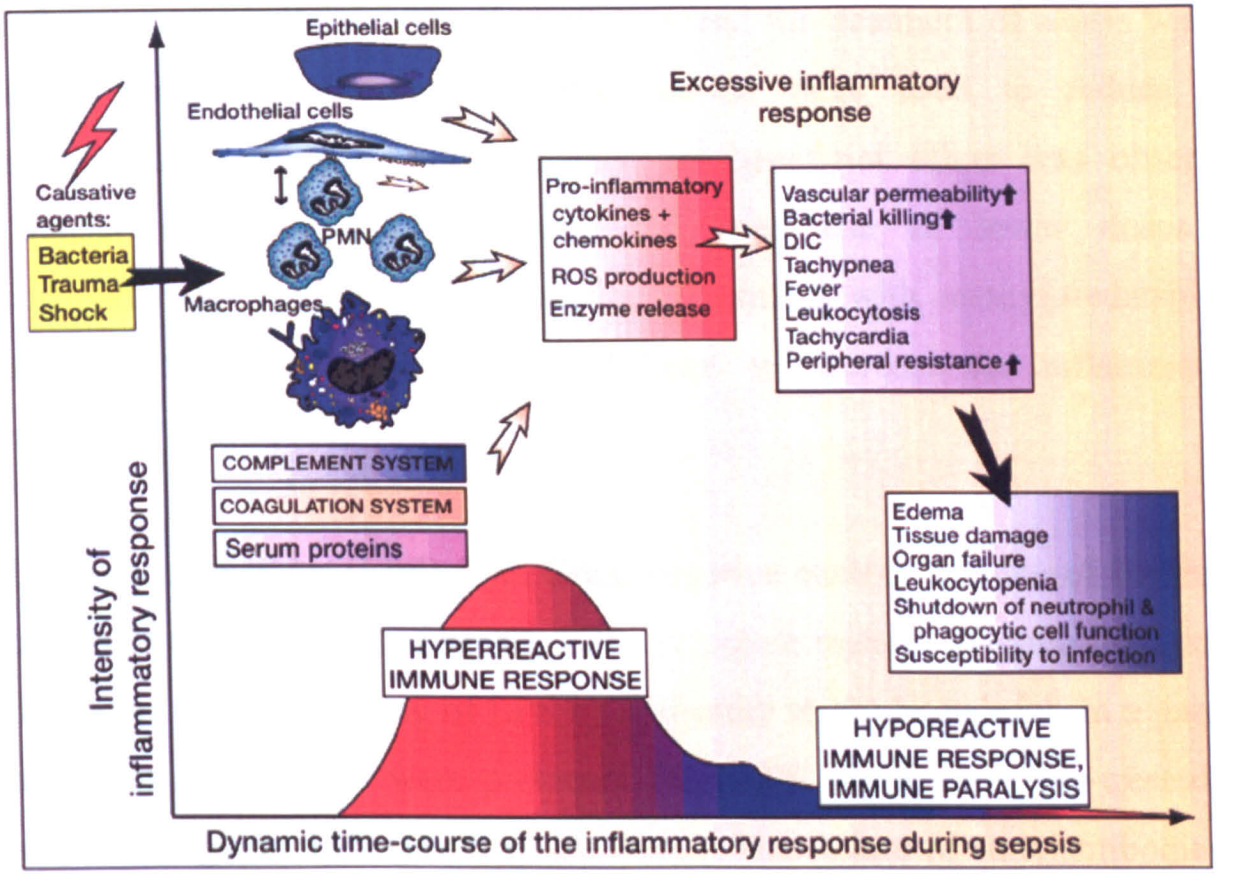


Figure 1.8: Excessive inflammatory mediator production during sepsis. Inflammatory response and activation of different type of cells in addition of complement and coagulator systems can be activated by different stimuli. This activation can lead to an exaggerated inflammatory response that stimulates leukocytes to release large amount of granular enzymes and reactive oxygen species (ROS) that cause tissue injury and may cause multi-organ failure. In parallel, the coagulatory system become activated and leads to disseminated intravascular coagulopathy (DIC) (Riedemann *et al.*, 2003b).

1.8.2 Strategies in treatment of septic peritonitis

The hyperactivity of the inflammatory system during sepsis leads to multi-organ failure, so most of the early strategies focused in the inhibition of pro-inflammatory mediators. The first clinical trial for treatment of sepsis was in 1963, when high doses of corticosteroids were used to reduce the inflammatory response. Unfortunately, no beneficial effect was observed (Bennet *et al.*, 1963). However, prolonged use of lower doses of corticosteroids showed minor benefits. Treatment with methylprednisolone lowers TNF-α and IL-6 levels and helps to decrease the inflammatory response (Meduri *et al.*, 1999).

Lipopolysaccharides (LPS) from Gram-negative bacteria are one of the major targets in the treatment of sepsis. LPS are potent inducer of proinflammatory mediators and so blocking of LPS by antiserum might be helpful. In a murine model of sepsis, mice were protected from LPS shock when pre-treated by antiserum against LPS (Davis *et al.*, 1969). The failure of such antibodies in subsequent clinical studies may be explained by the inability of the antibodies to inhibit LPS-induce cytokines from human monocytes *in vitro* (Warren *et al.*, 1993).

Another experimental approach targeted protein A (the inner protein core of LPS) showed that, using monoclonal antibodies against protein A in septic shock did not improve the survival (Bone *et al.*, 1995).

TNF-α and IL-1 are potent proinflammatory mediators that elevate during sepsis and their levels correlate with the clinical outcome. Pre-immunisation of mice with polyclonal antibodies against murine TNF-α improves the survival of mice and protects them from the lethal effect of LPS produced by

E.coli. Interestingly, the protective effect was found to be dose dependent and the maximum protection was achieved when the antibodies were administered prior to LPS injection (Beutler *et al.*, 1985). However clinical trials showed that the use of anti TNF-α strategy had no significant benefits (Reinhart and Karzai, 2001). In an animal model of septic shock, injection of rabbits with antibodies against IL-1 receptor prior to the injection of *E.coli* endotoxin improved the survival and gave a significant high level of protection. This finding indicates that endotoxin shock mediated by IL-1 in the rabbit model of septic shock can be inhibited by the use of IL-1 receptor antagonist (Ohlsson *et al.*, 1990). Unfortunately, continuous intravenous infusion of recombinant human anti IL-1 receptor antagonist was not successful in improving the clinical outcome of sepsis in humans (Fisher *et al.*, 1994).

During sepsis, the coagulation system becomes activated parallel to the activation of the inflammatory system. Application of anticoagulants is therefore important during sepsis to prevent blood clotting and act at the same time, to some extend, as anti-inflammatory.

Three different anticoagulants were tested during sepsis; tissue factor pathway inhibitor (TFPI), antithrombin (AT)-III and activated protein C (APC). Both TFPI (tifacogin) and AT-III were evaluated in to phase III clinical trials and the results showed no significant benefit (Warren *et al.*, 2001).

Activated protein C (APC) is produced after activation of plasma protein C by thrombin on the endothelial cell surface. APC in the presence of a cofactor protein S acts as an inhibitor of clotting factors Va and VIIIa and thereby as anticoagulant. APC was found to inhibit production of TNF-α, IL-1 and IL-6

from monocytes and so reduces the inflammatory response. Also it was found that APC reduces the adhesion between neutrophils and endothelial cells. Clinical trials in sepsis showed promising results; where APC-treated groups showed a significant improvement in survival and clinical outcome (Healy, 2002). Another therapeutic approach towards the treatment of sepsis depends on blocking the complement anaphylatoxin C5a and its receptor C5aR. C5a is an anaphylatoxin that has potent proinflammatory effects and is continuously produced during complement activation. C5a activates phagocytic cells to release the granular enzymes and stimulates super-oxide anion release from neutrophils. In addition, it causes vasodilation, increases vascular permeability and causes T cell apoptosis. Excessive production of C5a can lead to uncontrolled proinflammatory response and tissue damage that can lead to multi-organ failure.

C5a receptors are highly expressed by many cell types in all organs such as lung, liver, kidney and heart. C5 mRNA expression was found to be up-regulated during sepsis. In a mouse model of CLP, blocking of C5a receptors with specific polyclonal antibodies significantly improved the survival of mice in sepsis. In addition, the serum levels of TNF-α and IL-6 and the bacterial loads in blood and in different organs were significantly lower than in control mice (Riedemann *et al.*, 2003). Mice deficient in C3 and C5 were found to be more susceptible to sepsis when compared to mice deficient of C3 only with a significant higher rate of mortality and septicemia in mice with combined deficiency. C3 deficient mice were suffering from impaired bacterial clearance due to absence of C3b (which is the major opsonin) resulting in absence of opsonophagocytosis. A recent report demonstrated that C3 deficient mice were able to activate C5 in absence of C3 through a new complement

activation pathway where thrombin may directly cleave C5. On the other hand, mice deficient in C5 were found to have a significantly higher degree of bacteremia when compared to wild type control. This septicemia may be due to the inability of C5 deficient mice to generate C5b which is important component for the formation of the MAC (Flierl *et al.*, 2008). In a rat model of CLP, pre-immunisation of rats with antibodies against C5a improved the survival of septic rats and decreased the bacterial load in blood and in different organs (Czermak *et al.*, 1999).

Depending on the previous data, blocking of C5a and/or C5aR could be a useful therapeutic intervention during sepsis as this treatment will still allow formation of the MAC. At the same time, blocking of C5a or its receptors may decrease the inflammatory storm during sepsis especially after successful clinical trials showed that anti-C5a treatment can decrease complement mediated inflammatory responses following ischemia and reperfusion of the heart (Fitch *et al.*, 1999). In addition, reduced tissue damage in a model of intestinal ischemia/reperfusion in rats was also observed after pre-treatment of rats with anti-C5a (Wada *et al.*, 2001).

Aims of the work

The main aims of this study were to:

(i) Study the role of the lectin pathway of complement activation in *Streptococcus pneumonia* infection.

(ii) Study the role of the lectin pathway of complement activation in poly-microbial peritonitis in mouse using caecal ligation and puncture (CLP) a model of bacterial sepsis that resembles the clinical situation of an infection with a mixed bacterial flora of intestinal origin.

(iii) Express recombinant human and mouse MASP-2 for the establishment of MASP-2 specific monoclonal antibodies.

(iv) Assess the inhibitory activity of the antibodies raised in (iii) to block the lectin pathway functional activity *in vitro* and (mouse) *in vivo*.

Chapter 2

Materials & Methods

Chapter 2

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and media

1kb plus DNA ladder	Invitrogen
Agarose, electrophoresis grade	Melford
Ammonium persulphate	BDH laboratories
Ampcillin	Sigma
Azaserine-Hypoxanthine 50x	Sigma
Barbital	Sigma
Bovine serum albumin	Sigma
Brian heart infusion (BHI) medium	Oxoid
Calf intestinal alkaline phosphatase	Promega
Cell strainer (70μm)	BD falcon
Chinese hamster ovary serum-free II medium	Invitrogen
Coomassie Brilliant Blue R250	Serva
Deoxynucleotides, PCR grade	Promega
diethyl pyrocarbonate (DEPC)	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Disodium hydrogen phosphate	Sigma
DPX resin	BDH laboratories
Eosin	BDH laboratories
F12 nutrient mixture (Ham) with glutamax	Invitrogen
Foetal bovine serum	Sigma
Foetal calf serum	Harlan

Formalin	Fisher
Glutamine	Sigma
Glycine	Sigma
Haematoxylin	BDH laboratories
Hank's balanced salt solution (HBSS)	Gibco
High fidelity taq polymerase	New England Biolabs
Histopaque-1077	Sigma
Histopaque-1119	Sigma
Horse blood	Oxoid
Hygromycin B	Sigma
Imidazole	Sigma
Mannan 99.5%	Sigma
N,N,N',N, tetramethylenediamine (TEMED)	Sigma
Oligo $(dT)_{23}$ anchored primers	Sigma
Penicillin/streptomycin	Invitrogen
Phenol/Chloroform for RNA extraction	Sigma
poly ethylene glycol 1500 (PEG1500)	Roche
Polyacrylamide gel	Sigma
Proteinase K	Promega
QuantiTect SYBR Green Master Mix	Qiagen
RBC lysis buffer	Sigma
RNase H	Promega
RNasin	Promega
RPMI medium	Sigma
SeeBlue® Plus2 Pre-Stained Standard	Invitrogen
Sigma Fast p-Nitrophenyl Phosphate tablet	Sigma

Sodium dihydrogen mono phosphate	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Superscript II reverse transcriptase	Invitrogen
T4 ligase	New England Biolabs
Tissue-Tec OCT embedding solution	CellPath
Triazol	Invitrogen
Triton X-100	BDH laboratories
Trizma base	Sigma
Trypsin/EDTA for cell culture	Invitrogen
Tween 20	Sigma
β-mercaptoethanol	Sigma
Mannan	Sigma
fluothane	AstraZeneca
Horse blood	Oxoid
Human serum albumin	Statens serum institute, Kopenhagen, Denemark

2.1.2 Buffers and solutions

ELISA buffers

Coating buffer	15 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
Tris buffer saline (TBS)	10 mM Tris-HCL 140 mM NaCl pH 7.4
MBL- binding buffer	20 mM Tris-HCl 10 mM $CaCl_2$ 1 M NaCl 0.05% (v/v) Triton X-100 0.1% (w/v) HAS 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

Competent cell preparation buffers

TfbI solution	30 mM K-acetate
	50 mM $MnCl_2$
	100 mM KCl
	10 mM $CaCl_2$
	15% glycerol
	pH 7.4
TfbII solution	10 mM Na-MOPS
	75 mM $CaCl_2$
	10 mM KCl
	15% glycerol
	pH 7.4

Protein purification buffers

Phosphate buffer	6.5 mM Na_2HPO_4
	2.7 mM KCl
	1.5 mM KH_2PO_4
	pH 7.8
Loading buffer	2x Phosphate buffer
	100 mM NaCl
	5 mM Imidazole
Washing buffer	Phosphate buffer
	100 mM NaCl

	25 mM Imidazole
	pH 7.4
Elution buffer	Phosphate buffer
	100 mM NaCl
	500 mM Imidazole
	pH 7.4

SDS-PAGE and Western blot

Stacking gel (5%)	2.1 ml dH_2O
	0.5 ml of 30% Bis-acrylamide gel
	0.38 ml of 1M Tris-HCl (pH6.8)
	0.04 ml of 10% (w/v) SDS
	0.04 ml of 10% (w/v) ammonium per-sulphate
	0.006 ml TEMED
Resolving gel (12.5%)	4.8 ml dH_2O
	6 ml of 30% Bis-acrylamide gel
	3.8 ml of 1M Tris-HCl (pH6.8)
	0.150 ml of 10% (w/v) SDS
	0.150 ml of 10% (w/v) ammonium per-sulphate
	0.004 ml TEMED
Tris-Glycine buffer	25 mM Tris base
	192 mM glycine
	0.1% SDS
	pH 8.3
Coomassie staining solution	10% Gacial acetic acid

Coomassie destaining solution	40% Methanol 0.2% Coomassie brilliant blue R250 10% Glacial acetic acid 40% Methanol
SDS loading buffer	100 mM Tris-HCl 4% SDS 5% β Mercaptoethanol 10% Glycerol 0.2% (w/v) Bromophenol blue pH 6.8
SDS-transfer buffer	20 mM Tris base 150 mM glycine 0.038% SDS 20% (v/v) methanol

2.1.3 Kits

Coomassie protein assay kit	PIERCE
Mouse monoclonal antibody isotyping kit	Hycult
Nucleobond®Xtra Midi kit	Macherey-Nagel
Pierce ECL Western Blotting Substrate	PIERCE
ProPur kit MIDI G	Nunc
QIAquick gel extraction kit	Promega
Rat monoclonal antibody isotyping kit	AbD serotec
RESTAIN Quick Diff. Kit	REAGENA
Wizard genomic DNA purification kit	Promega
Wizard plus SV minipreps DNA purification system	Promega
Murine TNF-α ELISA kit	Diaclone

2.1.4 Oligonucleotides

All the primers used in this work were purchased from MWG Biotechnology (UK) at a concentration of 100 pmol/µl.

hM2_Bam+Xa	ggatccattgaaggtcgCACCCCCTTGGGCCCGAA
hM2A_R	GTGCCCCTCCTGCGTCACCTCTG
hM2K_R	CTCGAGGCAGACACGCAAGTTCAAAATCACTAATT
hM2A_F	CAGAGGTGACGCAGGAGGGGCAC
hM2K_F	GGATCCACCCCCTTGGGCCCGAAGTG
hM2_R6	GCCATGTCCACAGTAATGATGA
mM2_Bam+Xa	ggatccattgaaggtcgCACCCCCTTGGGCCCGAA
mM2A_R	CCCCCCCTGCGTCACCTCTGCA
mM2K_R	GCCCTCCAACTATCTTTCCTCCTATAGTG
mM2A_F	AGAGGTGACGCAGGGGGGGCATTA
mM2K_F	CTATAGGAGGAAAGATAGTTGGAGGGCA
mM2_R6	ctcGAGGAACAATGATTGGACGATGCAA
M 2screen_F1	CAT CTA TCC AAG TTC CTC AGA
M2wto_R1	AGC TGT AGT TGT CAT TTG CTT GA
Neo5_R1	CTG ATC AGC CTC GAC TGT GC
HB_link_F	AGCTTCATCATCATCATCACCATG
HB_link_R	GATCCATGGTGATGATGATGATGA

2.2 Methods

2.2.1 *In vitro* studies

2.2.1.1 Preparation of formalin-fixed *S. pneumoniae*

S. pneumoniae D39 was grown overnight at 37°C in brain heart infusion broth medium, spun down at 2000 xg for 10 minutes and the cell was pellet washed three times with PBS. After fixation with 0.05% formalin in PBS for 1 hour at room temperature the cell pellet was washed twice with PBS. Finally the bacteria were re-suspended in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) (Lynch *et al.*, 2004).

2.2.1.2 Enzyme Linked Immuno-Sorbent Assay (ELISA)

2.2.1.2.1 Binding ELISA

Micro-titer ELISA plates (Maxisorb, Nunc) were coated with *S. pneumoniae* D39 (OD_{550} = 0.6) and 10 μg/ml Mannan (Sigma) or 10 μg/ml N-acetyl albumin (Promega) as a positive control in coating buffer. After overnight incubation at 4°C, residual protein binding sites were blocked with 250 μl of 1% (w/v) BSA (Sigma) in TBS buffer (10mM Tris-HCl, 140 mM NaCl, 1.5 mM NaN_3, pH 7.4) for two hours at room temperature. The plates were then washed three times with TBS with 0.05% Tween 20 and 5 mM $CaCl_2$ (washing buffer).

Serial dilutions of serum in MBL binding buffer (20 mM Tris-HCl, 10 mM $CaCl_2$, 1 M NaCl, 0.05% (v/v) Triton X-100, 0.1% (w/v) HSA, pH 7.4) were added to the plates in duplicate starting from 1:80. Wells receiving only buffer were used as negative controls. Following overnight incubation at 4°C, plates were washed three times. Then either rat anti-mouse MBL-A (Hycult) diluted 1:5000, rat anti-mouse MBL-C (Hycult) diluted 1:5000 or rabbit anti-mouse Ficolin-A diluted 1:2000 (Kindly provided by Dr. V. Runza, University of Regensburg, Germany) were added. Following 2 hrs incubation at room temperature, plates were washed and MBL-A, MBL-C

or Ficolin-A binding was detected by addition of 100 µl of alkaline phosphatase (AP) conjugated goat anti-mouse (Sigma) or AP conjugated goat anti-rabbit diluted 1:10000 (Sigma) in washing buffer. After incubation for 90 minutes at room temperature plates were washed and the presence of AP was determined by addition of 100µl substrate solution (Sigma Fast p-Nitrophenyl Phosphate tablet sets, Sigma) and incubation at room temperature for 20 minutes. The absorbance was measured at 405nm using a BioRad ELISA micro-titre plate reader model 608.

2.2.1.2.2 C4 cleavage assay

Lectin pathway dependant C4 deposition on an activator surfaces was assayed by measuring the deposition of C4b without interference from the classical pathway using high salt concentration buffer (Petersen *et al.*, 2001).

Micro-titre ELISA plates were coated with *S. pneumoniae* and Mannan and blocked as previously described. After three times wash, serum dilutions (starting from 1:80) in MBL binding buffer were added to the plates. Wells receiving only buffer were used as negative controls. Following overnight incubation at 4°C and wash, 100 µl/well of 1 µg/ml human C4 (supplied by Dr. N. Lynch, University of Leicester, UK) were added in BBS (4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4). Plates were then incubated for 90 minutes at 37°C to allow deposition of C4b onto the surface of *S. pneumoniae* or on Mannan. Following another wash, deposited C4b was detected by addition of 100 µl of chicken anti-human C4c-AP conjugated antibody (Immunsystem AB, Uppsala, Sweden) diluted 1:1000 in washing buffer. After incubation for 90 minutes at room temperature, wells were washed three times and the presence of AP was determined by addition of 100 µl substrate solution

(Sigma Fast P-Nitrophenyl Phosphate tablet sets, Sigma). After incubation at room temperature for 20 minutes the absorbance was measured at 405 nm using the BioRad micro-titre ELISA plate reader model 608.

2.2.1.2.3 C3 cleavage assay

Micro-titre ELISA plates were coated with *S. pneumoniae* and Mannan (Sigma) and blocked as previously described. Following overnight incubation at 4°C with serum dilutions (starting from 1:80) in BBS and wash, 100 µl of rabbit anti-human C3c (Dako) diluted 1:5000 was added to each well and plates were incubated for 90 minutes at 37°C. After incubation, plates were washed three times and 100 µl of AP conjugated anti-rabbit IgG diluted 1:10000 in washing buffer was added to each well. After 90 minutes incubation at room temperature, plates were washed and the presence of AP was determined by addition of 100 µl substrate solution (Sigma Fast p-Nitrophenyl Phosphate tablet sets, Sigma). After 20 minutes incubation the OD at 405 nm was measured using the BioRad micro-titre ELISA plate reader model 608.

2.2.1.2.4 Measurement of murine TNF-α in mouse serum using sandwich ELISA

Murine TNF-α was measured in serum using a solid phase sandwich ELISA. 96 micro-titre well plate had been coated with monoclonal antibody against murine TNF-α which binds specifically to the murine antigen from mouse serum. A biotinylated polyclonal antibody was used as a recognition antibody to detect the mouse antigen. A Streptavidin-Horse Radish peroxidase was then incubated to bind with the secondary antibody, then the chromogenic substrate TMB was added and the developed colour was measured.

Procedures:

Murine TNF-α was measured using a commercial Murine TNF- α ELISA kit (Diaclone) according to the instruction manual. Two fold serial dilutions of the standard were prepared using the standard dilution buffer starting from 1000 pg/ml to 31.25 pg/ml. 100 µl of each standard and the serum samples from mice was loaded into the ELISA plate and incubated for 2 hrs at room temperature, diluent alone was used as a negative control. After incubation, the plate was washed three times with 300 µl of 1x washing buffer. 50 µl of the biotinylated polyclonal antibody against Murine TNF-α was then added to each well and incubated at room temperature for another one hour. After washing, 100 µl of streptavidin-HRP solution was added to all wells and incubated for 30 minutes at room temperature. After incubation the plate was washed again and 100 µl of TMB solution was added to each well and the plate was incubated in the dark at room temperature for 20-30 minutes. Finally, the colour development was stopped by addition of 100 µl of H_2SO_4. The absorbance was then read at 405 nm using Biorad micro-titre ELISA plate reader model 608.

2.2.1.2.5 Screening of monoclonal antibody producing hybridomas

ELISA technique was used to detect the antibody titre in mouse and rat sera or to screen the positive hybridomas producing specific monoclonal antibody.

ELISA plates were coated with 10 µg/ml of hMASP-2A or mMASP-2A in coating buffer for overnight at 4°C. Next day, the plates were blocked and washed as previously described. Samples were added undiluted from different hybridomas supernatants or in serial dilutions (mouse/rat sera) and the plates were incubated for two hours at 37°C then washed three times.

In order to detect specific monoclonal antibody production, 100 µl of AP conjugated rabbit anti-mouse (Sigma) or AP conjugated goat anti-rat (Sigma) diluted 1:10000 in washing buffer were added to each well and incubated for 90 minutes at room temperature. Finally, the plates were washed and the presence of AP was determined by addition of 100 µl of a substrate solution (Sigma Fast p-Nitrophenyl Phosphate tablet sets, Sigma). After incubation at room temperature for 20 minutes, the colour intensity in each well was measured at 405 nm using a BioRad micro-titre ELISA plate reader model 608.

2.2.1.3 Phagocytosis Assay

2.2.1.3.1 Isolation of polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (PMNs) were isolated from whole blood from healthy adult donors using a one step isolation procedure using Histopaque-1119 (sigma) and Histopaque-1077 (sigma) according to the instruction manuals. Briefly, 3 ml of histopaque-1119 was added to a 15 ml conical falcon tube, and then 3 ml of Histopaque-1077 was carefully layered onto the Histopaque-1119. 6 ml of heparinised blood was layered on top of the Histopaque 1077 layer and the PMNs were separated by centrifugation at 700 xg for 30 minutes at room temperature. After centrifugation the leukocytes were isolated by aspirating the layer formed between Histopaque-119 and Histopaque-1077. The isolated PMNs were washed twice by addition of 10 ml of Hank's balanced salt solution (HBSS) containing 1.2 mM Ca^{++} and 1.2 mM Mg^{++}, pH7.4 (GIBCO) and centrifuged at 200 xg for 10 minutes. Finally the isolated PMNs were re-suspended in HBSS to a final concentration of 10^7 cells /ml.

2.2.1.3.2 Opsonisation of *S. pneumoniae* D39

One ml of overnight culture of *S. pneumoniae* D39 (10^8 CFU/ml) were washed twice with HBSS and re-suspended into a final concentration of 10^6 CFU/ml. 100 µl of the bacterial suspension was opsonised by incubation at 37°C for 30 minutes under rotation with 20% $MASP2^{+/+}$ or $MASP2^{-/-}$ mouse serum.

2.2.1.3.3 Killing assay of pneumococci by PMNs

The killing assay of pneumococci by PMNs was estimated by measuring the decrease in number of the viable bacteria with time. 10^6 PMNs were incubated with 10^5 pre-opsonised or non opsonised living bacteria in a final volume of 250 µl in HBSS pH 7.4 at 37 °C under rotation. Samples were taken at 0, 30, 60, 120 and 240 minutes. The samples were serially diluted in HBSS and plated onto blood agar plates followed by incubation at 37 ^{O}C under anaerobic condition (Jounblat *et al.*, 2004).

2.2.1.3.4 Cytospins and staining of PMNs

25 µl samples of HBSS containing PMNs were centrifuged to attach the PMNs to glass microscope slide using a cytospin kit at 1500 xg for 3 minutes using a Cytospin 2 centrifuge (Shanon). The slides were air dried for 15 minutes and stained by RESTAIN Quick Diff. Kit (REAGENA) according to the instruction manual. The slides were dipped in the fixative solution A for 30 seconds then into solution B for 25 seconds and finally dipped into solution C for another 25 seconds. The slides were washed with water, air-dried then mounted in DPX resin.

2.2.1.3.5 Fixation of PMNs for examination by transmission electron microscope (TEM)

After 2 hrs incubation of neutrophils with opsonised *S. pneumoniae*, the cells were centrifuged for 5 minutes at 250 xg. Cells were washed twice with 500 µl of 0.1M PBS (pH 7.2) and then fixed by re-suspension into 250 µl of 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2). Fixed PMNs were then examined by TEM.

2.2.2 *In vivo* studies

2.2.2.1 Mice

MASP-2 deficient mice and their wild type littermates were used in all experiments. These mice are the only available mice completely deficient from any lectin pathway residual activity. The mice were backcrossed with C57BL/6 for eleven generations.

2.2.2.2 Streptococcal infection study

All the procedures used in this study followed the murine model of bronchopneumonia described by Kadioglu *et al.*, 2000.

2.2.2.2.1 Preparation of passaged pneumococci

Ten ml of BHI broth was inoculated with *S. pneumoniae* D39 overnight at 37 °C under anaerobic condition. Next day, bacteria were pelleted and re-suspended in 5 ml phosphate buffered saline (PBS, pH 7.4). 50 µl of the bacterial suspension (10^8 CFU/ml) was injected into the peritoneal cavity of MF1 mice. At 24 hours post infection, mice were deeply anaesthetised with 2.5% (v/v) fluothane (AstraZeneca, Macclesfield, UK) over oxygen (1.5 to 2 litre/minute). Blood samples were collected by cardiac puncture and mice were culled immediately by cervical dislocation. 100 µl blood sample was inoculated into 10 ml of BHI and incubated overnight at 37 °C under

anaerobic conditions. Next day, bacteria were pelleted and re-suspended into 30 ml of BHI supplemented with 20% foetal calf serum (Harlan) and incubated at 37°C for 5-6 hours. When the OD_{500} reached 0.6, 0.5 ml aliquots of the bacterial culture were placed in cryotubes and stored at -80 °C. The following day, the viable count was determined to check the viability of the pneumococcus.

2.2.2.2.2 Preparation of infectious dose

An aliquot of the passaged pneumococci was thawed at room temperature. *S. pneumoniae* was spun down, the supernatant was removed and the pellet was re-suspended into 400 µl of PBS. The bacterial suspension was diluted to give a final count of 10^6 CFU in 50 µl of PBS.

2.2.2.2.3 Infection of mice

Female $MASP2^{-/-}$ mice and their wild type littermates were used in this model of infection. All mice were 10 to 12 weeks old when infected. Mice were lightly anaesthetised with 2.5% (v/v) fluothane (AstraZeneca, Macclesfield, UK) over oxygen (1.5 to 2 litre/min), and 50 µl PBS containing 1×10^6 CFU of *S. pneumoniae* was then administered into the nostrils of the mice. The inoculum dose was confirmed by viable count after plating on blood agar plates.

2.2.2.2.4 Determination of lung and blood bacterial burdens

At pre-chosen time intervals following infection, groups of mice were deeply anaesthetised and blood was collected by cardiac puncture. Immediately afterwards, the mice were culled by cervical dislocation. Lungs were removed separately into 10 ml of sterile PBS, weighed, and then homogenised in a Stomacher-Lab blender (Seward Medical, London, UK). Viable counts in lung homogenates and blood were determined by

serial dilution in sterile PBS and plating onto agar plates supplemented with 5% (v/v) horse blood (Oxoid, Basingstoke, UK) and incubated for 18h at 37°C under anaerobic condition.

2.2.2.2.5 Freezing of lungs and livers for mRNA extraction

At pre-chosen time intervals following infection, groups of mice were culled and organs were collected immediately, snap-frozen in liquid nitrogen and stored at -80°C untill used.

2.2.2.3 Histology

2.2.2.3.1 Preparation of frozen lung tissues for cryostat sectioning

At pre-selected time points following infection, mice were killed and whole lungs were collected. Aluminum foil moulded around a bijoux tube was used as a container to freeze the lung tissues embedded in Tissue-Tec OCT (CellPath, UK). The aluminium cylinder containing the lung and the OCT compound was placed in a beaker of iso-pentane that was lying on the surface of liquid nitrogen. Lung samples were then stored at -80°C. Two days before sectioning, the lungs were removed to -20 °C (Kadioglu *et al.*, 2000).

2.2.2.3.2 Cryostat sectioning

Lung sections (10 μm) were cut at -25°C using a Bright Cryostat on top of super- premium microscope glass slide (BDH). The lung sections were allowed to air-dry at room temperature for 24 hrs.

2.2.2.3.3 Haematoxylin and Eosin tissue sections staining

Haematoxylin and Eosin staining procedures were done according to the protocol described in Wheather's Functional Histology, 3rd edition, 1993. The air-dried lung sections were stained for 25 seconds with Meyer's

Haematoxylin (DBH), and then washed with water. After that tissues were stained with Eosin (DBH) for 25seconds followed by another wash. The stained sections were then dried out into a series of alcohol washes (70%, 90% and 100%) for 30 seconds in each dilution. Finally, the stained sections were placed into xylene to dry for 10 seconds. The sections were then mounted in synthetic resin DPX solution, covered with a coverslip and examined under light microscope.

2.2.2.4 Poly-microbial model of peritonitis

2.2.2.4.1 Cecal ligation and puncture (CLP)

MASP-2 deficient mice and their wild type littermates were anaesthetised and the cecum was exteriorised and ligated 30% above the distal end. After that, the cecum was punctured once with a needle of 0.4 mm diameter. The cecum was then replaced into the abdominal cavity and the skin was closed with clamps (Echtenacher *et al.*, 1990). This procedure was done at University of Regensburg Germany in collaboration with Professor Daniela Maennel.

2.2.2.4.2 Bacterial counts

Mice were culled by cervical dislocation 16 hours post CLP. Peritoneal lavage was collected. Serial dilutions of the peritoneal lavage and organs homogenates were prepared in PBS and inoculated in Mueller Hinton plates with subsequent incubation at 37 °C under anaerobic condition for 24 hours.

2.2.3 Immunisation of mice and rats with hMASP-2A and mMASP-2A

Four female MF1 mice and two female wistar rats were immunised subcutaneously with 25 μg of purified recombinant hMASP-2A or 100 μg of purified recombinant mMASP-2A respectively in complete Freund's adjuvant. Animals were boosted three times with half the amount of the

antigen every week. After the last immunisation dose, serum samples were taken from each animal and the antibody titre was assessed by ELISA as previously described (see 2.2.1.2.5). Animals with the highest antibody titre were culled and spleens were aseptically removed and Splenocytes were isolated see (2.2.5.2.1).

2.2.4 Molecular Biology techniques

2.2.4.1 RNA methods

2.2.4.1.1 Extraction of total RNA

100 mg tissue samples were homogenised in 1 ml of Trizol reagent (Invitrogen), using Stomacher-Lab blender (Seward Medical, London, UK). Homogenised samples were left at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. After incubation, 0.2 ml of chloroform was added per 1 ml of Trizol reagent. The samples were mixed vigorously and then centrifuged at 10000 xg for 15 minutes at 4°C. Centrifugation separated the biphasic mixtures into the lower red phenol-chloroform phase and the upper colourless aqueous phase. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of iso-propanol (for each initial millilitre of Trizol reagent). Samples were incubated at room temperature for 10 minutes and centrifuged at 10000 xg for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. RNA pellet was then air dried and dissolved in diethyl pyrocarbonate (DEPC) treated water. RNA was quantitated by measuring the absorbance at A_{260} nm.

2.2.4.1.2 Purification of RNA

10 µg of RNA was digested with 2µl RNase-free DNase I (Promega), 5 µl DNase buffer in a final volume of 50 µl DEPC water (RNase free water) for 30 minutes at 37°C. After digestion, the final volume was increased to

200µl with DEPC water, and then extracted once with 200 µl of phenol/chloroform/iso-amyl alcohol mixture (Sigma). Samples were vigorously shaken for 15 seconds, followed by centrifugation at 10000 xg for 10 minutes. The upper aqueous phase containing RNA was carefully removed and the RNA was precipitated with 500 µl of absolute ethanol and 20 µl of 3 M sodium acetate followed by centrifugation at 10000 xg. The purified RNA was dried at room temperature for 15 minutes and re-dissolved in DEPC water (Lynch *et al.*, 2005).

2.2.4.1.3 Determination of concentration and Purity of RNA

8 µl of RNA sample was taken from the original stock and diluted to 800 µl with DEPC water. The diluted RNA sample was transferred into a clean Quartz cuvette and the absorbance was measured at 260 nm and 280 nm. One ml DEPC water was used as a blank and RNA concentration was determined using the following formula:

$$[\text{RNA } \mu g/\mu l] = A_{260} \times 40 \times \text{dilution factor} / 1000$$

The purity of the RNA sample was determined by calculating the ratio between A_{260}/A_{280}. Ratios of 1.7 to 2 represent good RNA.

2.2.4.1.4 Reverse transcriptase polymerase chain reaction (RT- PCR)

One μg of RNA was reverse transcribed into cDNA according to the instruction manual of superscript ^{tm} first strand cDNA synthesis system for RT-PCR (Invitrogen).

One μl of Oligo$(dT)_{23}$ anchored primers (Sigma) were added to 1μg of RNA in a final volume of 11.5 μl. The reaction mixture was incubated for 10 minutes at 70°C. After that, the temperature was decreased to 45°C.

One μl superscript II, 2 μl RT buffer (10x), 2 μl $MgCl_2$, 2 μl of 0.1M DTT, 1 μl dNTPs (10mM) and 0.5 μl RNasin (Promega) were added to each sample and incubated for another 60 minutes at 45°C, followed by 10 minutes incubation at 70°C. Finally, the temperature was dropped to 4°C. After cDNA preparation, the template RNA was digested by addition of 1 μl RNase H (Promega) into a final volume 50 μl with incubation at 37°C for 20 minutes.

2.2.4.1.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real time PCR was performed using the Light Cycler (Roche diagnostics, Mannheim) according to the instruction manual. SYBR green master mix. (Qiagen) was used to amplify the cDNA. SYBR green is a fluorescent dye that binds to double strand DNA and emits fluorescence. In solution, SYBR green that does not bind cDNA emits a minor fluorescence. After annealing of the primers, SYBR green binds to the newly formed double strand DNA and the fluorescence is enhanced. Amplification of cDNA can be monitored by measuring the increase in the fluorescence throughout the cycles.

2.2.4.1.6 Analysis of gene expression by qRT-PCR

Quantitative real-time PCR was performed to determine the levels of lung mRNA expression of C3, C4, C1q, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-6, MIP-2, IL-1β, IL-10 and liver mRNA expression of MBL-A and MBL-C using the Light Cycler. The expression levels were computed by normalising each cytokine or chemokine concentration to the concentration of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample. Each 15 µl of PCR reaction contained 1 µl of original cDNA synthesis reaction (corresponding to 30 ng of total RNA), 1.5 µM of each primer, and 7.5 µl of QuantiTect SYBR Green Master Mix. The samples were cycled according to the following experimental protocol (Fig. 2.1).

Sequences of all primers used in this study were listed in table (2.1).

Program	**Denaturation**			**Type**	**Quantification**	**Cycles**	**1**
Segment Number	Temperature Target (°C)	Hold Time (Sec)	Slope (°C/Sec)	2 Target Temp (°C)	Step Size (°C)	Step Delay (Cycles)	Acquisition Mode
1	95	900	20	0	0	0	None
Program	**Cycling**			**Type**	**Quantification**	**Cycles**	**1**
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	55	30	20	0	0	0	None
3	72	15	15	0	0	0	Single
Program	**Melt**			**Type**	**Quantification**	**Cycles**	**1**
Segment Number	Temperature Target (°C)	Hold Time (Sec)	Slope (°C /Sec)	2 Target Temp (°C)	Step Size (°C)	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**	**Quantification**	**Cycles**	**1**
Segment Number	Temperature Target (°C)	Hold Time (Sec)	Slope (°C /Sec)	2^{o} Target Temp (°C)	Step Size (°C)	Step Delay (Cycles)	Acquisition Mode
1	40	30	20	0	0	0	None

Figure 2.1: Experimental protocol for amplification of cDNA of different genes using Light Cycler.

2.2.4.1.7 qRT-PCR data analysis

Analysis of mRNA expression of TNF-α is used here as an example for the data analysis from the Light Cycler. The fluorescence signal was detected at the end of each cycle, and results were analysed using the Fit Points option in the LDCA software supplied with the machine. The fit point's option allows the baseline to be set above the noise bands (Fig. 2.2) to minimize the error in calculation of the standard curve. The standard curve is automatically generated and the crossing points of the samples were determined and the corresponding concentrations were automatically displayed on the screen (Fig. 2.3). Melting curve analysis was used to confirm the specificity of the products (Fig. 2.4).

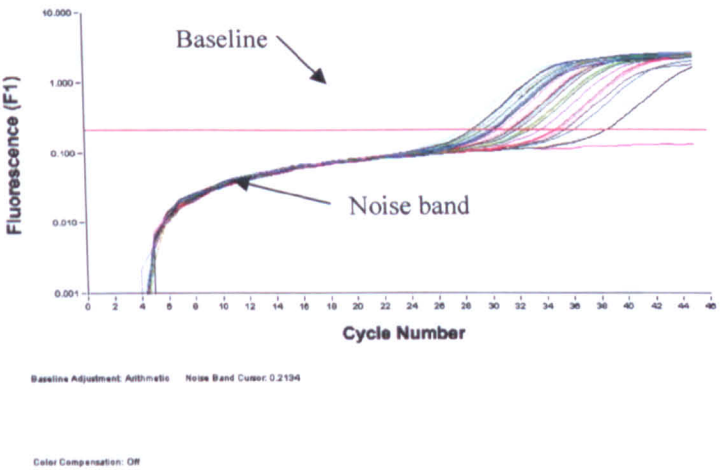


Figure 2.2: TNF-α amplification curves using the Light Cycler. Each colored line represents a sample. Curves were analysed by fit points methods where the baseline was adjusted to remove the noise band background.

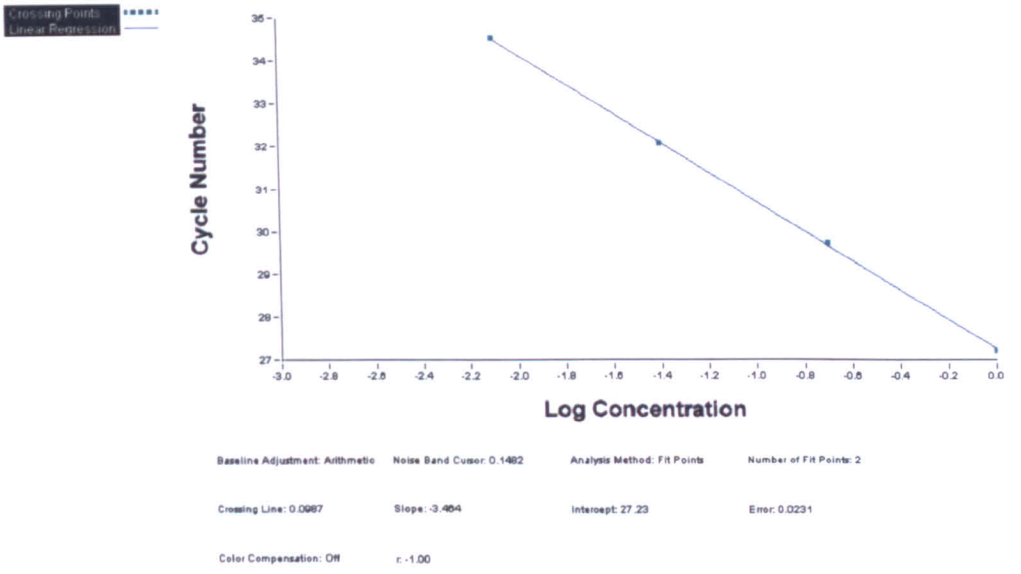


Figure 2.3: Standard curve used to calculate the relative concentration of TNF-α cDNA copies in each sample.

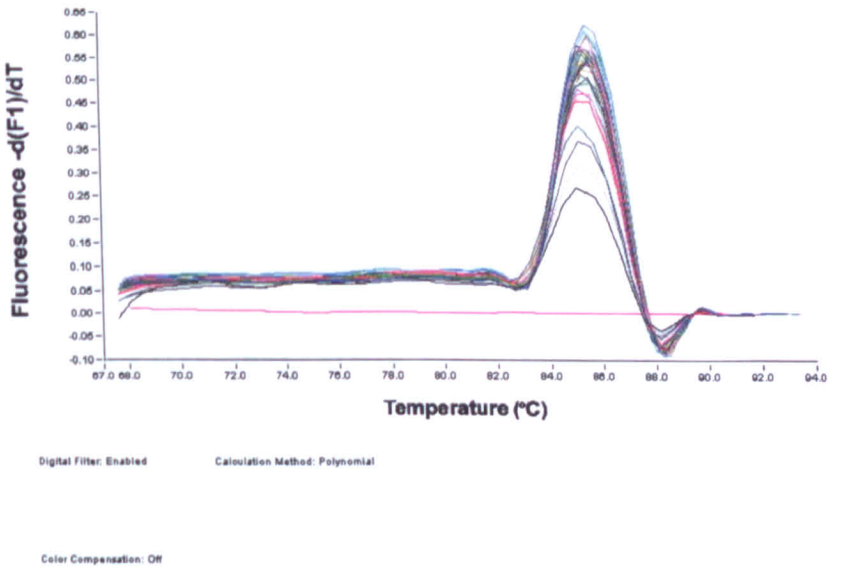


Figure 2.4: Melting curve analysis of TNF-α cDNA copies with a linear negative control line (red) showing absence of non-specific amplification.

Primer	Sequence (5' to 3')	Product size
rmC1qB_F	CTTCGAAAAGGTGATCACCA	266 bp
rmC1qB_R	CTGTGGCCTGCAGGTGA	
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	
C3_F	AGTGCTGACCAGTGAGAAGACA	136 bp
C3_R	GAAGTTTGCCACCACTGTCA	
C4_F	ATGCCCTTCAGGTGTTCCAG	156 bp
C4_R	ACTGGCCCAACTTCTCACTGAC	
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	90
MIP2_R	AAGGCAAACTTTTTGACCGCC	
MBL-A_F	CAGGGTCACAAACCTGTGAG	295 bp
MBL-A_R	TGCAACTTGTTGGTTAGCT	
BML-C_F	GACCTTAACCGAAGGTGTTCA	305 bp
MBL-C_R	CAGTTTCTCAGGGCTCTCAG	

Table (2.1): Sequence of oligonucleotides used in quantitative real time PCR.

2.2.4.2 DNA Methods

2.2.4.2.1 Polymerase Chain Reaction

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of a known sequence. Two synthetic oligonucleotide primers were synthesised; one is complementary to the antisense strand at the 5' end of the region to be amplified, and the other is complementary to the sense strand at the 3' end of the region to be amplified. Template DNA is mixed with a thermostable DNA polymerase and a molar excess of each of the primers and the four dNTP's. The reaction mixture is first heated to 95°C to denature the template, then cooled to a temperature that permits the primers to anneal to their target sequences and finally incubated at a temperature that is optimal for DNA synthesis. The cycle of denaturation, annealing and DNA synthesis is repeated 30-35 times using an automated thermal cycler. Since the products of each cycle serve as templates for the next, each cycle essentially doubles the amount of amplified DNA and the reaction proceeds exponentially. Thirty cycles of amplification result in a theoretical magnification up to 10^9.

Modifications of the oligonucleotide primers allow restriction sites, start codons, stop codons or point mutations to be engineered into the ends of the PCR product.

2.2.4.2.2 Generation of hMASP-2A and hMASP-2K mutations

The coding sequences for human MASP-2K and human MASP-2A were generated by site directed mutagenesis using two step PCR reactions. High fidelity taq DNA polymerase (Phusion) from New England Biolabs was used to generate the mutations (Fig. 2.4A and 2. 4B).

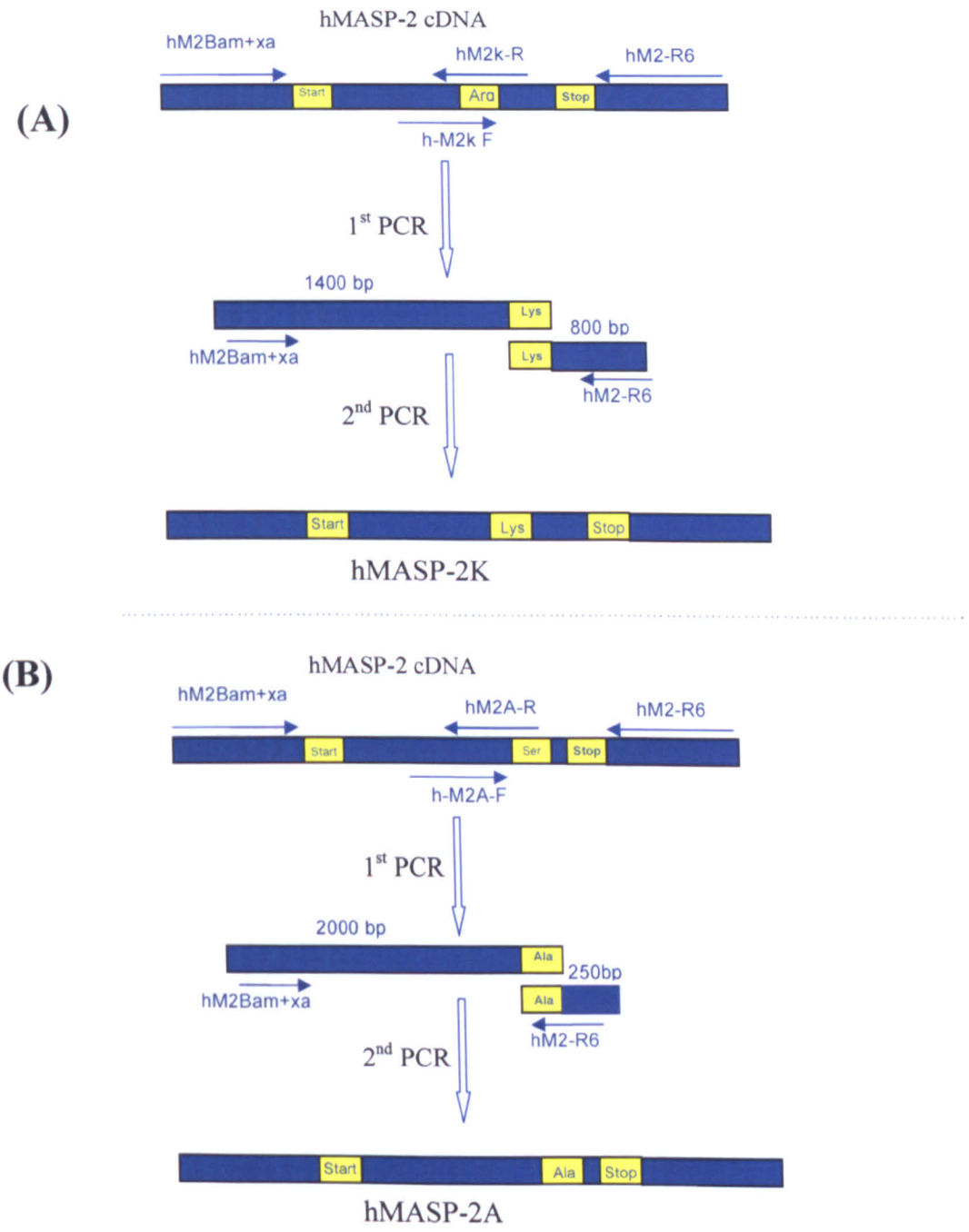


Figure 2.5: Schematic representation of the generation of hMASP-2K (A) and hMASP-2A (B) coding sequences using two step PCR procedures.

2.2.4.2.2.1 First step PCR

Mutation to generate the coding sequences for hMASP-2K and hMASP-2A were engineered using four primers each. Each of the two primer sets were used to synthesise a fragment of the mutagenised cDNA.

The first fragment was generated using the following protocol:

10 ng hMASP-2 cDNA	x µl
Phusion buffer 5X	5 µl
dNTP mix. (10 mM)	0.5 µl
phusion polymerase	0.25 µl
hM2-Bam+Xa (5 µM)	2.5 µl
hM2A-R / hM2K-R (5 µM)	2.5 µl
Distilled water	to 25 µl

The second fragment was created using the following protocol:

10 ng hMASP-2 cDNA	x µl
Phusion buffer 5X	5 µl
dNTP mix. (10 mM)	0.5 µl
phusion polymerase	0.25 µl
hM2K-F/ hM2A-F(5 µM)	2.5 µl
hM2-R6 (5 µM)	2.5 µl
Distilled water	to 25 µl

The cycling program was:

Initial denaturation	98°C	90 second	
Denaturation	98°C	15 second	15 cycles
Annealing	70°C {-0.8C/cycle}	30 second	
Elongation	72°C	40 second	
Denaturation	98°C	15 second	30 cycles
Annealing	58°C	30 second	
Elongation	72°C	40 second	
Final elongation	72°C	5 minutes	
Cooling down	4°C	∞	

2.2.4.2.2.2 Second step PCR

The two fragments corresponding to hMASP-2A or hMASP-K were ligated together under the following conditions:

Template DNA 10 ng		x µl
Phusion buffer 5X		5 µl
dNTP mix. (10 mM)		0.5 µl
phusion polymerase		0.25 µl
hM2_Bam+Xa(5 µM)		2.5 µl
hM2K_R / hM2_R6(5 µM)		2.5 µl
Distilled water	to	25 µl

The cycling program was:

Initial denaturation	98°C	90 seconds		
Denaturation	98°C	15 seconds		
Annealing	60°C	30 seconds		
Elongation	72°C	40 seconds	2	34
Final elongation	72°C	5 minutes		
Cooling down	4°C	∞		

2.2.4.2.3 Generation of mouse mMASP-2A and mMASP-2K mutations

mMASP-2A and mMASP-2K mutations were generated as previously described in generation of hMASP-2A and hMASP-2K using the corresponding primers suitable to create the mutation.

2.2.4.2.4 Purification of DNA from the agarose gel (band-Prep)

DNA bands separated on an agarose gel were purified using QIAquick Gel Extraction Kit (Promega). DNA fragments were excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed and 3 volumes of buffer QG were added to 1 volume of the gel and incubated at 50°C for 10 minutes with shaking until the gel completely dissolved. The dissolved DNA was applied on the QIAquick column and the column was centrifuged for 1 minute at 6000 xg. 500 µl of buffer QG was added to the column followed by centrifugation for 1 minute at 5000 xg. After that, 750 µl of washing buffer PE was added and the column was centrifuged for another 1 minute at 5000 xg. The column was then centrifuged again for another 1 minute to remove the residual ethanol of the washing buffer. DNA was then eluted with 50 µl buffer BE into a clean 1.5 ml eppendorf tube.

2.2.4.2.5 Chemically competent *E. Coli*

A competent culture of *E. coli* was generated according to the protocol of (Hanahan, 1983) who showed that, bacteria treated with ice cold solution of $CaCl_2$ and then briefly heated at 37°C could be transfected with plasmid DNA . The mechanism by which plasmid DNA enters the competent cells is unknown. Apparently, this treatment induces a transient state of competence during which the cells are able to take up the plasmid DNA.

2.2.4.2.5.1 Preparation of chemically competent *E. coli*

Sterile platinum wire was used to streak the *E. coli* strain directly from the frozen stock onto the surface of an LB agar plate. The plate was incubated for 16 hours at 37°C. After incubation, one colony was transferred into 5 ml of LB medium containing 20 mM $MgSO_4$ and the cells were grown overnight at 37°C. The following day, 100 ml of LB medium was inoculated with 1 ml of the overnight culture and cells were grown for 2.5-3.0 hours at 37°C until the OD at 550 nm was between 0.7-0.8. After that, cells were harvested by centrifugation at 2000 xg for 10 minutes and the cell pellet was re-suspended in 30 ml sterile ice cold TfbI buffer and incubated on ice for 5-30 minutes, depending on the strain chosen. 10 minutes work well for XL1-blue and TOP10F'. Cell pellet was collected by centrifugation at 2000 xg for 10 minutes at 4°C and carefully re-suspend in 4 ml TfbII buffer. 0.2 ml fractions were aliquoted into microcentrifuge tubes and transformed immediately or stored at -80°C.

2.2.4.2.5.2 Transformation of Chemically competent *E. coli*

Transformation of competent *E.Coli* (XL blue) with DNA was performed using chemical transformation. The cells were removed from -80°C and thawed slowly in ice, and then 2 µl of the ligation reaction and 2 µl of 2M β-mercaptoethanol were added to 50 µl of the competent cells and mixed gently, then incubated on ice for 20 minutes. After that cells were heat shocked at 37°C for 5 minutes then transferred again into ice for another 2 minutes then 450 µl of LB broth was added to the tubes and incubated at 37°C for 1 hour with gentle shaking. Two different volumes; 50µl and 200 µl were plated on LB ampicillin plates (50 µg/ml) and incubated overnight at 37°C.

2.2.4.3 Molecular cloning

2.2.4.3.1 DNA ligation

Ligation of foreign DNA molecules into vector DNA was accomplished using bacteriophage T4 ligase (New England Biolabs). This enzyme catalyse the formation of the phosphodiester bonds between neighbouring 3' hydroxyl groups and 5' phosphate ends of double stranded DNA molecules. Both blunt end ligation and cohesive ligation are possible using T4 ligase. T4 ligase works best at 37°C. However, at this temperature base pairing between the 3-4 nucleotide long protruding ends generated by restriction enzymes is very unstable, so temperatures ranging from 4-20 °C are preferred.

2.2.4.3.2 Cloning of PCR product into pGEM-T Easy vector

pGEM-T Easy vector (Promega) is an open vector with 3' terminal thymidine in both ends. These single 3' –T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing re-cyclisation of the vector and providing a compatible overhang for PCR products generated by certain thermo-stable polymerases.

PCR DNA product (ng) was ligated with pGEM-T Easy vector (50ng/ul) in presence of T_4 ligase and T_4 ligase buffer in a ratio given by (Sambrook *et al.*, 1989):

ng insert of DNA=

$$\frac{\text{(ng vector DNA) x (kb size of the insert DNA)}}{\text{(Kb size of the vector)}} \times \frac{\text{insert}}{\text{vector}} \text{ molar ratio}$$

The ligation was carried out in a final volume of 10 µl de-ionised water containing 50 ng of vector DNA, x ng of insert (calculated from the above equation), 1 µl of T_4 DNA ligase (New England Biolabs) and 1 µl (10X) T_4 DNA ligase buffer. The reaction mixture was incubated at 4°C for overnight.

2.2.4.3.3 Cloning into the expression vector pSecTag 2/hygroB

DNA constructs in pGEM-T (Promega) Easy and the expression vector pSecTag2/hygroB (Invitrogen) were digested by the same restriction enzymes and analysed by agarose gel electrophoresis. Purified constructs and the excised constructs were ligated according to the previous protocol (2.2.4.3.2).

2.2.4.3.4 Restriction digestion of Plasmid DNA

Isolated and purified Plasmid DNA and DNA fragments were digested as follow:

DNA 1 µg	x µl
BSA 10 µg/µl	2 µl
Restriction buffer 10X	2 µl
Restriction enzyme (R1) 10 µg/µl	1 µl
Restriction enzyme (R2) 10 µg/µl	1 µl
Deionised distilled water up to	20 µl

The reaction mixture was incubated at the 37°C for two hours.

2.2.4.3.5 De-phosphorylation of linearised plasmids

Linearised plasmid DNA was de-phosphorylated using calf intestinal alkaline phosphatase (CIAP) according to the manufacturer's protocol.

0.05 U of CIAP	5 µl
50 ng of digested plasmid DNA	x µl
10x reaction buffer	10 µl
Nano-pure distilled water to	100 µl

The reaction mixture was incubated at 37°C for 30 minutes. The de-phosphorylated DNA was then purified using Qiagen nucleotide removal kit.

2.2.4.3.6 Isolation and purification of plasmid DNA

2.2.4.3.6.1 Mini scale purification of plasmid DNA (Miniprep)

Single colonies were picked and cultured in LB medium containing 100 µg/ml ampicillin at 37°C overnight with shaking at 2000 xg.

Plasmid DNA was purified using Wizard plus SV Minipreps DNA purification system (Promega). 5 ml of overnight bacterial culture was centrifuged for 10 minutes at 13000 xg and the pellet was re-suspended in 250 µl of cell re-suspension solution. After that, 250 µl of cell lysis solution was added and mixed by inverting the tube 4 times and incubated at room temperature for 3 minutes, and then 10 µl of alkaline protease solution was added and mixed and kept at room temperature for 5 minutes. After that 350 µl of neutralisation solution was added and mixed well. Cell debris was removed by centrifugation at 13000 xg for 10 minutes. The clear lysate was decanted into a spin column and washed with 750µl of washing buffer then plasmid DNA was eluted using 50 µl of nuclease free water.

2.2.4.3.6.2 Large scale purification of plasmid DNA

Large scale purification of plasmid DNA from competent cells was carried out using Nucleobond®Xtra Midi kit (Macherey-Nagel). 100 ml of LB medium containing ampicillin 100µg/ml was inoculated with 10 µl of the starter culture and incubated at 37°C with shaking for 12-16 hours. Cells were harvested by centrifugation at 6000 xg for 15 minutes at 4°C. The cell pellet was re-suspended in buffer S1 + RNase. Cells were lysed by addition of lysis buffer (S2) with gentle mixing by inverting the tube several times. The mixture was incubated at room temperature for 3 minutes. Pre-cooled neutralising buffer (S3) was added to the suspension with gentle mixing by inverting the tube several times and the suspension was incubated on ice for 5 minutes. The bacterial lysate was clarified by centrifugation at 13000 xg for 40 minutes followed by filtration using Nucleobond filter paper. The

clear lysate was transferred to a Nucleobond column pre-equilibrated with buffer N2 and the column was allowed to empty by gravity. The column was washed twice using 10 ml of buffer N3 and the plasmid DNA was eluted using 3 ml of buffer N5. 3 ml of iso-propanol was added to precipitate the eluted DNA and the DNA was obtained by centrifugation at 16000 xg for 30 minutes at 4°C. The precipitated DNA was washed with 3 ml of 70% ethanol and the DNA was again obtained by centrifugation at 16000 xg for 10 minutes at room temperature. Finally, the DNA pellet was re-dissolved in an appropriate volume of TE buffer (Promega).

2.2.4.4 Isolation of genomic DNA from mouse ear snips

Genomic DNA was isolated from mouse ear snips using Promega Wizard Genomic DNA kit. Mice ear snips approximately (0.3 cm) were digested overnight at 55 °C in 300 µl lysis buffer (25 0µl nuclei lysis solution + 60 µl 0.5 M EDTA) and 10 µl of 20 mg/ml proteinase K (Invitrogen) with gentle shaking. Next day, 1.5µl of Rnase A solution (4 mg/ml) was added to each sample and mixed by inverting the tubes several times. The tubes were incubated at 37°C for 15 minutes and samples were allowed to cool to room temperature for 5 minutes before proceeding. After that, 100 µl of protein precipitation solution was added and mixed by vortexing at high speed for 20 seconds. The samples were chilled in ice for 5 minutes and the precipitated protein was removed by centrifugation at 3000 xg for 4 minutes. The clear supernatant containing DNA was removed carefully and transferred into 1.5 ml eppendorf tubes. 300 µl of room temperature iso-propanol was added and mixed by inverting the tubes several time to precipitate the genomic DNA. The precipitated DNA was obtained by centrifugation at 10000 xg for 5 minutes at room temperature. The clear supernatant was removed carefully and DNA pellet was washed by 300 µl of 70% ethanol then DNA was re-

precipitated by centrifugation at 10000 xg for 5 minutes. The DNA pellet was air dried for 10-15 minutes and 50 µl of DNA re-hydration solution was added and incubated for overnight at 4°C. The prepared genomic DNA was then stored at 2-8°C until further use.

2.2.4.5 Genotyping of MASP-2 deficient mice with PCR

MASP-2 deficient mice and their wild type litter mates were identified using the standard PCR reaction. Each PCR reaction mixture consisted of:

Genomic DNA (200ng/µl)	1 µl
Reaction buffer (10x)	1.5 µl
$MgCl_2$ (2.5mM)	1.5 µl
dNTP mix. (10 mM)	0.3 µl
M2screen_F1	1.5 µl
M2wto_R1	1.5 µl
Neo5_R1	1.5 µl
Taq-DNA polymerase	0.12 µl
Nanopure distilled water	6.08 µl

Three primers were used of identification of homozygous, heterozygous and wild type mice. These primers were:

M2screen_F1: 5'-CAT CTA TCC AAG TTC CTC AGA-3'

M2wto_R1 : 5'-AGC TGT AGT TGT CAT TTG CTT GA-3'

Neo5_R1 : 5'-CTG ATC AGC CTC GAC TGT GC-3'.

2.2.5 Cell Culture techniques

2.2.5.1 Transfection of Chinese hamster ovary (CHO-K1) cell line

CHO-K1 cells were grown in F12 nutrient mixture (Ham) with glutamax medium (Invitrogen) supplemented with 10% foetal calf serum (Harlan) and 100u/ml Penicillin/Streptomycin (GIBCO) untill 40-60% confluent growth. 2 μg of plasmid DNA were diluted in 1 ml of sterile distilled water, and 120 μl of (2 M) $CaCl_2$ was added drop wise with gentle shaking. This mixture was added drop by drop with bubbling to another tube containing 1 ml of 2x HBS (pH 7.4) and 40 μl of 100x phosphate buffer saline pH 7.4 and kept at room temperature. After 30 minutes 1 ml of the already prepared plasmid $CaCl_2$ mixture was added to the CHO-K1 cell line and incubated in CO_2 incubator for 24 hours. Next day, cells were washed twice with PBS buffer and supplemented with F12 nutrient mixture (Ham) with glutamax serum medium and the cells were incubated for another 24 hrs. On the third day, cells were washed twice with PBS and trypsinised with 1 ml of 1x trypsin-EDTA solution (GIBCO). Cells were resuspended in F12 nutrient mixture serum medium containing 300 μg/ml of Hygromycin B and platted onto 96 well plates. The selection medium was changed every 48 hrs. Expression of the specific proteins was screened by dot plot and confirmed by western blot techniques.

2.2.5.2 Generation of fusion cell line

2.2.5.2.1 Separation of splenocytes

Each spleen from immunised animals was aseptically cut into small pieces and gently strained through 70 μm cell strainer (BD). The cell strainer was washed with 20 ml of RPMI serum free medium (SFM) (Sigma). Splenocytes were centrifuged at 1200 xg for 5 minutes and washed twice with 10 ml of RPMI SFM. RBCs were lysed by 5 ml of RBC lysis buffer (Sigma) and splenocytes were re-suspended into 10 ml of SFM. 35 million

splenocytes were used to prepare the fusion and the rest of the cells were frozen down and stored in liquid nitrogen.

2.2.5.2.2 Preparation of hybrodoma

Spleen cells were mixed with NS0 myeloma cells in a ratio 1:4 in RPMI SFM (Sigma) and pelleted at 1200 xg for 5 minutes. After centrifugation, the supernatant was completely removed. Splenocytes and NS0 cells were then fused together by addition of 0.8 ml of polyethylene glycol 1500 (Roche) through a period of 1 minute with gentle stirring. After that, 10 ml of RPMI-SFM was added stepwise with gentle stirring over a period of 5 minutes. Fused cells were then pelleted and re-suspended into 50 ml of RPMI medium supplemented with 15% FCS (Sigma), 200 u/ml Penicillin/Streptomycin (Sigma), 1 mM pyruvic acid (Sigma), β-mercaptoethanol 0.05 μM (Sigma), hydrocortisone 0.5 μg/ml (Sigma) and L-glutamine 0.4 mM (Sigma). Hybridoma cells were finally plated into 96 well plates and incubated at 37 °C and 5 %CO_2, as a negative control NS0 myeloma cells were added to the last two rows of each plate. Next day, hypoxanthine and azaserine (Sigma) were added to each well at a final concentration of 100 μM hypoxanthine and 5.7 μM azaserine. The hybridomas were fed every 3 days by removing 100 μl of the old medium and replacing it with fresh RPMI medium containing 15% FCS. When the hybridomas reached 30-50% confluence, supernatant samples were taken for screening using ELISA as previously described in section (2.2.1.2.5). Positive clones were selected and transferred into 24 well plates and finally into 25cm^2 flasks (James *et al.*, 1993).

2.2.5.3 Large scale production of monoclonal antibodies

The two-compartment bioreactor celline flask (IBS Integra Bioscience) was used to maintain hybridoma growth for large scale production of antibodies

(Fig. 2.5). The flask is divided into a medium compartment that is separated from cell compartment by 10 kDa semi-permeable membrane. This membrane allows a continuous supply of nutrients into the cells and removal of toxic metabolites from the cell compartment through the semi-permeable membrane. Individual accessibility to each compartment allows change the medium without affecting the growth of the hybridomas.

In order to maintain the hybridomas in this flask, one liter of complete RPMI medium (Sigma) supplemented with 1% FCS (Sigma) was placed in the medium compartment while, $25x10^6$ hybridomas cells were placed in the cell compartment and fed with 15 ml of RPMI complete medium supplemented with 15% FCS. Hybridomas were allowed to grow for 7 days before harvesting and collection of supernatant for antibodies purification.

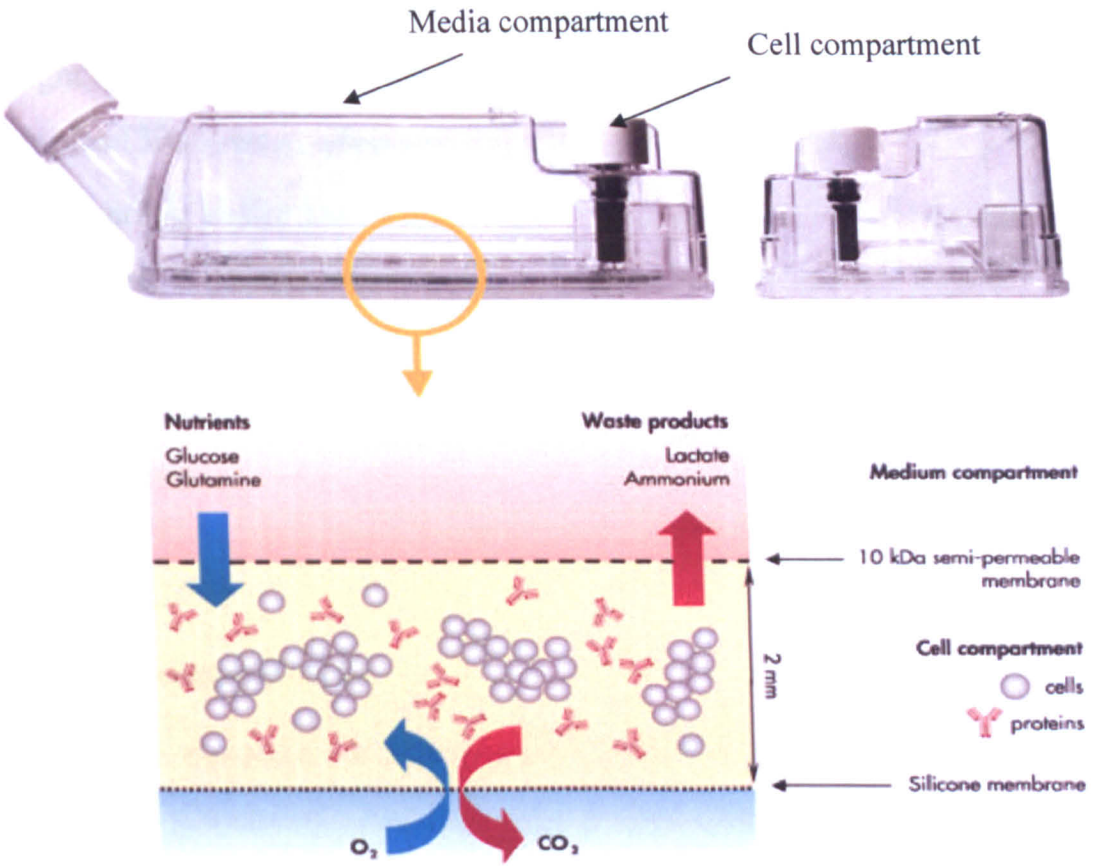


Figure 2.6: Schematic representation showing the two-compartment bioreactor celline (IBS Integra Bioscience website).

2.2.5.4 Monoclonal antibodies isotyping

Antibodies isotyping was performed using mouse monoclonal antibodies isotyping kit (Hycult) and rat monoclonal antibodies isotyping kit (AbD Serotec) according to the manufacturer's manuals.

2.2.6 Protein methods

2.2.6.1 SDS poly-acrylamide gel electrophoresis (SDS-PAGE)

Protein characterisation was assessed by SDS-PAGE under reducing and non reducing conditions. In the first case the protein was heated for 5 minutes at 95 °C with loading dye containing SDS and strong reducing agent (β-Mercaptoethanol). The denaturated polypeptides bind to SDS and become negatively charged thus the protein migrates only according to the molecular weight. Therefore, by using a marker of known molecular weight, the size of the protein can be estimated.

Samples (30µl) were denaturated by heating for 5 minutes at 95 °C in 10µl of 4x loading buffer and loaded onto a 12.5% SDS-gel. The gel was run in 1x SDS-gel running buffer at 150 V. The protein first migrated through the stacking gel of high porosity and deposited as a thin layer on the resolving gel where it will be resolved according to their molecular weight. The size of the fragments was compared with a broad range molecular weight standard.

2.2.6.2 Coomassie stain

In order to visualize the protein bands of poly acrylamide gel, the gel was soaked in Coomassie brilliant blue R-250 (Serva) solution with gentle shaking. After 30 minutes, the gel was decolorised using de-staining solution untill the background become clear and protein bands could be easily visible.

2.2.6.3 Immunobloting

2.2.6.3.1 Western blot

In order to identify the size of the proteins of interest, samples were subjected to immuno-blotting. The proteins were separated by SDS page gel electrophoresis. The casting gel was removed and the resolving gel was placed in western blot buffer. Cellulose membrane and two sheets of 3mm whatman filter paper were cut to the size of the gel and soaked into the blotting buffer for 10 minutes. The plastic cassette was opened and scotch brite pad previously soaked in the blotting buffer was placed on one side of the cassette followed by one filter paper membrane and then the gel was placed. The nitrocellulose membrane was carefully placed on top of the gel and entrapped air was removed. The second filter paper membrane was added followed by the second scotch brite pad. Finally the cassette was closed and placed into the transblot tank, in orientation that the nitrocellulose membrane side was directed towards the anode. The trans-blot was carried out at 250 mA for 1.5 hr.

After blotting the nitrocellulose membrane was removed and placed into the blocking solution (5% skimmed milk powder in PBS) with shaking for 1 hour at room temperature. The membrane was then washed once with washing buffer (0.05% tween 20 in PBS). The primary antibody, specific for the expressed protein diluted in blocking solution was added for 1.5 hrs with shaking at room temperature. After three 5 minutes washing steps the secondary HRP-conjugated antibody diluted in blocking buffer was added for 1 hr with shaking at room temp. After wash, the membrane was incubated with Horse Radish Peroxidase substrate (1:1mixture of solutions A and B of ECL kit) (PIERCE) for one minute and then, exposed to an autoradiography film (Fuji film) for 1 to 30 minutes according to the signal intensity.

Alternatively, the primary conjugated monoclonal antibody (anti-histidine-tag antibodies) (Sigma) diluted 1:4000 in blocking solution was added for 1.5hrs with shaking at room temperature. After washing, the antibody binding can be detected by ECL kit as described before.

2.2.6.3.2 Dot blot

Dot blot is a simple and fast technique that was used for screening of positive clones expressing hMASP-2A/mMASP-2A or antibodies producing hybridomas.

In this technique, 20 µl of culture supernatant from different clones or 1µg of purified protein were spotted onto nitrocellulose membrane and allowed to dry at room temperature the membrane was then blocked with 5% skimmed milk powder in PBS for 1hour. The next detection steps were carried out as previously described in western blot technique (2.2.6.3.1).

2.2.6.4 Protein purification

2.2.6.4.1 Purification of hMASP-2A and mMASP-2A

For large scale protein purification, each clone of the successfully transfected clones were grown in large triple flasks (Fisher) in F12 Ham medium (Invitrogen), supplemented with 10% fetal calf serum (Harlan), 100 u/ml Penicillin/Streptomycin (GIBCO) and 300 µg/ml of Hygromycin B until 60% confluent. Once confluence was reached, the cells were washed 3 times with PBS and the medium was replaced with Chinese hamster ovary serum-free II medium (Invitrogen) containing 100 µl/ml Penicillin/Streptomycin (GIBCO) and 300 µg/ml of Hygromycin B. After 72 hrs incubation at 37 °C, medium was harvested and the cell debris was removed prior to purification by centrifugation at 3000 xg for 10 minutes. Mutated MASPs were purified by loading 200 ml of cell supernatant

diluted with an equal volume of 2x loading buffer (phosphate buffer pH 7.4, containing 100mM NaCl and 5mM imidazole) on top of a HisGravi Trap column (GE Healthcare) and allowed to flow through the column by gravity. After that, the column was washed with 20 ml of washing buffer (phosphate buffer pH 7.4, containing 100 mM NaCl and 25 mM imidazole). Protein was eluted in one ml fractions in elution buffer (phosphate buffer pH 7.4, containing 100 mM NaCl and 500 mM imidazole). Protein containing fractions were identified by SDS-polyacrylamide gel electrophoresis followed by western blotting.

2.2.6.4.2 Purification of monoclonal antibodies

Mouse and rat IgG was purified using ProPur kit MIDI G (Nunc) according to the instruction manual. A protein G sepharose column (Nunc) was equilibrated with 10 ml binding buffer (0.1 M PBS with 140 mM NaCl, pH 7.4) and spun down at 150 xg for 10 minutes. After equilibration, the column was loaded with culture supernatant diluted 1:1 with binding buffer and spun down at 500 xg for 30 min. none specifically binding protein was washed away by 20 ml of binding buffer. Monoclonal antibodies were then eluted by 10 ml of elution buffer (0.1M Glycine, pH 3.1) and the eluted antibodies were immediately neutralised by addition of 1.3 ml of neutralising solution (1M Tris, pH 11). The eluted antibodies were dialysed against water and concentrated to 1 ml using Amicon Ultra-4 centrifugal filter unit with ultracel-10 membrane (Millipore). SDS–PAGE was used to assess to degree of purity and the activity of the antibodies was confirmed by ELISA and western blot analysis.

Unfortunately, IgM has no binding affinity to the commercially available protein G or protein A sepharose that are normally used to purify antibodies. Instead, IgM was partially purified by Amicon ultra-4 centrifugal filter unit with ultracel-100 membrane (Millipore). This type of

spin columns has a membrane cut off 100 kDa that allows removal of all FCS contaminations from the antibodies and concentration of the sample to the required volume.

2.2.6.4.3 Determination of protein concentration

Protein concentration was measured using Coomassie protein assay kit (PIERCE) according to the instruction manual.

Different concentrations of the standard albumin (BSA) were prepared in distilled water to give serial concentrations from 2 mg/ml to 25 µg/ml. 5 µl of standards and samples were pipetted into a 96 micro-titre plate and mixed well with 250 µl of the Coomassie reagent. The plate was incubated for 10 minutes at room temperature and the absorbance was read at 595nm using micro-plate reader (BioRad). The standard curve was prepared by blotting the concentration of each standard against the corresponding absorbance. The protein concentration of each sample was calculated from the standard curve.

Chapter 3

Role of lectin pathway of complement activation in S. pneumoniae infection

Chapter 3

Role of lectin pathway of complement activation in *S. pneumoniae* infection

3.1 Results

3.1.1 Genotyping of MASP2$^{-/-}$ mice

MASP-2 deficient mice were generated and bred in the University of Leicester Biomedical Services facility in a pyrogen free environment. Mice were backcrossed to pure C57BL/6 background for eleven generations (F11). A multiplex PCR protocol was used for genotyping of these mice. For this, three primers were used, one forward primer and two reverse primers. One of the reverse primers is located inside the neomycin cassette which disrupts the *MASP2* gene in the targeting construct and the other reverse primer is hybridising to the genomic sequence area of the wild type gene replaced by the neomycin cassette in the targeting construct. In the absence of the neomycin cassette the forward and the second reverse primer will give a band with a size of 750 bp to identify the wild type allele. The targeted allele disrupted by the neomycin cassette will be amplified by the forward primer and the neomycin specific reverse primer and give a band of approximately 500 bp. If a mouse homozygous for the wild type allele (MASP2$^{+/+}$), one band of 750 bp will be amplified. A mouse homozygous of the disrupted allele is (MASP2$^{-/-}$) and can be identified by a single band of 500 bp. Whereas, DNA from a heterozygous mouse two bands of 750 and 500 bp will be amplify respectively (Fig. 3.1).

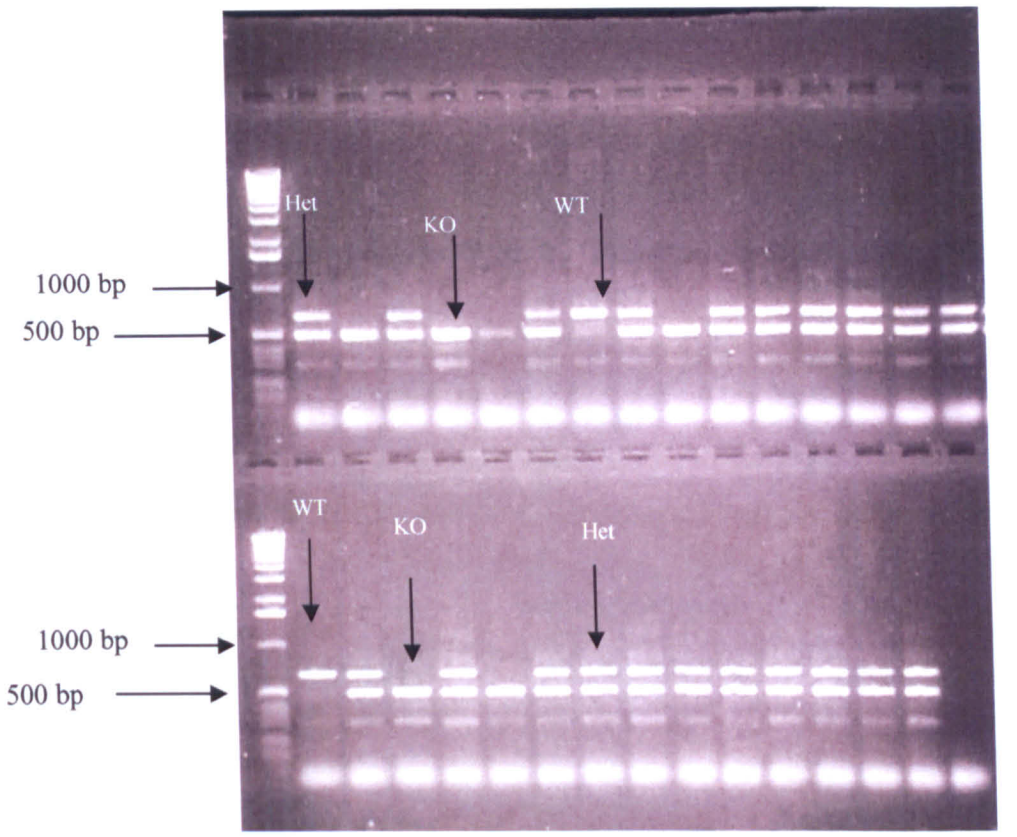


Figure 3.1: Genotyping of MASP2$^{-/-}$ and MASP2$^{+/+}$ mice. Wild type mice and KO mice give bands of a molecular weight equal to 750 pb and 500 bp respectively, while heterozygous mice give two bands at 750 bp and 500 bp. A DNA ladder was run at the outer left lane as a molecular weight marker.

3.1.2 Binding of carbohydrate recognition molecules of lectin pathway to *S. pneumoniae* D39

Activation of the lectin pathway of complement is initiated by the lectin pathway carbohydrate recognition ligands MBL or ficolins. The mouse has two types of MBL; i.e. MBL-A and MBL-C and two types of ficolins; ficolin-A (which is a serum protein) and ficolin-B (which is found in the bone marrow). The results showed that ficolin-A binds to *S. pneumoniae* D39 (Fig. 3.2) while, only MBL-C but not MBL-A showed a week binding affinity to *S. pneumoniae* (Fig. 3.3).

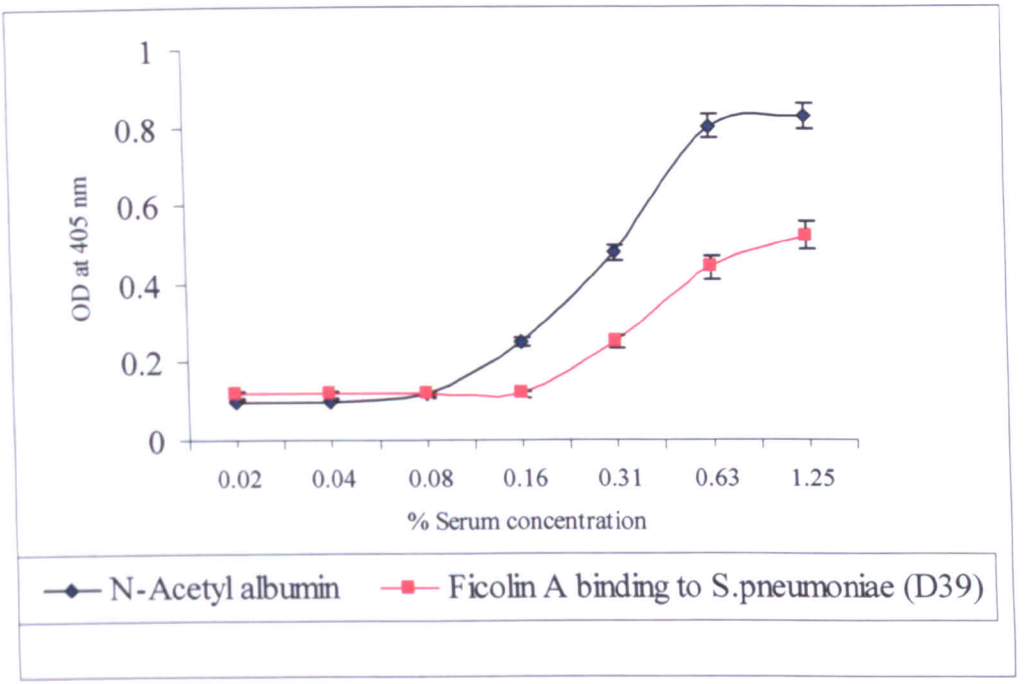


Figure 3.2: Binding of ficolin-A to *S. pneumoniae* D39. N-acetyl albumin was used as a positive control for Ficolin-A binding. Results are means ((±SEM) of 3 different experiments.

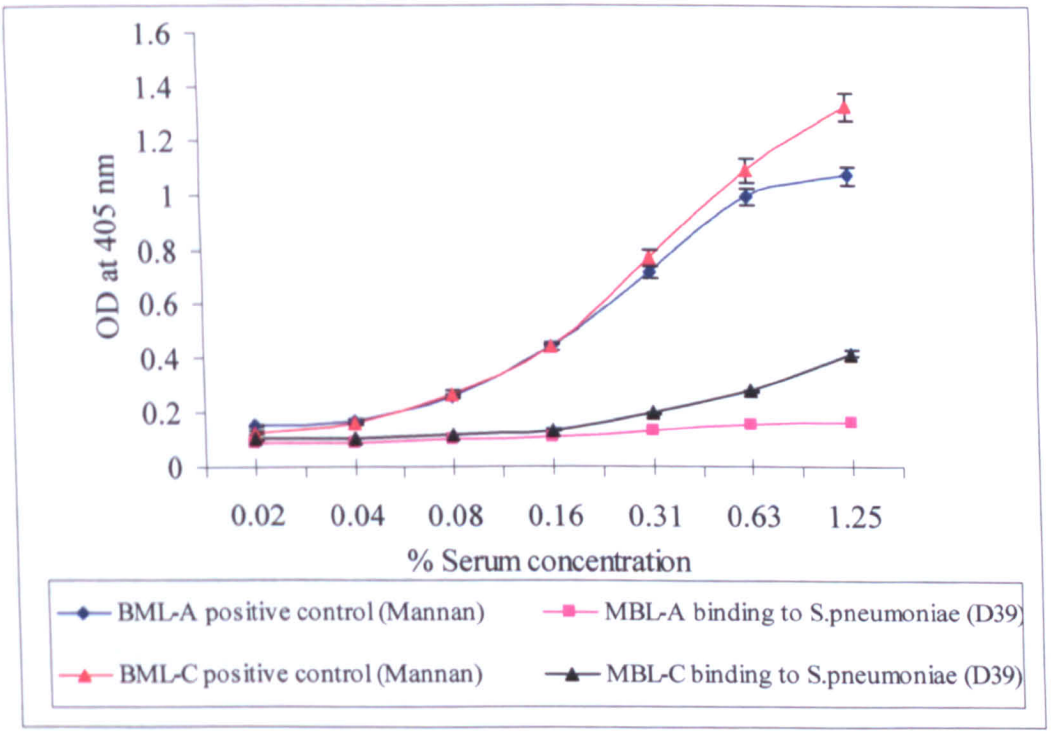


Figure 3.3: Binding of MBL-C but not MBL-A to *S. pneumoniae* D39.
Results are means ((±SEM) of 3 different experiments.

3.1.3 C4 and C3 deposition on *S. pneumoniae*

Activation of C4 on *S. pneumoniae* D39 was assessed by the deposition of C4b following cleavage through either the classical or the lectin pathway.

In order to detect C4 cleavage via the lectin pathway, MBL binding buffer was used. The high salt concentration of this buffer prevents activation of endogenous C4 and dissociates the C1 complex (composed of C1q, C1r, and C1s) this allowing monitoring activation via the lectin pathway without classical pathway activation. Likewise, the use of barbital buffer saline with low salt concentration allows to specifically targeting C4 deposition via the classical pathway. The results show that no C4 deposition was detectable through the lectin (Fig. 3.4) or through the classical pathway (Fig. 3.5) on different strains of *S. pneumoniae*. Interestingly, C3 deposition was observed on D39 and on the other five different *S. pneumoniae* serotypes tested when using serum from wild type mice (Fig. 3.6). In contrast, C3 deposition on D39 and the other serotypes was completely inhibited when using MASP-$2^{-/-}$ serum (Fig. 3.7).

In order to further define the role of MASP-2 in C3 deposition and opsonisation of *S. pneumoniae* D39, C3 deposition was also measured and quantified using sera from $C1q^{-/-}$, Factor B ($fB^{-/-}$), MASP1/$3^{-/-}$ and $C4^{-/-}$ mice. These assays were run with plasma/serum dilutions which were too high for the alternative pathway to contribute to C3 deposition. Interestingly, substantial C3 deposition was observed in sera of $C1q^{-/-}$, $fB^{-/-}$, $C4^{-/-}$ and MASP1/$3^{-/-}$ mice while no C3 deposition was observed in sera from MASP$2^{-/-}$ mice (Fig. 3.8).

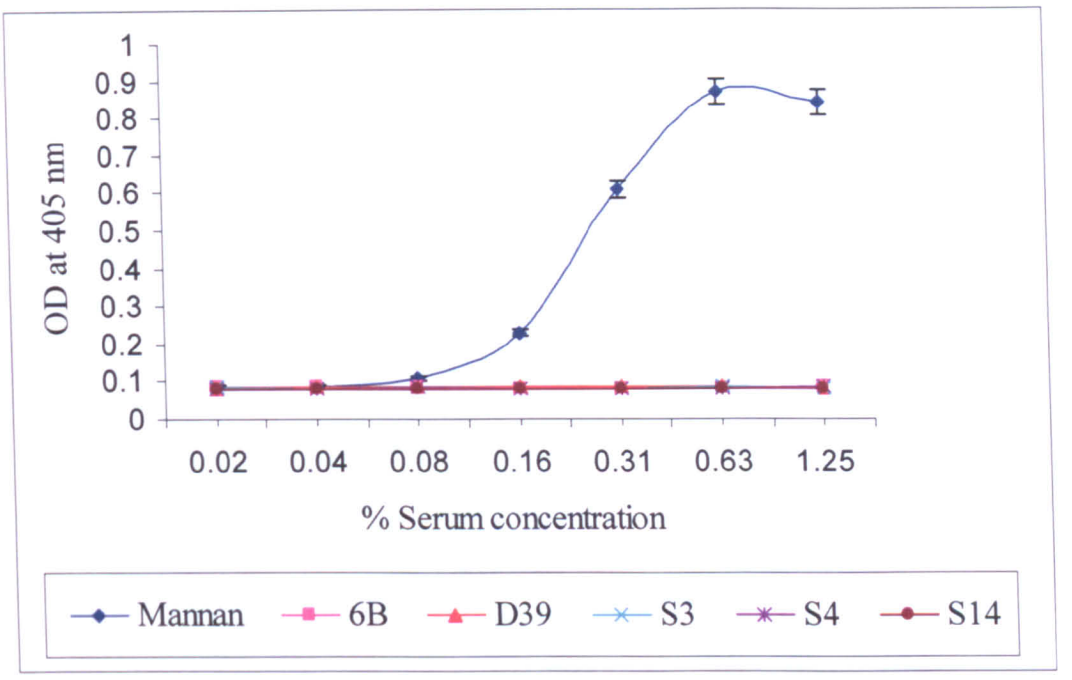


Figure 3.4: Lectin pathway mediated C4 deposition on different strains of *S. pneumoniae* Results are means ((±SEM) of 3 different experiments
Mannan was used as a positive control for C4 deposition.

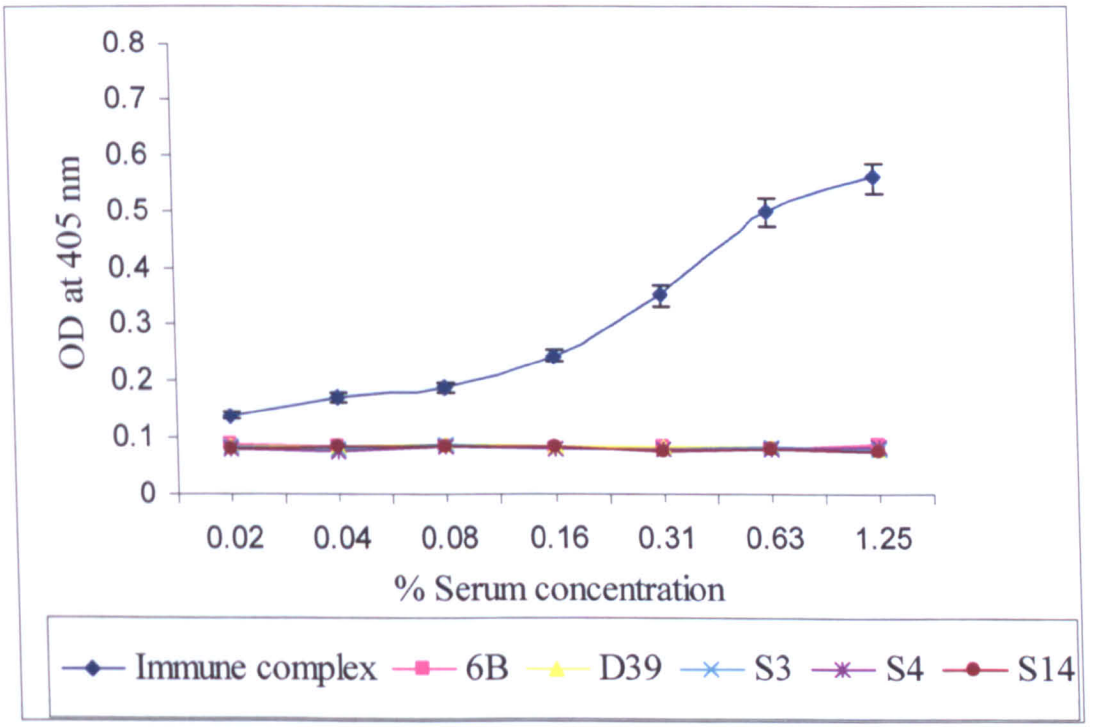


Figure 3.5: Classical pathway mediated C4 deposition on different strains of *S. pneumoniae* using mouse wild type serum. Results are means ((±SEM) of 3 different experiments. Immune complex was used as a positive control for C4 deposition.

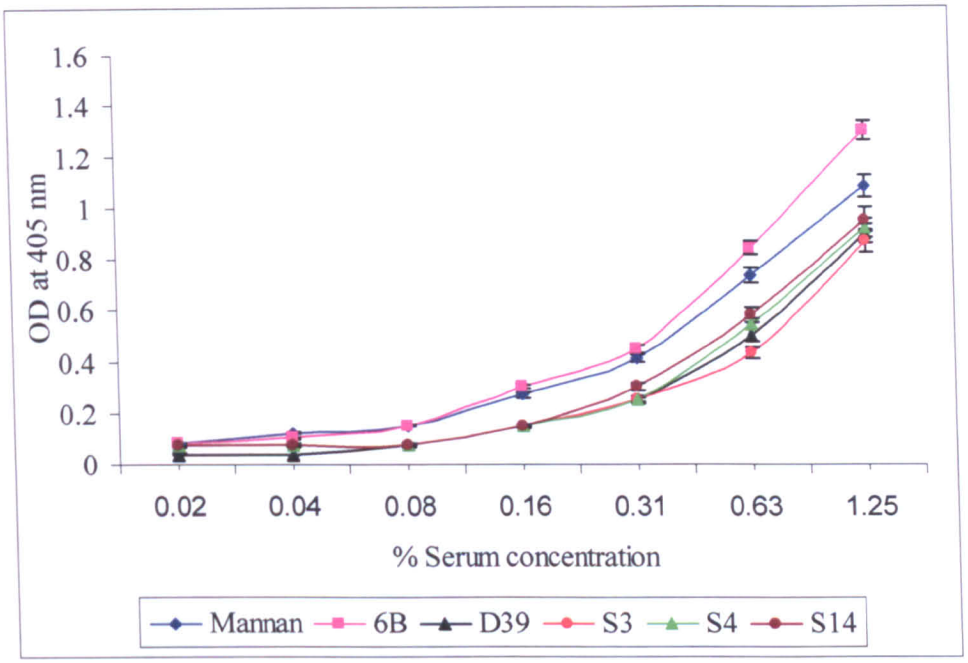


Figure 3.6: Lectin pathway mediated C3 deposition on different strains of *S. pneumoniae* using wild type serum. Results are means (±SEM) of 3 different experiments. Mannan was used as a positive control for C3 deposition.

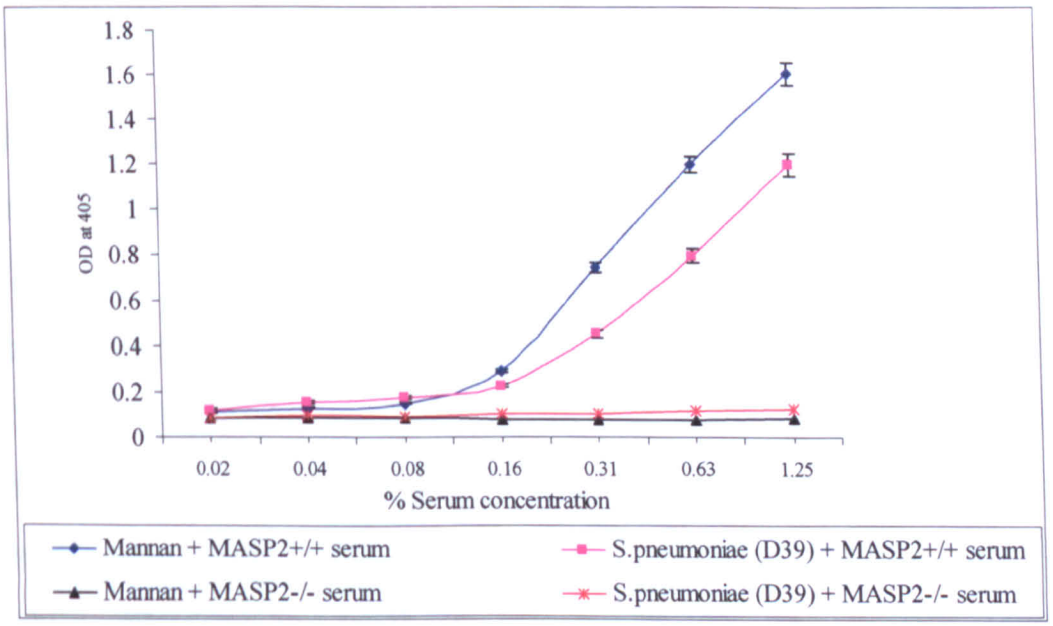


Figure 3.7: Lectin pathway mediated C3 deposition on *S. pneumoniae* D39 is MASP-2 dependent. The same result was observed with different *S. pneumoniae* serotypes (6B, S3, S4 and S14). Results are means ((±SEM) of 3 different experiments. Mannan was used as a positive control for C3 deposition.

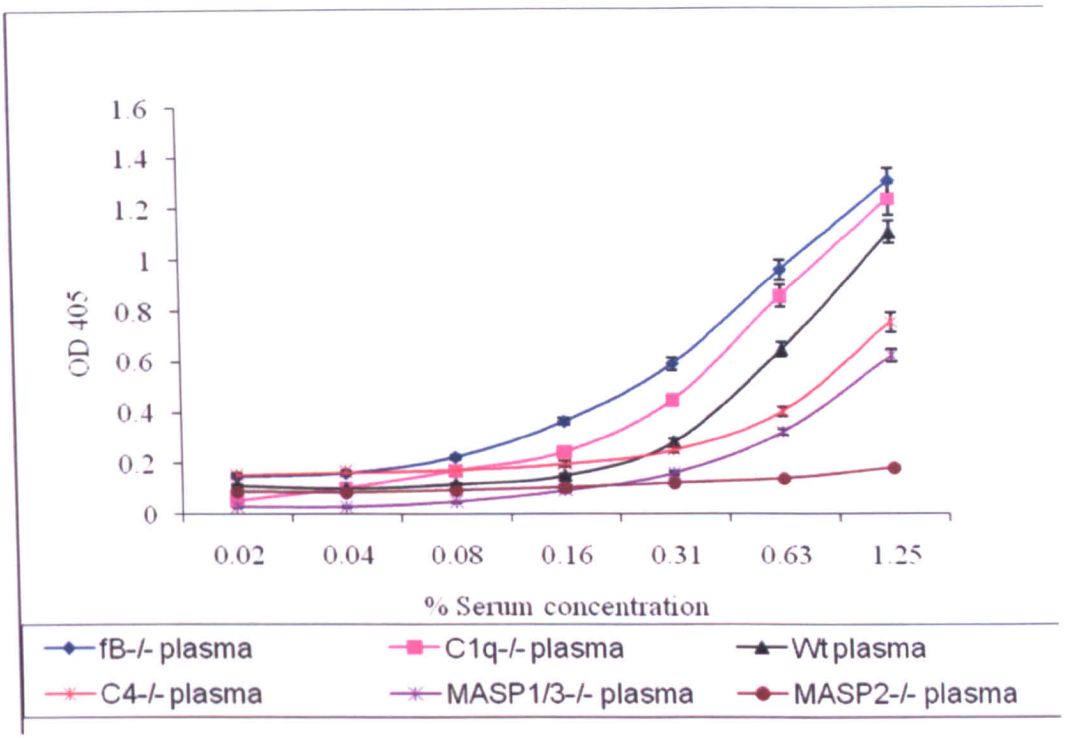


Figure 2.8: C3 deposition on *S. pneumoniae* D39 using sera/plasma of different transgenic mouse strains with complement deficiency. C3 deposition was observed with all sera except MASP-$2^{-/-}$ serum indicating that C3 deposition is MASP-2 dependent. Results are means (±SEM) of 3 different experiments.

3.1.4 Neutrophil killing assay of *S. pneumoniae*

In order to assess whether MASP$2^{-/-}$ mice are compromised in their ability to clear *S. pneumoniae* D39 from blood through phagocytosis by neutrophils, I analysed phagocytosis of *S. pneumoniae* after incubation in either MASP-$2^{-/-}$ or MASP-$2^{+/+}$ mouse serum. Serum opsonised *S. pneumoniae* were incubated with freshly isolated human neutrophils. As shown in Fig. 3.9, bacteria opsonised with wild type serum were efficiently internalised by the neutrophils. Phagocytosis and bacterial internalisation was confirmed by transmission electron microscopy (TEM) (Fig. 3. 10). In contrast, the majority of bacteria opsonised with MASP-$2^{-/-}$ serum adhered to the surface of neutrophils without being internalised (Fig. 3.11). Non-opsonised bacteria (not incubated with serum) were used as a negative control and were not

phagocytosed by neutrophils (Fig. 3.12). Bacteria incubated in the absence of neutrophils were used as a control for bacterial viability. To assess the survival of streptococci during 240 minutes of exposure to isolated human neutrophils, samples were taken at different time points and viable counts were determined (Fig. 3.13).

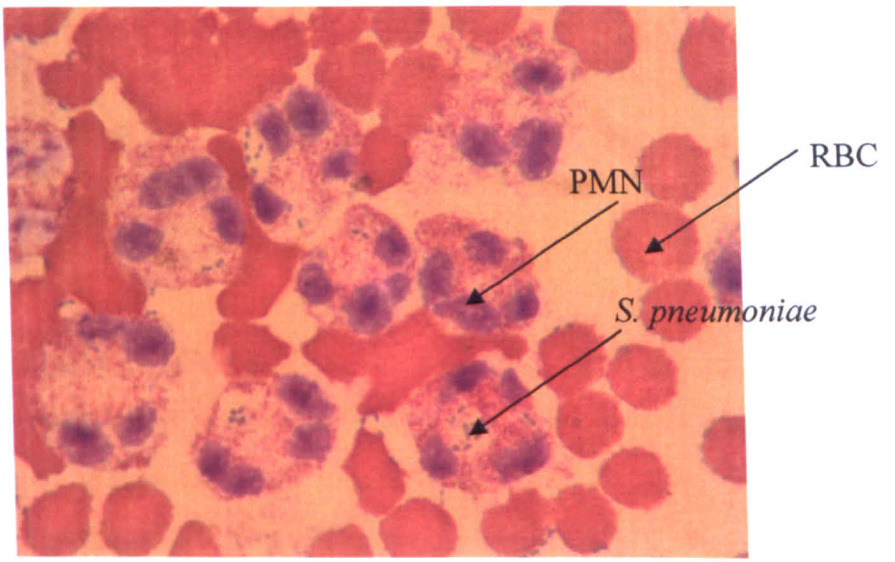


Figure 3.9: Neutrophils show enhanced phagocytosis of *S. pneumoniae* after opsonisation with MASP-$2^{+/+}$ serum and incubation with human PMNs for 2 hours.

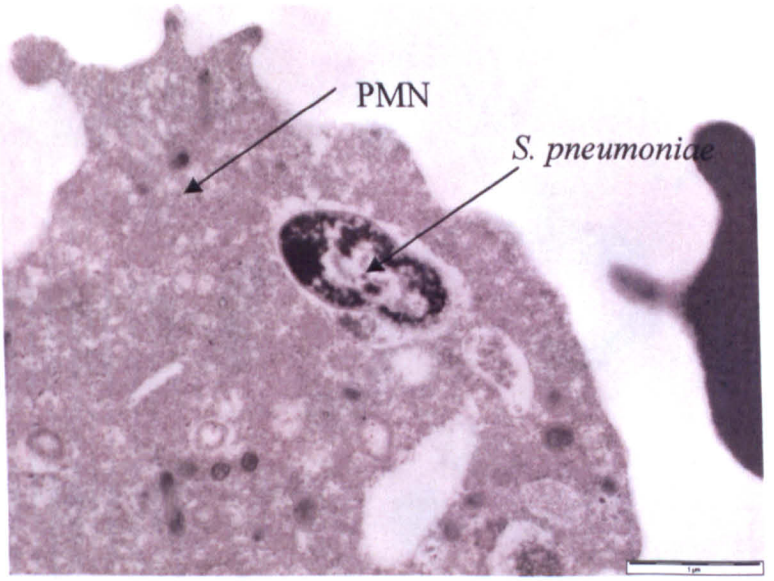


Figure 3.10: TEM image showing active phagocytosis and internalisation of *S. pneumoniae* by neutrophils after being opsonised with MASP-$2^{+/+}$ serum and incubation with human PMNs for 2 hours.

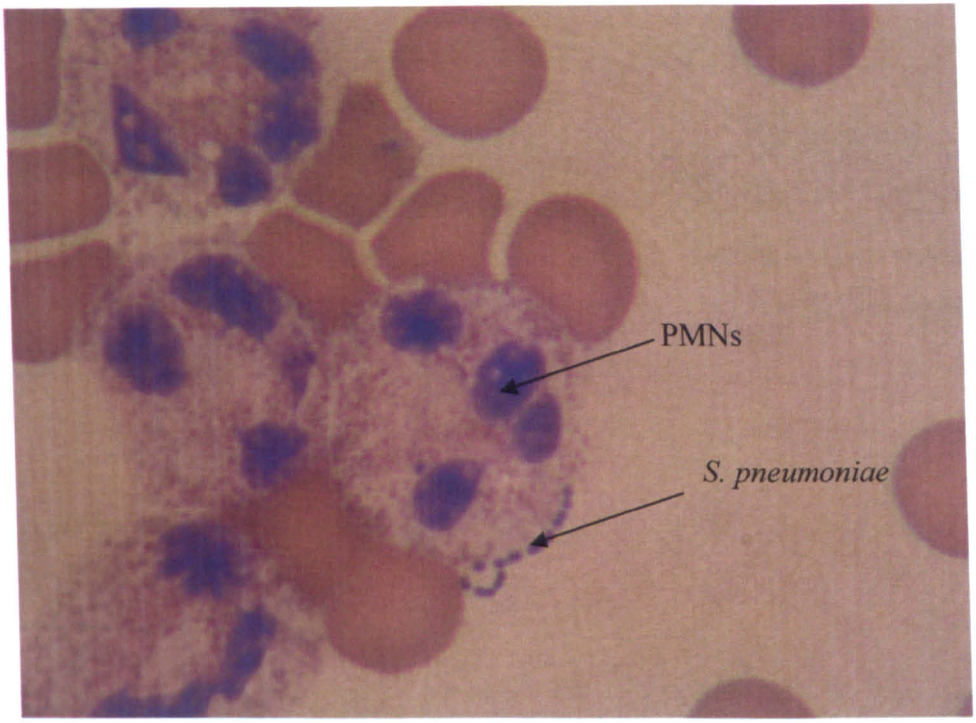


Figure 3.11: Neutrophils show no internalisation of *S. pneumoniae* after opsonisation with MASP-2$^{-/-}$ serum and incubation with human PMNs for 2 hours, indicating that lectin pathway deficiency results in defect of phagocytic activity.

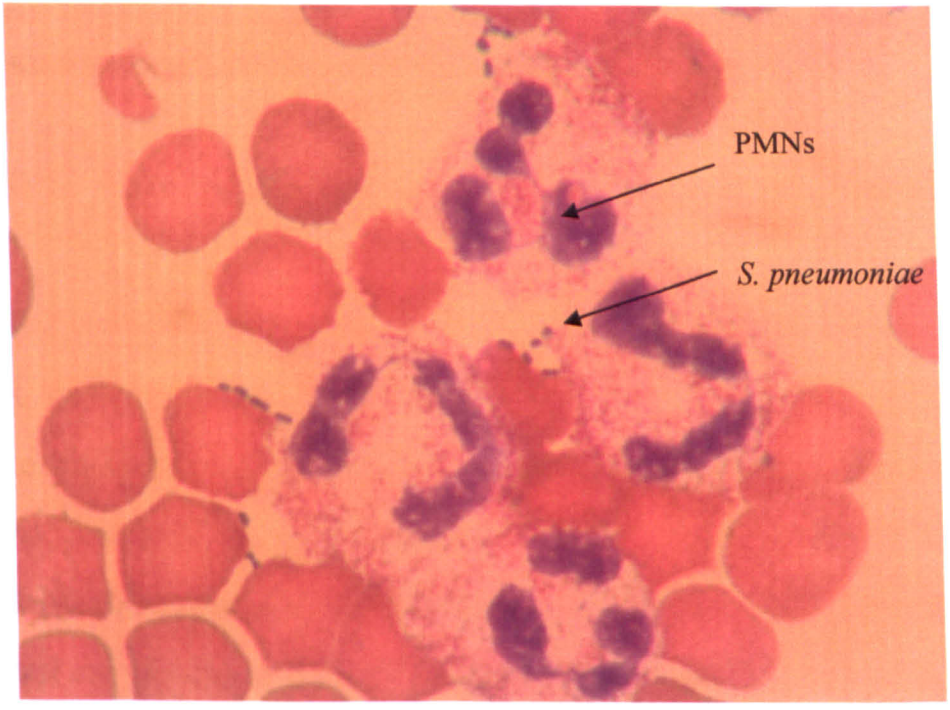


Figure 3.12: Neutrophils showing no phagocytosis of non-opsonised *S. pneumoniae* after incubation with human PMNs for 2 hours.

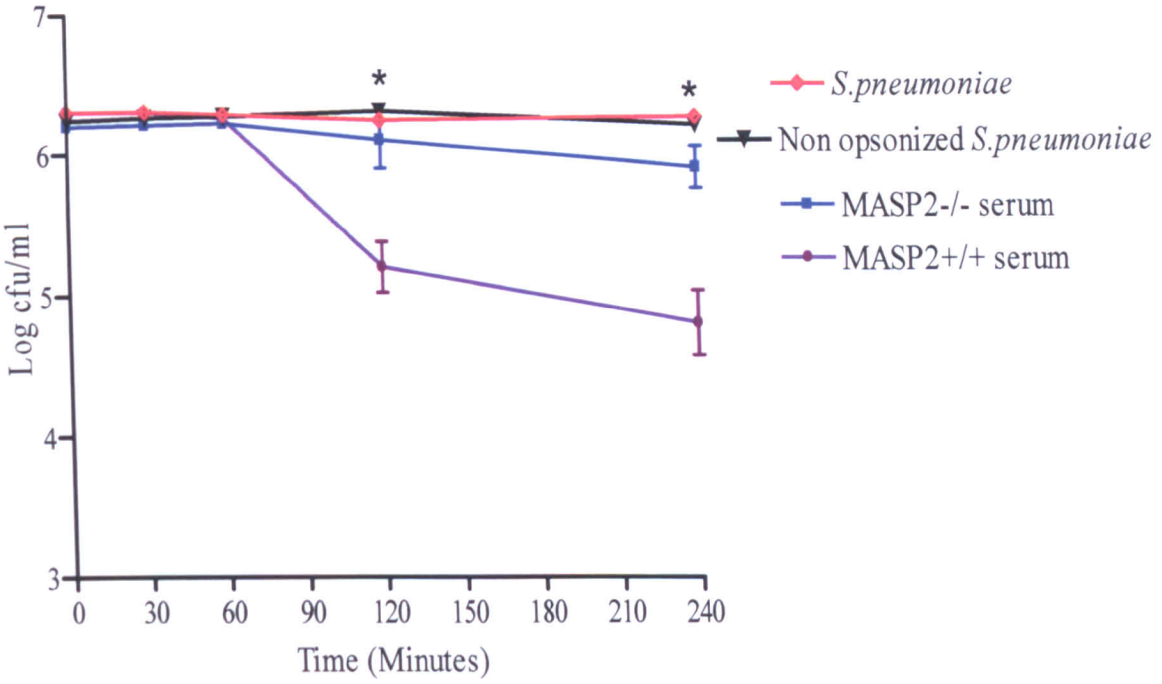


Figure 3.13: Neutrophil killing assay of *S. pneumoniae* D39 exposed to either MASP-2 deficient or MASP-2 sufficient sera or buffer only. The graph is showing the recovered CFUs in samples taken at the indicated time points following incubation with neutrophils. *S. pneumoniae* incubated in absence of PMNs was used as a control for the bacterial viability. Results are means (±SEM) of triplicate experiments ($^{*}P< 0.05$).

3.1.5 MASP2$^{-/-}$ mice exhibit increased mortality after intranasal infection with *S. pneumoniae* D39

The MASP-2 deficient mouse strain is the only presently available strain with a complete deficiency of the lectin pathway of complement activation. To assess the contribution of lectin pathway activation towards the host immune response to pneumococcal infection, MASP2$^{-/-}$ and MASP2$^{+/+}$ mice were challenged by intranasal infection with *S. pneumoniae* D39 dose (10^6 CFU). The survival of MASP-2 deficient mice was monitored and compared with that of MASP-2 sufficient littermate controls. At 72 hrs post infection, 85% of MASP2$^{-/-}$ mice died compared to only 25% of MASP2$^{+/+}$ mice (Fig. 3.14).

This result clearly demonstrates that lectin pathway plays a vital role in the host defense against *S. pneumoniae* infection.

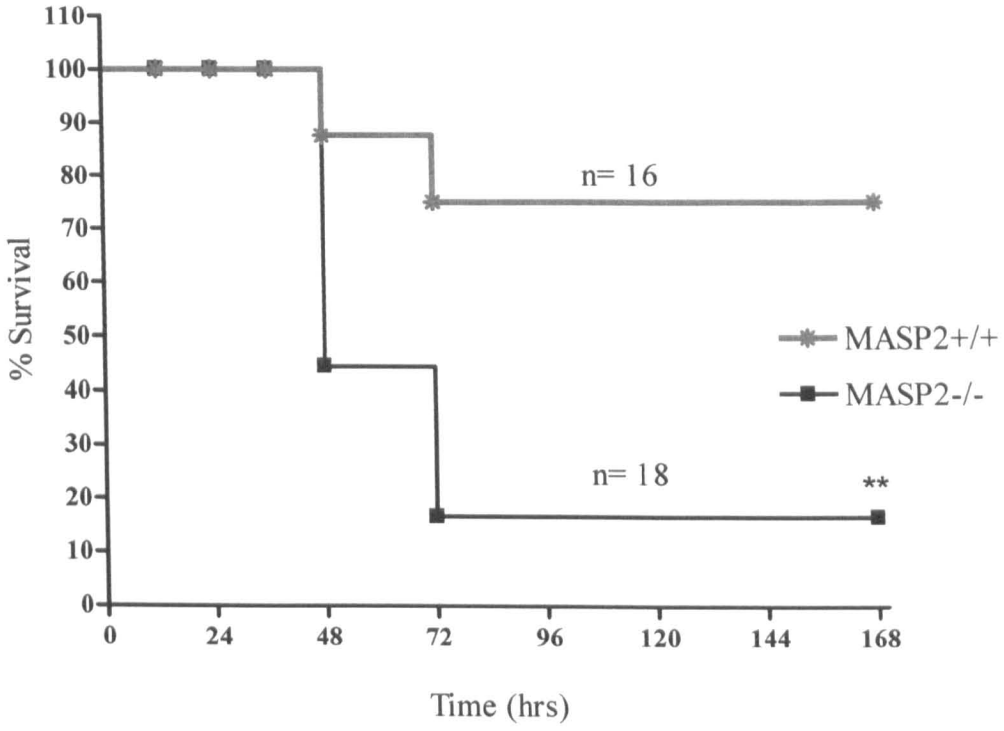


Figure 3.14: Kaplan Mayer survival curve for MASP-2 deficient mice and their wild-type littermates after intranasal infection with $1x10^6$ CFU of *S. pneumoniae* (D39). $^{**}P<0.01$.

3.1.6 Lung and blood infection of MASP2$^{-/-}$ and MASP2$^{+/+}$ mice after intranasal challenge with *S. pneumonia* D39

Following intranasal challenge with wild type D39 *S. pneumoniae,* mice were culled over different time points (12, 24, 48 and 72 hrs) post infection. MASP-2 deficient mice showed a significantly higher bacterial load in both lung tissue and blood compared to wild type littermates. In wild type mice, the bacterial counts decreased in lung tissue shortly after infection, then slightly increased at 48 and 72 hrs post infection when compared to the bacterial count recovered at zero time point. In contrast, the bacterial counts in lungs of

MASP-2 deficient mice increased continuously post infection up to the end of the observation period. Due to the high degree of mortality in MASP2$^{-/-}$ mice, observation and counting of recoverable CFUs was terminated at 48 hrs (Fig. 3.15). Likewise, the bacterial counts were significantly higher in blood of MASP2$^{-/-}$ mice compared to their wild type littermate controls at all time points after infection (Fig. 3.16). MASP2$^{-/-}$ mice developed the symptoms of severe infection at 48 hrs. In contrast, their wild type littermate controls recovered from the clinical symptoms of infection at this time. At 72 hrs, the MASP-2 sufficient mice started to clear the infection while the majority of MASP2$^{-/-}$ were moribund by 48 hrs post infection and had to be culled in line with the Home Office regulations.

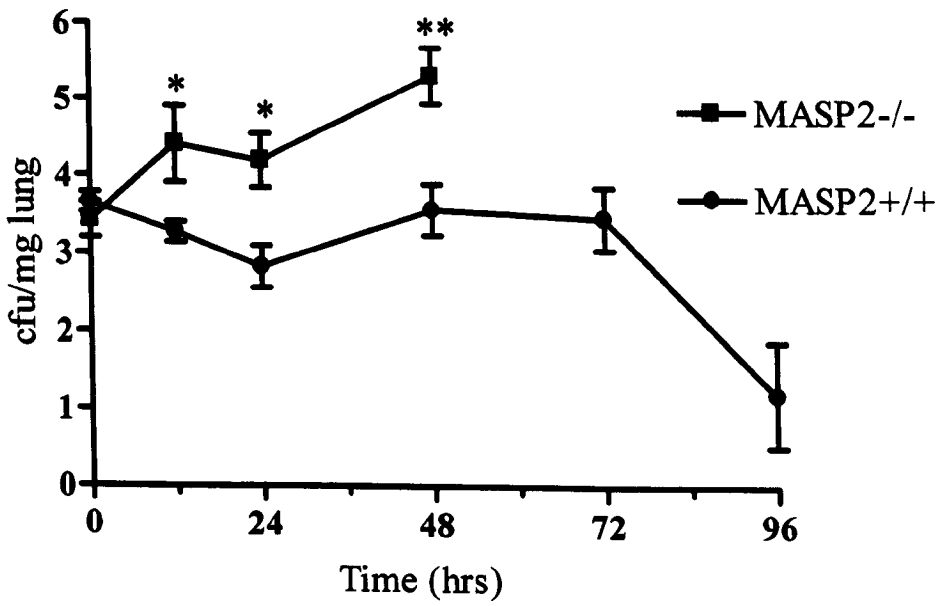


Figure 3.15: CFU counts in lung homogenates of MASP2$^{+/+}$ and MASP2$^{-/-}$ mice post intranasal infection with 10^6 CFU *S. pneumoniae* (D39). Ten mice (5 WT and 5 MASP2$^{-/-}$) were sacrificed at each time point. Results are means (±SEM). *$p<0.05$; **$p<0.01$.

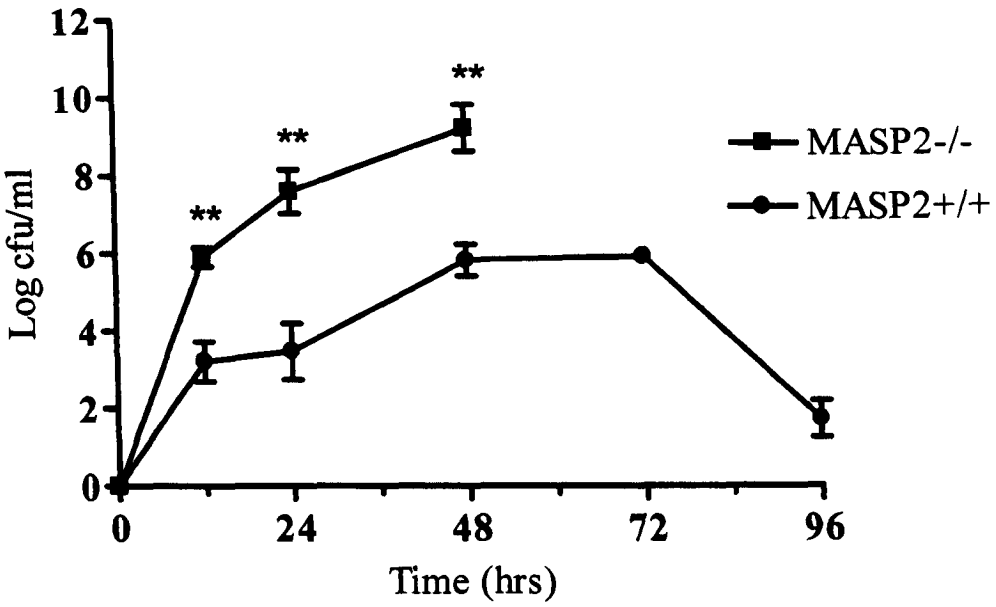


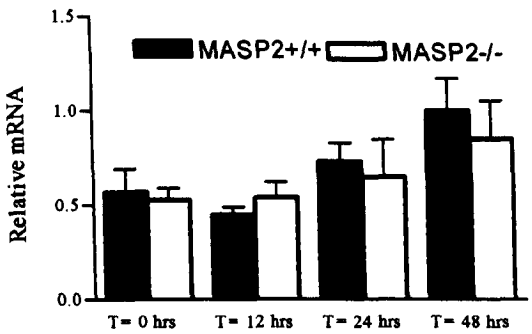
Figure 3.16: CFU counts in blood of MASP-$2^{+/+}$ and MASP-$2^{-/-}$ mice post intranasal infection with 10^6 CFU *S. pneumoniae* (D39). Ten mice (5 WT and 5 $MASP2^{-/-}$) were sacrificed at each time point. Results are means (±SEM). **$p<0.01$.

3.1.7 mRNA expression profiles in mouse lung tissues post *S. pneumoniae* infection

3.1.7.1 mRNA expression for complement components

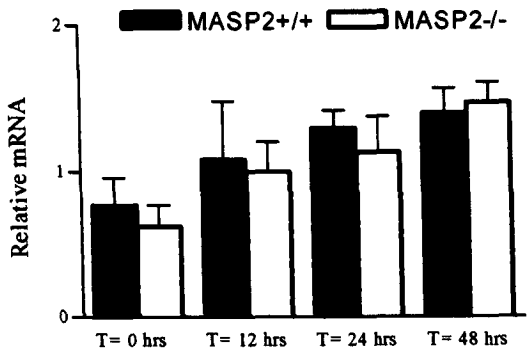
mRNA expression profiles of complement genes in tissue samples taken from organs of experimental animals following *S. pneumoniae* infection including C3, C4, C1q, MBL-A and MBL-C were quantified at different time points after infection using a Light Cycler based qRT-PCR technique. The results revealed that C3, C4 and C1q mRNA were slightly up-regulated 24 hrs post infection with no significant difference observed between $MASP2^{+/+}$ and $MASP2^{-/-}$ mice. While MBL-C mRNA level showed no marked increase during the infection, MBL-A showed a significant increase 12 hrs post infection with no significance difference between $MASP2^{+/+}$ and $MASP2^{-/-}$ mice (Fig. 3.17).

A



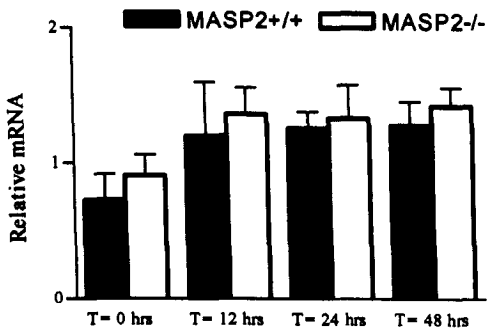
Relative C1q mRNA expression in mouse lung tissue after *S. pneumoniae* infection

B



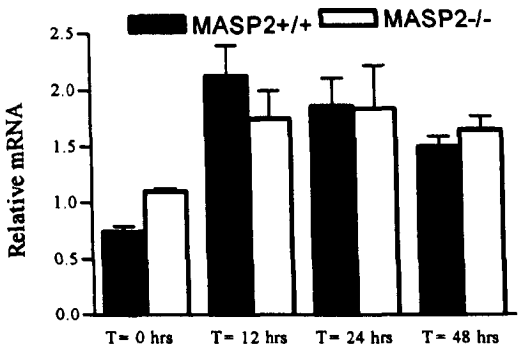
Relative C3 mRNA expression in mouse lung tissue after *S. pneumoniae* infection

C



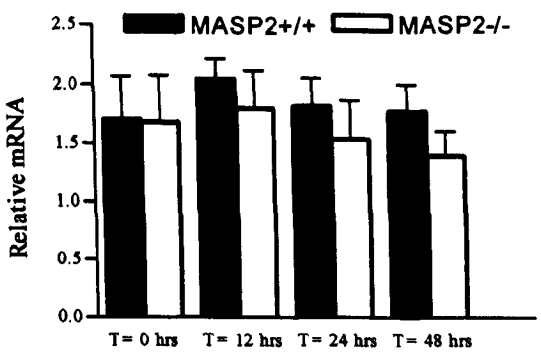
Relative C4 mRNA expression in mouse lung tissues after *S. pneumoniae* infection

D



Relative MBL-A mRNA expression in mouse liver tissues after *S. pneumoniae* infection

E

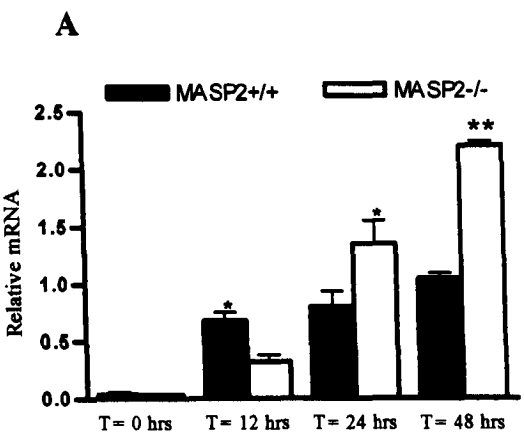


Relative MBL-C mRNA expression in mouse liver tissues after *S.pneumoniae* infection

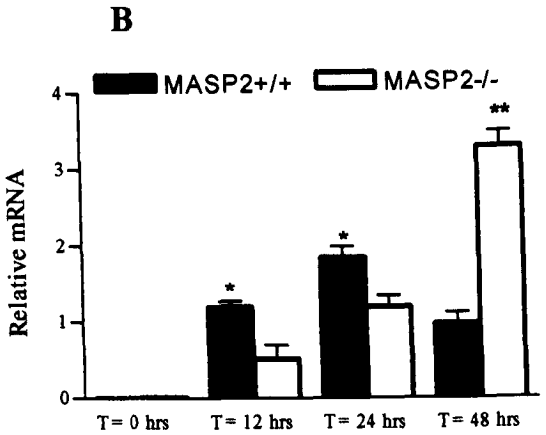
Figure 3.17: mRNA expression profile of complement genes in lung and liver tissues of wild type and MASP2$^{-/-}$ mice after intranasal infection with *S. pneumoniae* D39 over different time points. n=5 per each time point. Results are means (±SEM).

3.1.7.2 mRNA expression profiles of inflammatory cytokines

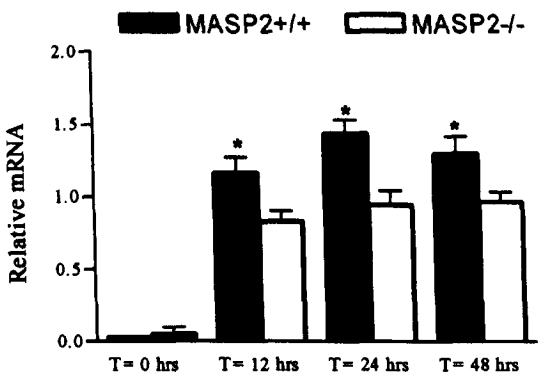
The results of the real time PCR analysis of mRNA expression profiles of cytokines in lung tissues showed that TNF-α expression was significantly higher in wild type mice at 12 hrs than the $MASP2^{-/-}$. However, levels of expression at 24 and 48 hr were significantly higher in $MASP2^{-/-}$ mice. MIP-2 mRNA expression significantly increased in lungs of wild type mice at 12 and 24 hrs while the levels of MIP-2 mRNA significantly increased in $MASP2^{-/-}$ mice compared to the wild type controls at 48 hrs. Interestingly, the expression levels of IL-1β mRNA were significantly higher in wild type mice than in $MASP2^{-/-}$ mice over all time points. IL-6 mRNA levels in lungs of $MASP2^{-/-}$ mice were significantly higher than those of $MASP2^{+/+}$ mice at 24 and 48 hrs post infection. The levels of INF-γ mRNA expression increased directly post infection in both $MASP2^{-/-}$ and $MASP2^{+/+}$ mice with no significant difference between both groups. Finally, no significant difference in the expression level of IL-10 mRNA was observed between both groups through out the course of infection (Fig. 3.18).



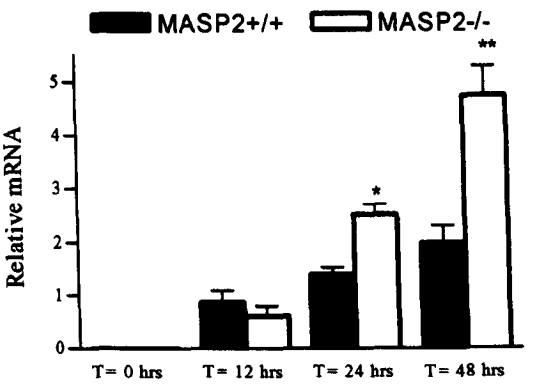
Relative TNFα mRNA expression levels in mouse lung tissues after infection



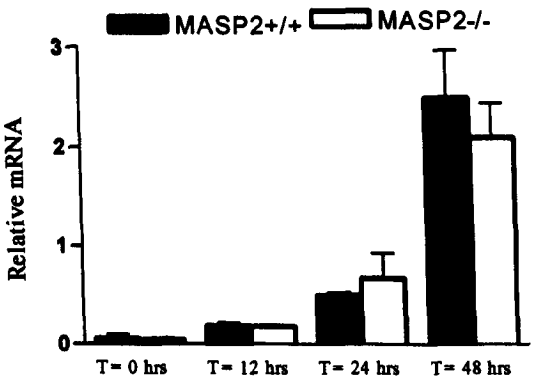
Relative MIP-2 mRNA expression levels in mouse lung tissues after infection



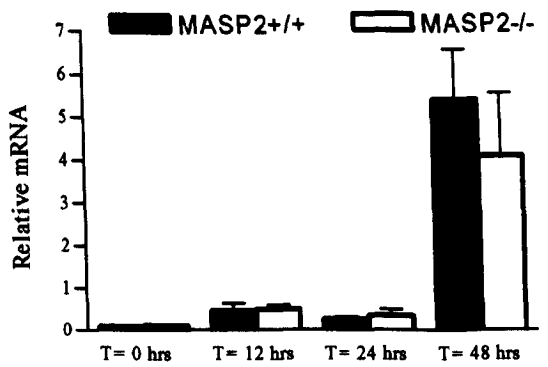
Relative IL-1β mRNA expression levels in mouse lung tissues after infection.



Relative IL-6 mRNA expression levels in mouse lung tissues after infection.



Relative INF-γ mRNA expression levels in mouse lung tissues after infection.



Relative IL-10 mRNA expression levels in mouse lung tissues after infection.

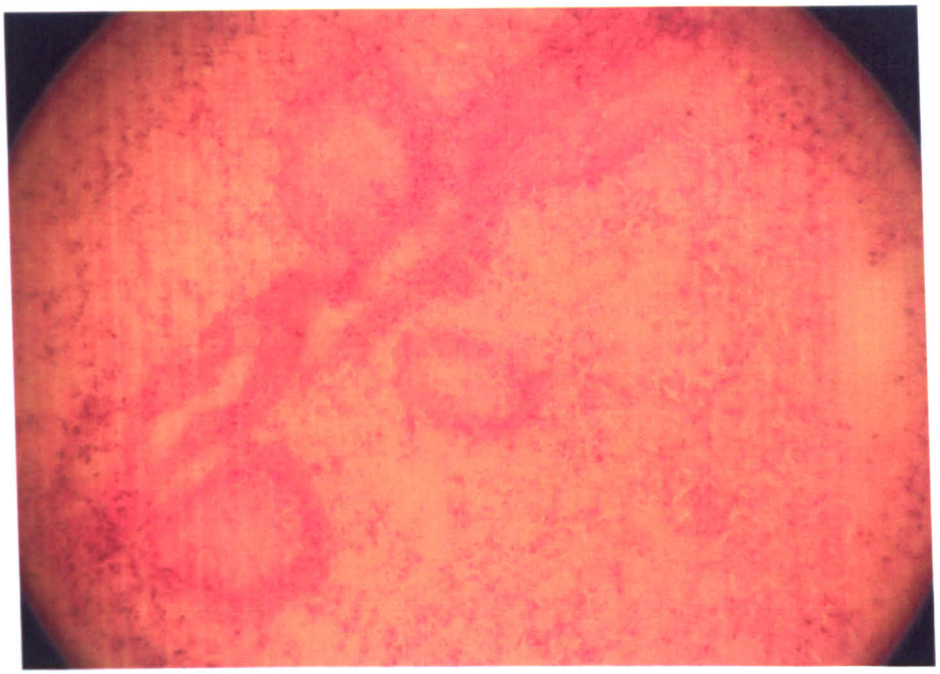
Figure 3.18: Relative mRNA expression of inflammatory mediators in wild type and MASP2$^{-/-}$ mice lung tissue over different time points after intranasal infection with *S. pneumoniae* D39. n=5 per each time point. Results are means (±SEM). *p<0.05; **p<0.01

3.1.8 Histological examination of lung tissue

Histological analysis of lung tissue sections from wild type and MASP2$^{-/-}$ mice infected with *S. pneumoniae* showed no significant difference in the pathological and histological examination at time zero (Fig. 3.19). However, at 24 hours, rapid and early recruitment of leukocyte into the lungs of wild type mice was evident when compared to the lungs of MASP2$^{-/-}$ mice. The inflammatory cells infiltrated into the bronchioles and the peri-vascular areas close to these bronchioles spread into the lung tissues of the wild type mice. In contrast, the lung section from MASP2$^{-/-}$ mice showed restricted infiltration of leukocyte into the infected bronchioles and peri-vascular areas (Fig. 3.20).

By 48 hrs post-infection, the histological changes seen in the lungs of MASP2$^{-/-}$ mice showed heavy neutrophil infiltration compared to the lungs of wild type mice. Bronchiole wall thickening was significantly increased in MASP2$^{-/-}$ mice, and solid fibrous tissue and exudates filling the bronchioles and alveolar spaces had appeared. Additionally, cellular infiltration at this time point was increased, with infiltration of inflammatory cells from bronchioles and peri-vascular areas into the surrounding lung parenchyma with several focal areas of consolidation becoming larger and more diffuse. During this period of infection, inflammation and tissue injury had encompassed nearly the entire lung surface. On the other hand, at 48 hrs post infection, the lungs of wild type mice exhibited a limited infiltration of the inflammatory cells into the lung bronchioles and into the lung parenchyma. This limitation was associated with a lower level of pathological tissue damage (Fig. 3.21).

A



B

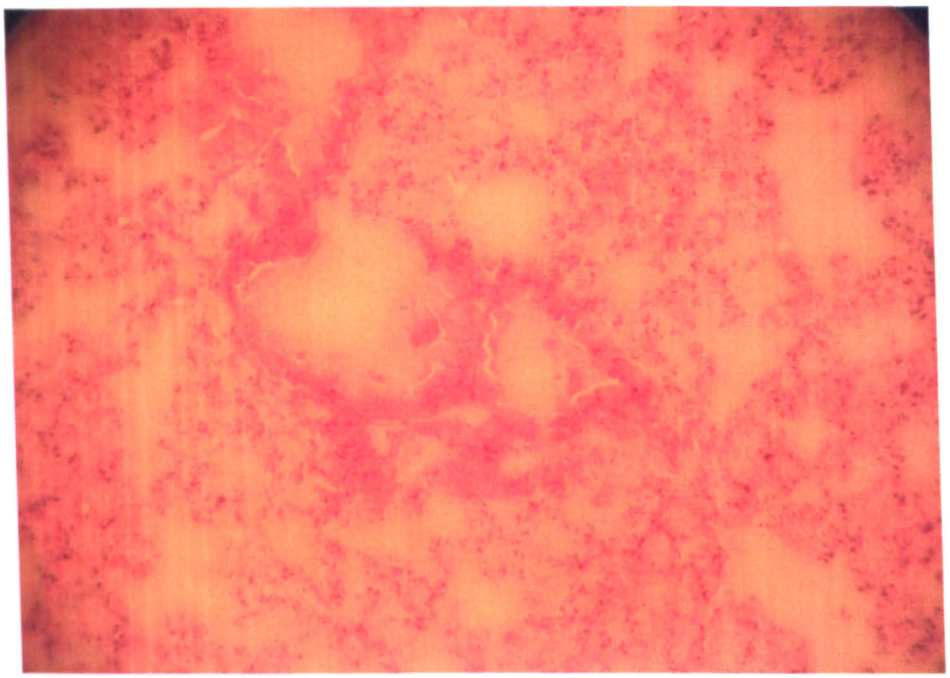


Figure 3.19: Light microscopy of lung tissue stained by Eosin & Haematoxylin from (A) $MASP2^{+/+}$ mice and (B) $MASP2^{-/-}$ mice infected with 10^6 CFU of *S. pneumoniae* at zero hrs post-infection. Magnification x 100.

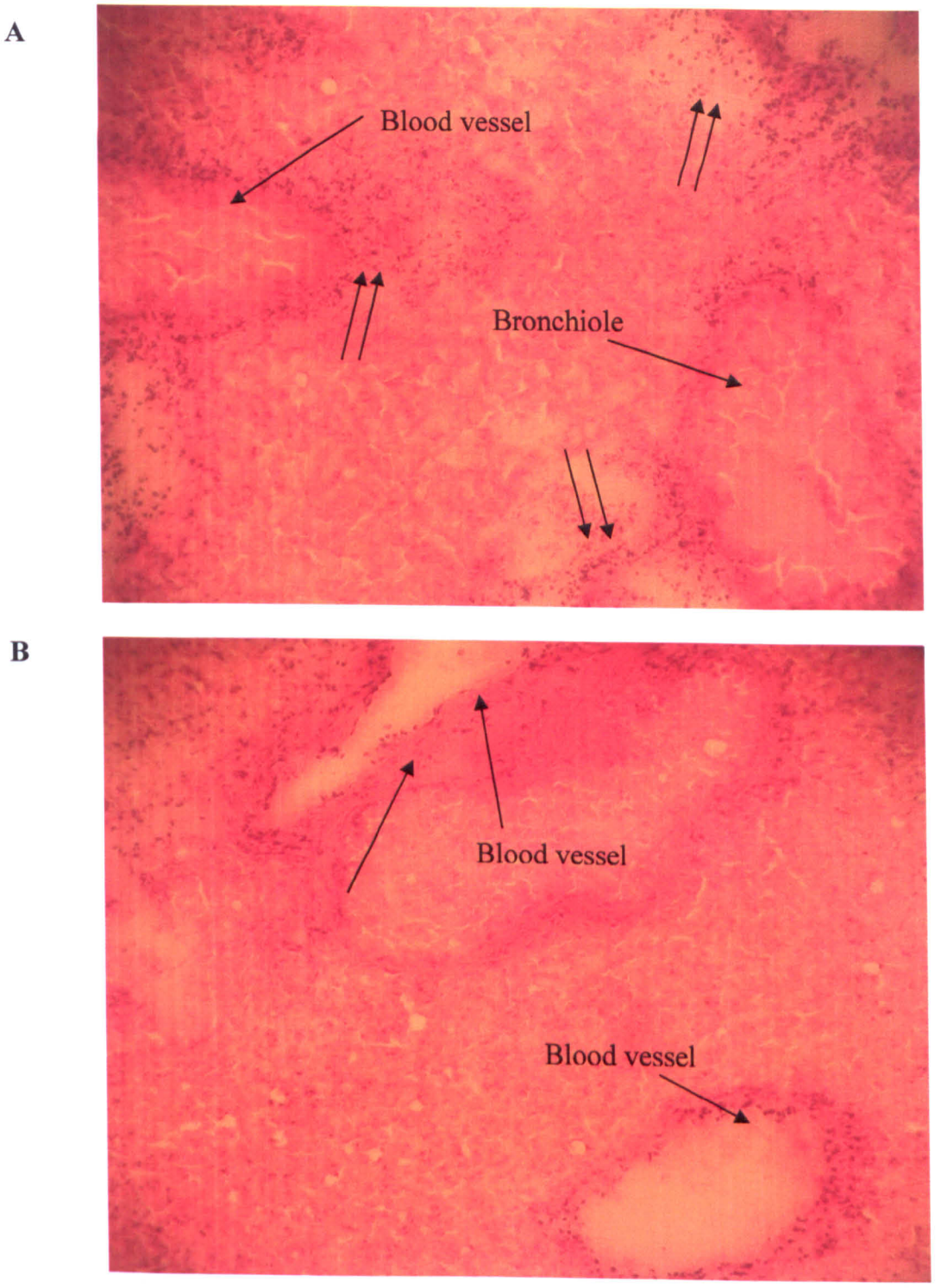


Figure 3.20: Light microscopy of lung tissue stained by Eosin & Haematoxylin from (A) $MASP2^{+/+}$ mice and (B) $MASP2^{-/-}$ mice infected with 10^6 CFU of *S. pneumoniae* at 24 hrs post-infection. Magnification x 100.

Single arrows indicate slight leukocyte infiltration into infected bronchioles.

Double arrows indicate medium leukocyte infiltration into infected bronchioles and lung tissues.

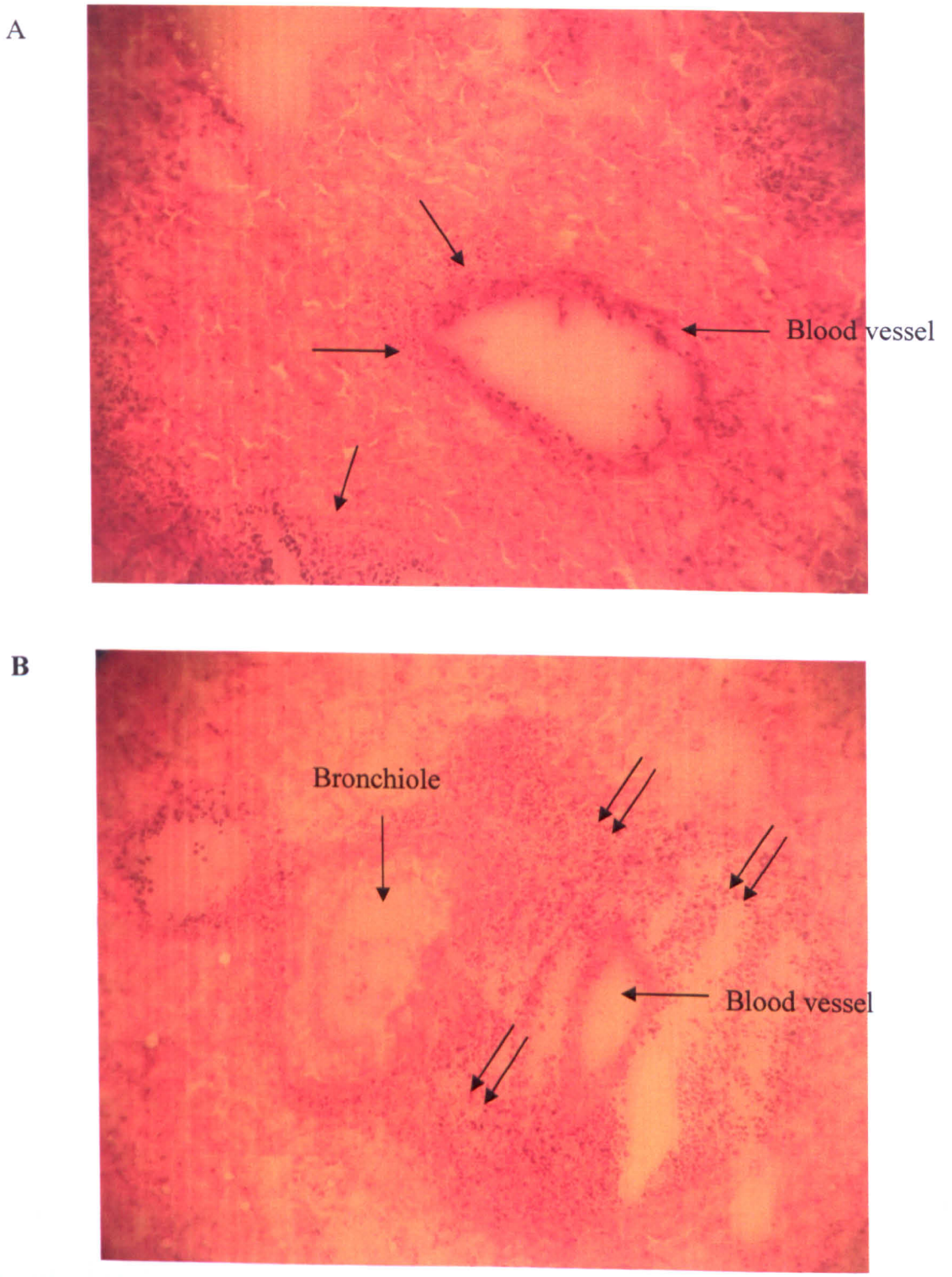


Figure 3.21: Light microscopy of lung tissue stained by Eosin & Haematoxylin from (A) $MASP2^{+/+}$ mice and (B) $MASP2^{-/-}$ mice infected with 10^6 CFU of *S. pneumoniae* at 48 hrs post-infection. Magnification x 100.

Single arrows indicate slight leukocyte infiltration of infected bronchioles

Double arrows indicate heavy leukocyte infiltration in infected bronchioles and lung tissues

Discussion

Streptococcus pneumoniae (the pneumococcus) is an important bacterial pathogen causing serious infectious diseases in man including pneumonia, septicemia, and meningitis (Obaro and Adegbola, 2002). The innate immune response provides a critical first line of defense against infection with bacterial pathogens, such as *S. pneumoniae.*

Several studies have reported the essential role of complement in protection against *S. pneumoniae* infection (Tu *et al.*, 1999; Brown *et al.*, 2002; Yuste *et al* 2005). Due to the lack of available models of lectin pathway deficiency the role of the lectin pathway in pneumococcus infection has not been investigated.

This study aimed to characterise the role of the lectin pathway of complement activation in experimental models of *S. pneumoniae* infection and of poly-microbial peritonitis using the only available murine model with complete lectin pathway deficiency, a mouse strain with a targeted deletion of the lectin pathway specific serine protease MASP-2. In addition, the interaction between the murine lectin pathway recognition molecules MBL-A, MBL-C and ficolin-A and S. *pneumoniae* was studied.

The results indicated that MBL-A did not bind to *S. pneumoniae* while MBL-C showed only low binding affinity. The weak binding of MBL-C and the inability of MBL-A to bind *S. pneumoniae* D39 was in parallel to absence of human MBL binding to different serotypes of *S. pneumoniae* (Krarup *et al.*, 2005). Absence of MBL binding to *S. pneumoniae* capsular polysaccharides is possibly due to absence of free 3-OH and 4-OH groups in hexose rings, which

form the MBL-binding motifs (Krarup *et al.*, 2005). In contrast, Ficolin-A showed a strong binding capacity to *S. pneumoniae* D39 and this finding was also in parallel with human L-ficolin binding to different strains of *S. pneumoniae* (Krarup *et al.*, 2005). The observed binding may be due to presence of N-acetylated glucosamine residues in the pneumococcus capsule of all strains (Sahly *et al.*, 2008). Opsonisation of bacteria by ficolins has been shown to increase complement C4 and C3 deposition which will potentially help in the phagocytosis of bacteria by leukocytes (Matsushita *et al.*, 2000). Binding of either MBL-C or Ficolin-A to *S. pneumoniae* may be critical step towards the clearance of these invading bacteria.

Interestingly, C4 deposition could not be detected on *S. pneumoniae* D39 and on several other strains (S3, S4, S6, and S14). This finding was in agreement with previous studies reporting the absence of C4 deposition on *S. pneumoniae* (Krarup *et al.*, 2005; Li *et al.*, 2007). The observed C4 inhibition on the surface of *S. pneumoniae* is mainly due to pneumococcal surface protein A (PspA) which is a potent virulence factor produced by all *S. pneumoniae* strains (Li *et al.*, 2007). In a recent study C4 deposition was completely inhibited on the surface of *S. pneumoniae* D39. But when using a mutant strain deficient on PspA a strong C4 deposition was observed indicating the vital role of this virulence protein in protecting the pneumococcus against complement attack (Li *et al.*, 2007).

Surprisingly, even in absence of C4 deposition it was possible to detect a strong C3 deposition on the surface of different strains of pneumococci. Due to the high serum dilution used (1/80), this C3 deposition could not have been achieved via the alternative pathway activation, as the alternative pathway functional activity is rendered dysfunctional at dilutions higher than 1/20. This

indicates that C3 is activated in a C4 independent manner. To confirm this finding, C3 deposition on *S. pneumoniae* D39 was assessed using $C1q^{-/-}$, $fB^{-/-}$ and $C4^{-/-}$, $MASP2^{-/-}$, $MASP1/3^{-/-}$ and wild type sera. As expected, a significant C3 deposition from all sera included in this study was detected except $MASP\text{-}2^{-/-}$ serum which showed no C3 deposition. This finding demonstrated for the first time that C3 deposition on *S. pneumoniae* is MASP-2 dependent, indicating that C4 deficiency can be bypassed via a novel MASP-2 dependent C3 cleavage mechanism.

The diminished role of the alternative pathway in the protection against *S. pneumoniae* infection can be explained through a mechanism by which the bacterial surface component pneumococcal surface protein C (PspC) binds to the complement regulatory protein factor H which decays the alternative pathway C3bBb and C3bBb (Bb)n complexes and serves as a cofactor in the factor I mediated conversion of C3b to iC3b (Dave *et al.*, 2001; Jarva *et al.*, 2002). In addition, it appears that pneumococci protect themselves from the classical pathway mediated complement activation through PspA which inhibits C3 deposition through the classical pathway by either inhibiting C1q binding to *S. pneumoniae* (Li *et al.*, 2007) or inhibiting C4b deposition in close proximity to the bacterial surface (this work). Hence, the pathogen is protected from the complement attack through both the classical and the alternative pathway.

Opsonophagocytosis and killing of pathogens by resident alveolar macrophages is a major mechanism in the clearance of invading bacteria from the lung. As resident macrophages can not phagocytose most strains of *S. pneumoniae*, recruited PMNs play the main role in clearance of all strains

of *S. pneumoniae* from the alveolar area (Coonrod *et al.*, 1987). Several previous studies showed that C3 deposition on *S. pneumoniae* plays a vital role in opsonisation of the pneumococcus which crucially facilitates internalisation and bacterial killing by neutrophil (Brown *et al.*, 1981; Gordon *et al.*, 2000; Martner *et al.*, 2008). Impaired complement activation on the surface of the pathogens results in increase susceptibility to infection, due to reduced C3b-mediated clearance of bacteria by opsonophagocytosis or decrease proinflammatory response following infection (Brown *et al.*, 1981; Guo *et al.*, 2005). The results obtained from the neutrophil killing assay of *S. pneumoniae* showed that in serum from MASP2$^{-/-}$ mice, the bactericidal activity of neutrophils was significantly impaired when compared to pneumococci opsonised with MASP-2 sufficient serum. These results were somewhat not surprising as the opsonisation study showed that C3 deposition on *S. pneumoniae* is lectin pathway dependent and hence, no C3b would be deposited on the pneumococcus in MASP-2$^{-/-}$ serum resulting in C3b mediated phagocytosis being significantly impaired.

Several previous studies established the crucial rule of the complement system to innate immune response against *S. pneumoniae* infection either in animal models with selective gene targeted complement deficiencies or in humans with inherited complement deficiencies. In regard to this, an increased susceptibility to *S. pneumoniae* has been demonstrated in patients with C2 deficiency (Sampson *et al.*, 1982) and in patients with a low serum level of C3 (Dee *et al.*, 1977).

Intra-peritoneal injection of mice with cobra venom factor (CVF) resulted in a rapid depletion of complement components in plasma and CVF treated mice

failed to clear *S. pneumoniae* from blood stream following intra-peritoneal infection compared to the control mice (Winkelstein *et al.*, 1975).

Different gene targeted mouse models with specific complement deficiencies, helped to study the role of complement in the protection against *S. pneumoniae*. Brown *et al.* reported in 2002 that C1q deficient mice showed a significant increase in the rate of mortality when compared to control mice using a model of intranasal infection with *S. pneumoniae*. $C1q^{-/-}$ mice died from a significantly higher bacterial load in blood and lung tissues. The authors also observed that deficiency in $C4^{-/-}$ (deficient in classical and lectin pathway) showed no significant difference in mortality when compared to $C1q^{-/-}$ mice after *S. pneumoniae* infection and therefore they concluded that the classical pathway is the dominant complement pathway in the protection against *S. pneumoniae*.

In this study, intranasal challenge of $MASP2^{-/-}$ and $MASP2^{+/+}$ mice with *S. pneumoniae* clearly demonstrated that, $MASP2^{-/-}$ mice have a dramatically significant higher rate of mortality when compared to wild type mice after intranasal infection with *S. pneumoniae* D39 with a mortality of approximately 85% compared to 25% of their MASP-2 sufficient control littermates after 72 hrs of infection. In line with this, $MASP2^{-/-}$ mice showed a significantly higher bacterial load in blood and lung tissues during the observation time of this experiment. In contrast, the majority of MASP-2 sufficient mice survived the infection and started to clear the bacteria after the 3rd day following infection. These results showed that, the lectin pathway of complement activation has a major role in the immune defence against pneumococcal infection. The results of the phagocytosis assay underlined the

in vivo finding as bacteria opsonised with MASP-$2^{+/+}$ serum showed a significant increase in phagocytosis and killing by PMNs while bacteria opsonised by MASP-$2^{-/-}$ serum were not internalised and killed by PMNs. In addition, the ELISA results showed that C3 deposition on *S. pneumoniae* D39 was greatly impaired in MASP-$2^{-/-}$ serum so it was concluded that, the inefficient opsonisation of *S. pneumoniae* by the lectin pathway deficient serum is the molecular reason responsible for the failure of MASP$2^{-/-}$ mice to clear bacteria from blood and lung tissues which in it self may easily explain the severe phenotype of MASP-2 deficiency in *S. pneumoniae* infection.

The data from C4 and C3 deposition on *S. pneumoniae* challenge the conclusion of Brown *et al* that the classical pathway provides by far the most important protective mechanism against *S. pneumoniae* infection (Brown *et al.*, 2002) and the previous publication suggesting that the defence mechanism to *S. pneumoniae* is C4 dependent (Mold *et al.*, 2002). The present study demonstrated that C4 deposition on the surface of *S. pneumoniae* is not essentially required for the complement dependent clearance of *S. pneumoniae*. The most surprising and novel result is that C3 deposition via the lectin pathway activity is actually not strictly C4 dependent but is MASP-2 dependent. According to this, the results indicate the crucial role of the lectin pathway of complement activation in the immune response to *S. pneumoniae* infection and challenge the previous hypothesis published by Brown *et al.* (Brown *et al.*, 2002) according to which the classical pathway is the predominant protective pathway against *S. pneumoniae* infection.

The strong conclusion that the lectin pathway only has a marginal role in the fight against *S. pneumoniae* infection (Brown *et al.*, 2002) was based on the

observation that C1q deficient mice and C4 deficient mice showed no or little differences in their susceptibility to infection and according to the text book view both, the classical and the lectin pathway utilise C4 to subsequently form C3 and C5 convertases. As the phenotype of C1q and C4 deficiency shows only a marginal difference in severity of infection, so it was concluded that the additional deficiency of the lectin pathway in C4 deficient mice was accounted for a marginal difference (if at all) and therefore the lectin pathway can not be all that important in fighting *S. pneumoniae* infection. The data however, clearly indicate that this conclusion is incorrect as; (i) the severity of pneumococcal infection is dramatically increased in lectin pathway deficient mice (ii) $C4^{-/-}$ mice showed a residual lectin pathway dependent activation of C3 (iii) An effective lectin pathway activation of complement occurs in total absence of C4 in a MASP-2 dependent manner and MASP-2 participates in a novel lectin pathway mediated C4-bypass activation of complement C3.

Proinflammatory responses are known to play a major role in antimicrobial immune defence within the lung (Kerr *et al.*, 2002). These results showed that $MASP2^{-/-}$ mice respond to *S. pneumoniae* infection with a lower mRNA expression of the proinflammatory mediator IL-1β when compared to the wild type mice indicating that the deficient lectin pathway of complement significantly reduces the complement-mediated inflammatory response and thereby decreases the clearance of bacteria. This finding is in agreement with a previous work which compared the cytokine levels in the serum of susceptible (CBA/Ca) and resistant (BALB/C) mice after intranasal infection with *S. pneumoniae* D39, showing that the resistant mice had a higher level of IL-1β in lung tissues and in BALF (Kerr *et al.*, 2002). Low expression levels of IL-1β were also observed in infected C3-deficient mice that did not survive

the infection when compared to their C3 sufficient controls (Rupprecht *et al.*, 2007). The results showed here are also in agreement with a recent study reporting that IL-1β deficient mice showed a higher rate of mortality when compared to their wild type controls with lung showing diffuse pneumonia with severe lung injury and impaired PMN recruitment (Kafka *et al.*, 2008).

TNF-α production is a critical upstream cytokine in pneumococcal pneumonia which in combination with IL-1 isoforms mediates neutrophil recruitment in murine models of pneumococcal pneumonia (Jones *et al.*, 2005). In a similar model of infection, TNF-α production peaks at 12 h after infection and contributes to bacterial clearance and survival (Bergeron *et al.*, 1998 and Rijneveld *et al.*, 2001). The intranasal model of infection showed significant elevated mRNA levels of TNF-α and macrophage inflammatory protein 2 (MIP-2) expression in lung tissues of wild type mice after 12 hours in comparison to $MASP2^{-/-}$ mice. Early expression of TNF-α and MIP-2 accelerates leukocyte infiltration into lung tissues which facilitate bacterial clearance (Neumann *et al.*, 1996). On the other hand, the majority of $MASP2^{-/-}$ mice died with significantly elevated levels of TNF-α at day three post infection in comparison to wild type mice. In agreement with Kerr *et al.* 2002, elevated level of TNF-α at the late stage of infection is a sign of tissue destruction and in most cases likely to be fatal. Excessive TNF-α production, via its effects on neutrophil activation, has been observed to contribute to lung injury in a variety of diseases (Windsor *et al.*, 1993; Linden *et al.*, 2005). TNF-α also stimulates the release of chemokines such as MIP-2 which has a well established role in the immune defence against bacterial pneumonia. Early expression of MIP-2 in lungs of $MASP2^{+/+}$ mice stimulates PMN influx into lung tissues after infection and promotes the clearance of bacteria from

lung tissues (Greenberger *et al.*, 1996). In addition, MASP2$^{-/-}$ mice showed a higher mRNA abundance of IL-6 and MIP-2 at a late stage (48 hrs) post infection. In a previous study, using a similar model of *S. pneumoniae* infection but different *S. pneumoniae* serotypes, those mice that survived the infection showed lower levels of IL-6 and MIP-2 at 48 hrs, while mice that succumbed to infection showed a higher levels of IL-6 and MIP-2 and increasing numbers of PMNs in the lung tissues with accelerated tissue injury and lung damage (Dallaire *et al.*, 2001).

Early recruitment of inflammatory cells into lung tissues is an important factor in controlling and clearing of *S. pneumoniae* infection (Martner *et al.*, 2008). Early expression of TNF-α, MIP-2 and IL-1β were observed in lungs of wild type mice after 12 hours from *S. pneumoniae* infection in comparison to MASP2$^{-/-}$ mice. Induction of inflammatory cytokines and chemokines immediately after infection mediated neutrophil recruitment into the lung tissues which then started to diffuse into the infected lung within 24 hours post infection and mediated clearance of bacteria. In contrast, MASP2$^{-/-}$ mice started to elicit a significant inflammatory response a long time after the wild type mice. Leukocyte recruitment into the lung tissues occurred as late as 24 hours post infection. At this time, the bacterial load in MASP2$^{-/-}$ mice was uncontrollable and the viable count started to increase dramatically. After 48 hours, the wild type mice showed a significant reduction in neutrophil recruitment into the lung, while the MASP2$^{-/-}$ mice showed a significantly higher level of neutrophil infiltration that released TNF-α which in turn caused lung injury and tissue damage.

To ensure that the lectin pathway deficiency in MASP-2 deficient mice did not affect the expression levels of mRNA of C1q, C3, C4, MBL-A and MBL-C before and after *S. pneumoniae* infection, the abundance of these complement component were quantified using Light Cycler based real time PCR. The results showed that there was no significant difference in the expression level between MASP-2 deficient mice and their littermates' sufficient mice before and during the time of infection.

In a clinical study, a low level of MBL was shown to have a limited role in the protection against *S. pneumoniae* infection (Endeman *et al.*, 2008). In contrast, another clinical study showed that the level of MBL was not correlated with the severity of *S.pneumoniae* infection during acute episodes or in the recovery phase (Perez-Castellano *et al.*, 2006). Also in an additional study the absence of the role of MBL in *S. pneumonia* infection was confirmed as the distribution of variant MBL alleles related to low MBL serum concentrations was similar among both the patients and healthy individuals and the MBL genotype was not associated with the infection outcome (Kronborg *et al.*, 2002).

In conclusion, the lectin pathway of complement was shown to have a pivotal role in innate immune response against *S. pneumoniae* infection. Deficiency in the lectin pathway significantly increased the susceptibility to infection and impaired the bacterial clearance from blood and lung. The results pointed to the conclusion that C3 opsonisation of *S. pneumoniae* is significantly impaired in absence of lectin pathway functional activity resulting in a phagocytic defect. My results also demonstrate that MASP-2 deficiency reduced the complement mediated inflammatory response to *S. pneumoniae* infection causing a crucial and significant delay in neutrophil recruitment into the lung tissues of $MASP2^{-/-}$ mice at the early stage of infection.

Chapter 4

Role of complement activation in poly-microbial septic peritonitis

Chapter 4
Role of complement activation in poly-microbial septic peritonitis

4.1 Introduction

A number of animal models of sepsis have been developed to study the course of septic peritonitis and severe systemic inflammation (Rittirsch *et al.*, 2007). Lipo-polysaccharides (LPS) are the outer membrane component of Gram-negative bacteria. Systemic administration of LPS either by intravenous infusion or intra-abdominal injection has been shown to cause sepsis-like syndrome similar to the pathological changes in patient with sepsis (Rittirsch *et al.*, 2007) and triggers high expression levels of proinflammatory cytokines through TLR-4 signalling (Remick *et al.*, 2000).

A widely used infection model is that of fecal pellet peritonitis, which induces sepsis by intra-abdominal administration of faeces (Browne and Leslie, 1976). This model has been replaced by a bacterial inoculum model, which induces peritonitis by intra-peritoneal administration of bacteria of single species such as *Bacteroides fragilis* or mixed number of bacteria such as *E. coli* plus *B. fragilis*. This model is more controllable and reproducible than the model of fecal administration (Nakatani *et al.*, 1996).

Colon ascendens stent peritonitis (CASP) is another experimental model of peritonitis used in mice. In this model, a stent is implanted in the ascending colon, which leads to continuous leakage of the bacteria into the abdominal cavity causing bacterial infection and sepsis. The rate of mortality of this

model depends on the size of the stent, the use of different stent sizes results in controlling the rate of mortality (Zantl *et al.*, 1998).

Caecal ligation and puncture (CLP) model is a model in which the cecum is ligated and punctured by a needle, leading to continuous leakage of bacteria into the abdominal cavity which reach the blood through the lymph drainage and then distributed into all the abdominal organs leading to multi-organ failure and septic shock (Eskandari *et al.*, 1992). CLP model mimics the course of sepsis observed in patients and induces an early hyper-inflammatory response followed by a pronounced hypo-inflammatory phase. During this phase, the animals are highly sensitive to bacterial challenges (Wichterman *et al.*, 1980).

The CLP model has been used as a model of sepsis in this study because it considered to be a clinically relevant model of peritonitis with bacteraemia (Stover *et al.*, 2008).

4.2 Results

4.2.1 Survival of MASP2$^{-/-}$ and their wild type littermate controls after CLP

MASP2$^{+/+}$ and MASP2$^{-/-}$ mice were subjected to CLP and the survival of the mice was monitored over a period of 14 days. No significant difference was observed in the survival time between the two groups with an over all mortality rate reaching approximately 75% (Fig. 4.1).

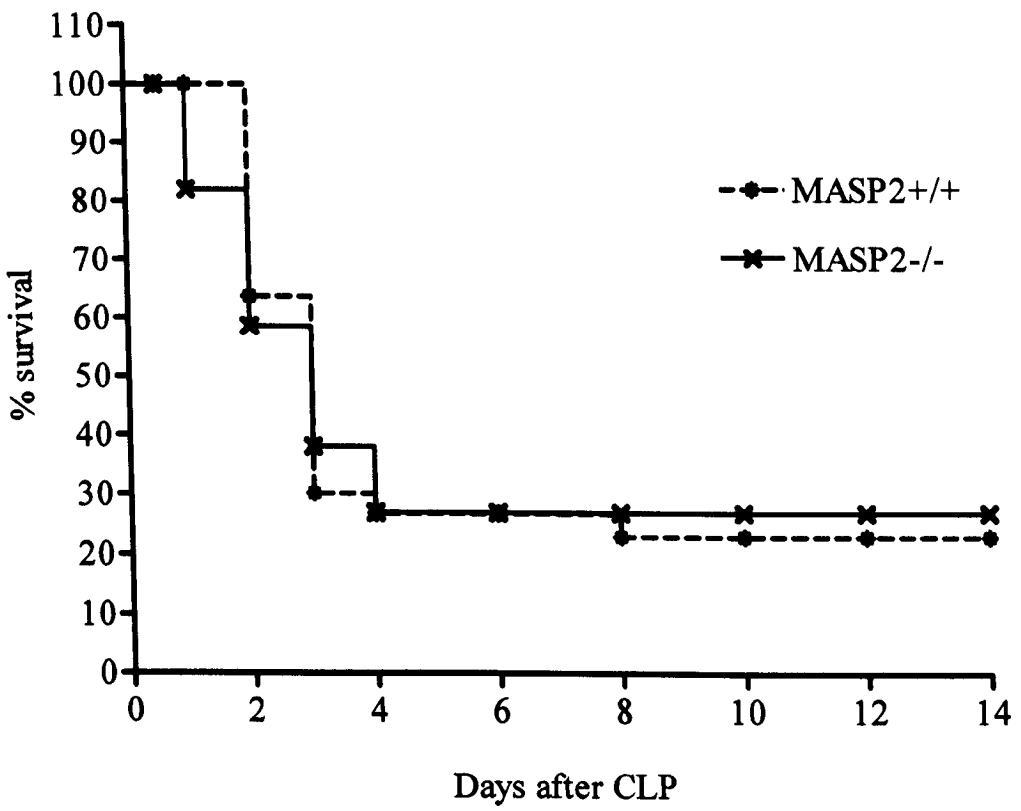


Figure 4.1: Kaplan Mayer Survival curve of MASP2$^{-/-}$ (n= 16) and wild type mice (n= 18) after CLP.

4.2.2 Bacterial load in peritoneal lavage after CLP

Despite the fact that both groups of mice tested showed no significant difference in their overall survival rate, the bacterial load in peritoneal lavage of MASP2$^{-/-}$ mice showed a significant higher number of recoverable bacteria than that of their wild type littermate controls (**p<0.01) (Fig. 4.2).

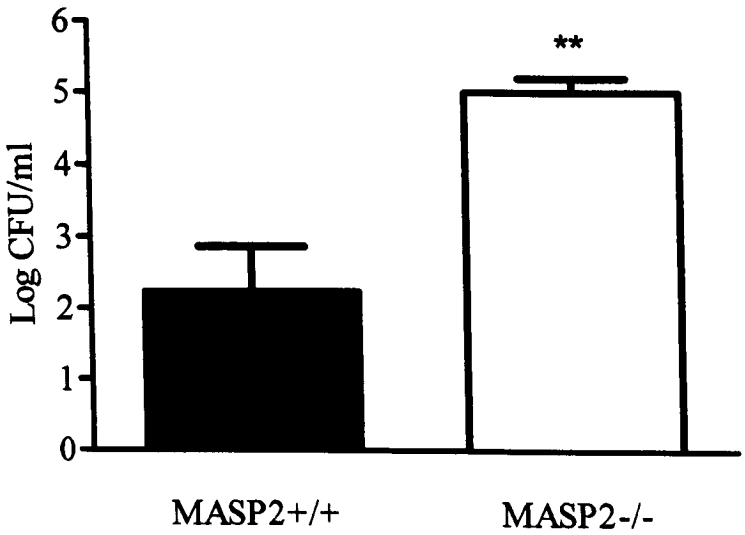


Figure 4.2: Bacterial load in peritoneal lavage from wild type (n=4) and MASP2$^{-/-}$ (n=4) after CLP. **p<0.01

4.2.3 Lectin pathway activation after CLP

In order to investigate the involvement of the lectin pathway in defence against poly-microbial peritonitis after CLP, a mannan dependent C4 cleavage assay was performed. This test specifically measures the degree of C4 deposition via the lectin pathway without interference from the classical pathway. C4 cleavage activity in serum from wild type mice showed a significant diminished activity after 16 hours of CLP in comparison to the control wild type mice (Fig. 4.3).

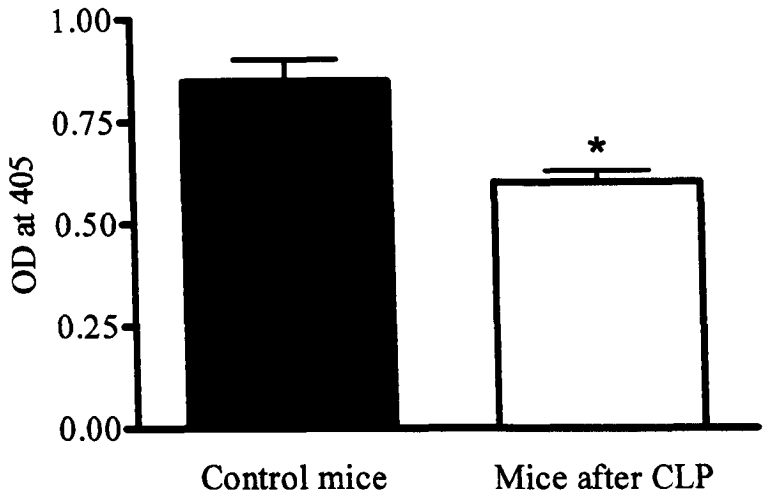


Figure 4.3: C4 deposition on mannan 16 hours after CLP.

Mannan dependent C4 deposition from control mice serum (n=3) was compared with C4 deposition from $MASP2^{+/+}$ mice after CLP (n=3). *p <0.05

4.2.4 mRNA expression profiles after CLP

4.2.4.1 mRNA expression profiles of complement genes

In order to investigate whether the decrease in Mannan dependent C4 deposition was due to impaired expression of C4 or due to low expression of the lectin pathway carbohydrate recognition molecules MBL-A and MBL-C, mRNA expression levels of these proteins were measured after CLP by Light Cycler analysis as described in the materials and methods. The expression level of C4 was significantly increased after CLP in spleen and liver whereas the expression level in lung, kidney and brain did not change (Fig. 4.4). Likewise, the expression level of both MBL-A and MBL-C in mouse liver was significantly increased after CLP (Fig. 4.5).

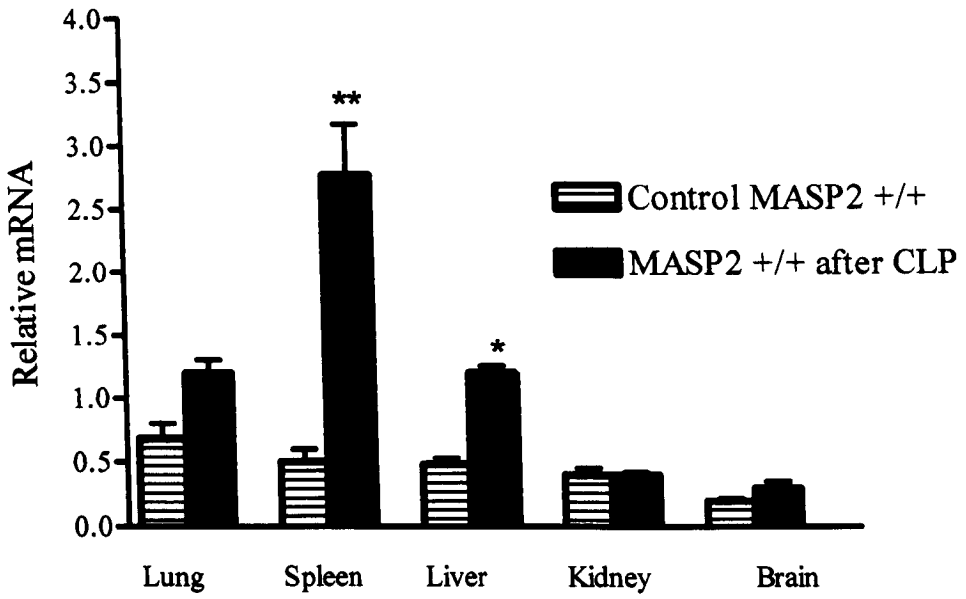


Figure 4.4: C4 mRNA expression levels in different wild type mice organs without CLP (n=3) and after CLP (n=3).*p<0.05, **p<0.01

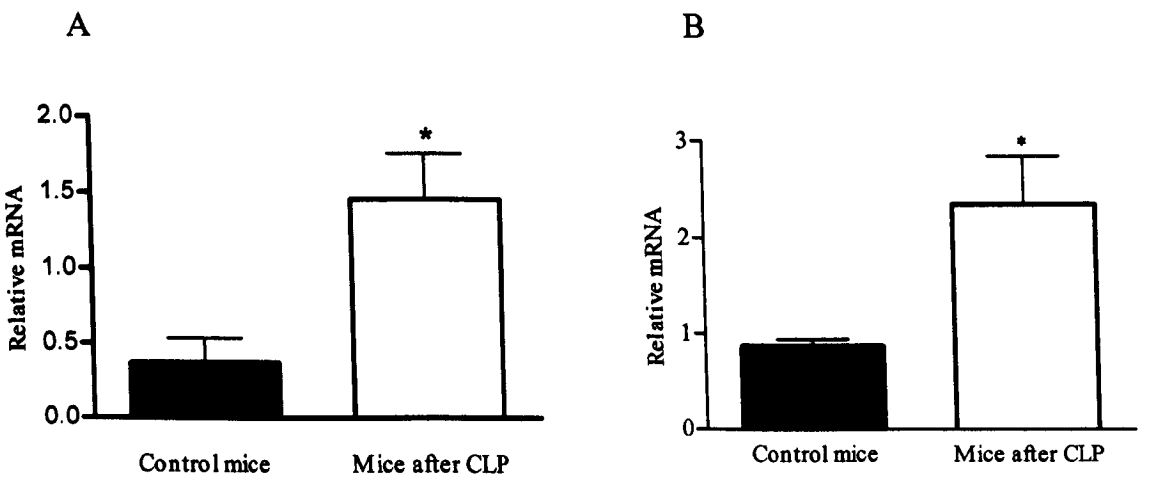


Figure 4.5: mRNA expression levels of MBL-A (A) and MBL-C (B) in liver tissues of control mice and mice after CLP. n= 3, *p<0.05.

4.2.4.2 Inflammatory cytokines mRNA expression profiles during sepsis

The high level of mortality between wild type mice (even though they showed a lower bacterial load than the MASP2$^{-/-}$ mice), encouraged us to investigate whether the inflammatory response may play a significant role in inducing mortality of these mice. Therefore mRNA expression levels of inflammatory cytokines were determined during sepsis using Light Cycler based qRT-PCR. The abundance of IL-1β, MIP-2 and TNF-α mRNA significantly increased 16 hrs after CLP in lungs and spleens of wild type mice compared to MASP2$^{-/-}$ mice, while the expression levels of these inflammatory mediators was not changed in liver, kidney and brain in both groups of mice tested. On the other hand, the expression level of the sepsis cytokine IL-6 was significantly increased in lung and spleen of MASP2$^{-/-}$ mice following CLP. Again the level of IL-6 in the liver, kidney and brain of both groups of mice remained unchanged. In this model of sepsis INF-γ mRNA expression was not induced in liver, kidney and brain of MASP2$^{-/-}$ and MASP2$^{+/+}$, while its level was significantly increased in lung and spleen of MASP2$^{-/-}$ when compared to their wild type littermate control mice during the course of CLP induced peritonitis (Fig. 4.6).

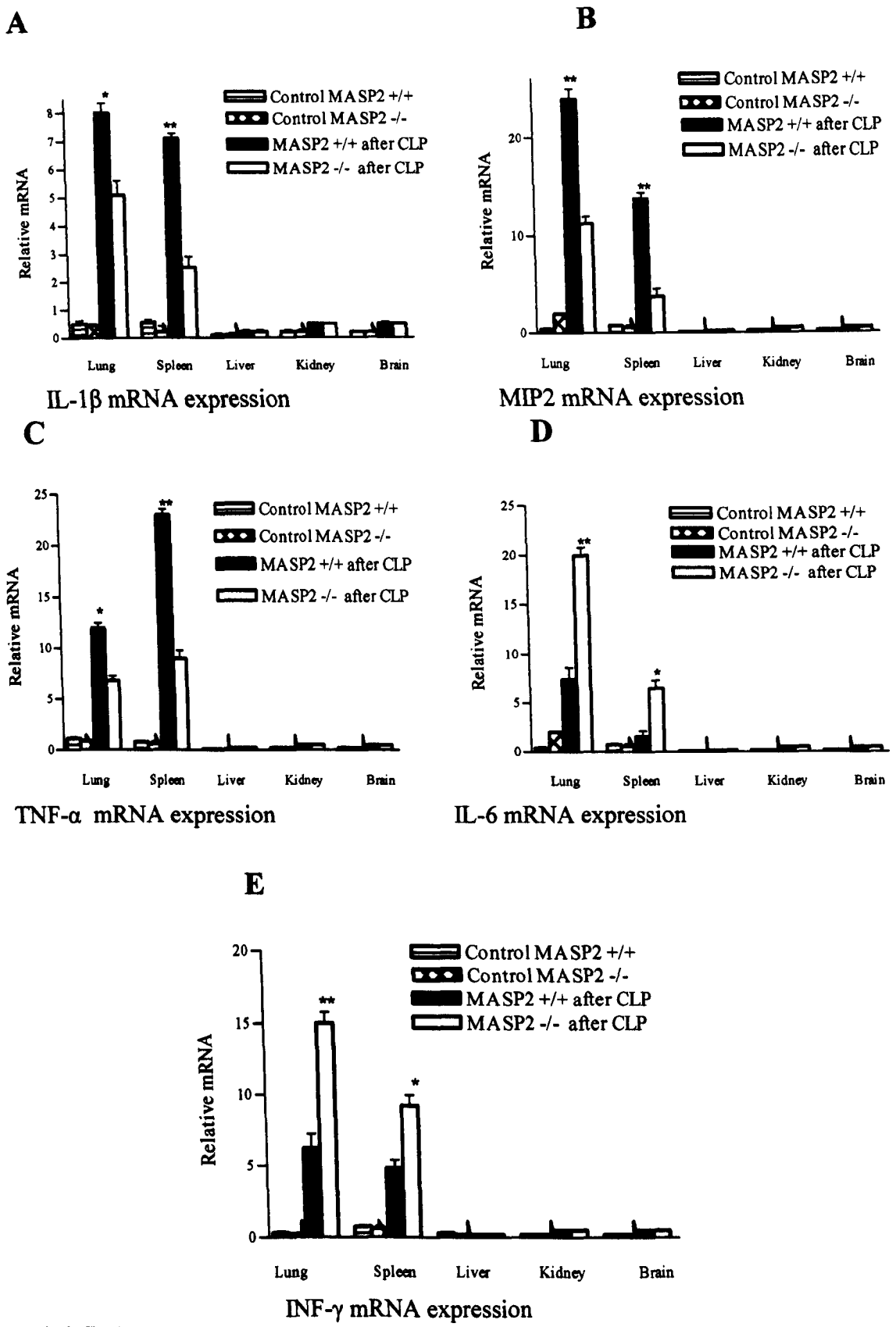


Figure 4.6: Relative mRNA expression of inflammatory mediators in different organs of $MASP^{+/+}$ and $MASP^{-/-}$ mice after CLP in comparison to control mice. n=3. Results are means ±SEM. *p<0.05; **p<0.01.

4.2.5 Serum concentration of TNF-α in mouse serum after CLP

To investigate whether the high level of TNF-α mRNA expression in lung and spleen of $MASP2^{+/+}$ mice after CLP was associated with a correspondingly increased serum concentration, the serum level of TNF-α was quantified by sandwich ELISA from the same mice assessed for TNF-α mRNA expression. The results showed a significantly higher serum concentration of TNF-α in $MASP2^{+/+}$ after CLP in contrast to the $MASP2^{-/-}$ mice, where the TNF-α serum level remained nearly unaltered (Fig. 4.7).

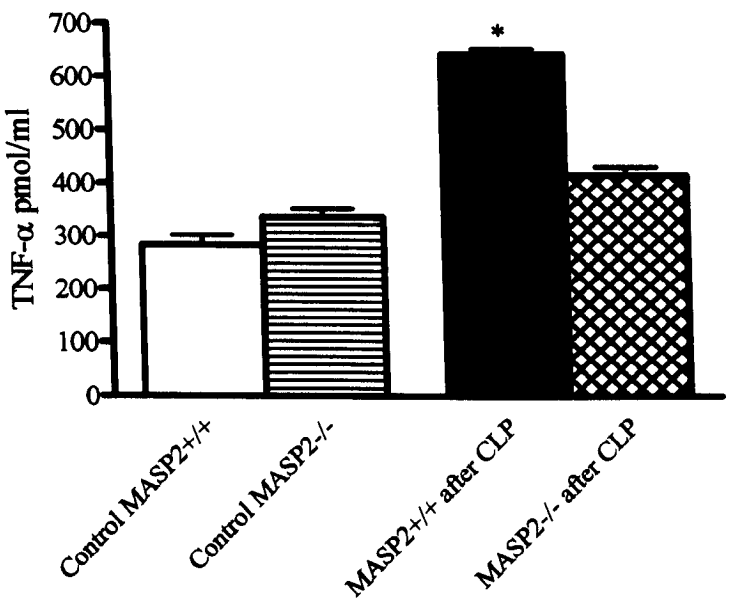


Figure 4.7: Serum concentration of TNF-α in $MASP2^{-/-}$ and $MASP2^{+/+}$ 16 hrs after CLP. (n= 3), *$p<0.05$

Discussion

Poly-microbial peritonitis induced by CLP is considered to be a relevant animal model to investigate the role of different mechanisms of the innate immune response during bacterial sepsis (Stover *et al.*, 2008). The murine CLP model mimics the course of bacterial peritonitis in humans with an early hyper-inflammatory phase followed by a subsequent hypo-inflammatory phase during which the animals are extremely sensitive to bacterial super-infections (Echtenacher *et al.*, 1996). Sepsis is characterised by massive infiltration of neutrophils into the peritoneum which aim to fight the infection through clearance of the invading pathogens. When the innate immune defence fails to clear the invading bacteria from the peritoneum, the pathogens enter into the blood stream and disseminate to the host organs, causing an exaggerated inflammatory response (Riedemann *et al.*, 2003). The inflammatory response in mice following CLP mimics the clinically critical stage of immuno-paralysis observed in patient during poly-microbial sepsis (Volk *et al.*, 1996).

Several previous studies showed the importance of an intact complement system during sepsis. Mice deficient in properdin, the only positive regulator of the alternative pathway of complement activation, showed a significantly impaired survival time and significant higher bacterial load in blood, lung, spleen, liver and kidney when compared to their wild type littermates. This finding demonstrates the participation of the alternative pathway of complement activation in the immune response during sepsis (Stover *et al.*, 2008).

C1q deficient mice showed 92% mortality in comparison to only 27 % of wild type mice after CLP indicating the importance of the classical pathway activation for effective antimicrobial immune defense in poly-microbial peritonitis (Celik *et al.*, 2001).

Mice deficient in C2 and factor B are deficient in complement activation via the classical, the alternative, and the lectin pathway exhibited a significant higher mortality rate when compared to their wild type littermate controls indicating the important role of an intact complement system in the protection against poly-microbial septic peritonitis (Celik *et al.*, 2001; Windbichler *et al.*, 2004).

The results showed that approximately 75% of both $MASP2^{-/-}$ and $MASP2^{+/+}$ mice died within the first three days after CLP with no significant difference in mortality between the two groups. However, wild type mice showed a significantly lower bacterial load in peritoneal lavage after CLP when compared to their $MASP2^{-/-}$ littermates. The reduced bacterial clearance in MASP-2 deficient mice in this model may be due to an impaired C3b mediated phagocytosis as it was previously demonstrated that C3 deposition is MASP-2 dependent.

In a previous study, mice lacking the classical pathway ($C1q^{-/-}$) showed no significant effect in mortality rate in comparison to wild type mice after three days following CLP (Windbichler *et al.*, 2004). Recognition of bacterial polysaccharides by carbohydrate recognition molecules of the lectin pathway such as MBL-A and MBL-C activate the lectin pathway that in turn provides a significant protection for $C1q^{-/-}$ mice (Liu *et al.*, 2001). On the other hand, MBL-A and MBL-C has been shown to bind CR1 on leukocytes and hence

this facilitates bacterial clearance but this mechanism alone is not sufficient to protect the mice from CLP (Ghiran *et al.*, 2000). Mice deficient in $fB^{-/-}$ and $C2^{-/-}$ still posses both molecules but these mice were highly sensitive to CLP and suffered from impaired bacterial clearance (Windbichler *et al.*, 2004).

The high rate of mortality in wild type mice (75 %) compared to only 27 % in mortality of wild type mice in a CLP model described by Windbichler *et al.* may be due to the difference in the genetic background of the mouse strain used. Where, the mice used in this study were on the more susceptible C57BL/6 genetic background while Windbichler *et al.* used mice of the more resistant 129/SV strain.

The unexpectedly high degree of mortality of wild type mice even with a relatively low bacterial load may be due to physiological causes other than septicemia. It could be assumed that, these mice might have died from a severe inflammatory response that leading to multi-organ failure and death (Meduri *et al.*, 1999). In order to investigate the effect of inflammatory responses in the survival of wild type and MASP-2 deficient mice, the mRNA expression levels of inflammatory mediators were analysed and compared in both groups 16 hrs after CLP. Interestingly, the wild type mice showed significant higher levels of mRNA expression for TNF-α, IL-1β and MIP-2 at the same time these mice had a very low bacterial load. In contrast, MASP-2 deficient mice showed a significantly lower mRNA expression levels for the inflammatory mediators TNF-α, IL-1β and MIP-2 while a very high bacterial load was found in the peritoneal lavage. This was in line with the finding of a significant higher serum level of TNF-α in the wild type mice compared to the MASP-2 deficient animals. MBL-A deficient mice which were previously challenged using the same model of peritonitis showed a significant higher

survival rate and lower bacterial load when compared to the wild type littermates controls. Again the surviving MBL-deficient mice showed a significantly lower level of TNF-α (Takahashi, *et al.*, 2002).

The results further showed that MASP-2 deficient mice have a significantly higher abundance of IL-6 mRNA when compared to wild type controls. The high expression levels of IL-6 in $MASP2^{-/-}$ mice were associated with the high bacterial load in peritoneal lavage of these mice, while wild type mice showed a significant lower expression level of IL-6 after CLP associated with the reduced bacterial load. IL-6 is a proinflammatory mediator and its high serum level is considered as a biological biomarker for sepsis and high rate of lethality in mice after CLP (Osuchowski *et al.*, 2007). Previous studies showed that neutralisation of IL-6 using specific antibodies prior to CLP procedures greatly improve the survival of mice in a dose dependent manner (Montz *et al.*, 1991; Mack *et al.*, 2001).

To investigate the role of the lectin pathway of complement activation after CLP, lectin pathway dependent C4 cleavage was measured on Mannan coated plate. This assay measured the ability of MBL oligomers to bind Mannan on the ELISA plates and its ability to bind functionally active MASP-2. This assay did not depend on the availability of endogenous C4 as a fixed amount of human C4 was added to all wells to be cleaved by mouse MASP-2 complexed with MBL. The results showed a significant decrease in C4 cleavage after CLP, indicating a decreasing level of MBL, most likely due to consumption. I assumed that this decrease in C4 cleavage activity was due to low serum levels of MBL oligomers which may have been consumed by binding to the carbohydrate molecules in the surface of invading pathogens after CLP.

It has been previously established that MBL-A and MBL-C concentrations in serum decreased after CLP due to their consumption (Windbichler *et al.*, 2004). To confirm that the decrease in MBL-A and MBL-C levels was not due to lower expression levels, mRNA was isolated from mouse liver and mRNA expression of both MBL-A and MBL-C was measured following CLP using a Light Cycler based qRT-PCR analysis. The results showed a significant increase in the mRNA expression profiles in mice after CLP.

Several clinical studies have highlighted the importance of the lectin pathway in controlling sepsis in humans. Patients with MBL variant alleles are more susceptible to development of sepsis and septic shock (Garred *et al.*, 2003). In addition, low serum levels in humans due to *MBL-2* exon 1 polymorphism were shown to be with a greater risk to develop sepsis in pediatric patients (Fidler *et al.*, 2004).

The novel finding of this study is that MASP-2 deficiency modulates the inflammatory immune response and reduces the expression levels of inflammatory mediators during sepsis. This lectin pathway mediated regulation of inflammatory response may offer a new therapeutic window in the treatment and protection from muti-organ failure during sepsis. Inhibitory monoclonal antibodies directed against MASP-2 could be used to block the lectin pathway activity and may serve as a new tool to modulate the severe inflammatory response during sepsis and septic shock.

A similar clinical approach was used by treating sepsis patients with activated protein C (APC). Interestingly, APC inhibited production of TNF-α, IL-1 and IL-6 by monocytes and reduced the inflammatory response. This clinical trial

showed promising results; where APC-treated groups showed a significant improvement in survival and the clinical outcome (Healy, 2002).

Overall, these findings showed that MASP-2 deficient mice showed no significant increase in mortality after CLP when compared to their MASP-2 sufficient littermates. Bacterial clearance however was significantly compromised in MASP-2 deficient mice. It was also demonstrated that a significant difference in the cytokine expression profiles between MASP-2 deficient and MASP-2 sufficient animals was also observed with TNF-α, IL-1β and MIP-2 expression being significantly reduced in MASP-2 deficient animals following CLP. In conclusion, MASP-2 deficiency may reduce the bacterial clearance but limit the inflammatory response to septic peritonitis considerably, thus leading to a relative reduction of the inflammation driven mortality during septic shock.

Chapter 5

Generation of inhibitory antibodies against human and murine MASP-2

Chapter 5

Generation of inhibitory antibodies against human and murine MASP-2

5.1 Results

5.1.1 Cloning and expression of recombinant human and mouse MASP-2A and MASP-2K

The generation of inhibitory monoclonal antibodies against human and mouse MASP-2 requires large quantities of highly pure MASP-2 zymogen. MASP-2 is a serine protease zymogen that easily converts into its proteolytically active form during the purification process. The enzymatic activity is located on the B-chain of activated MASP-2 and formed of a catalytic triad composed of serine, histidine and aspartic acid residues. Upon activation, the single chain MASP-2 zymogen is cleaved to the A chain (heavy chain) and B chain (light chain). Both fragments stay attached to each other through a disulphide bridge (Fig. 5.1) (Schwaeble *et al.*, 2002). To avoid auto-activation of MASP-2 during the purification process, a recombinant enzmatically inactive form of MASP-2 (MASP-2A) was generated by site directed mutagenesis of the MASP-2 coding sequence leading to the substitution of the serine residue in the catalytic domain with an alanine residue (Fig. 5.2). Another variant form retaining but slowing down the rate of autoactivation (MASP-2K) was also engineered by the substitution of the arginine residue at the cleavage site of the zymogen with a lysine residue (Fig. 5.3). This substitution would reduce the cytotoxicity during the expression of the recombinant protein and facilitate the purification process.

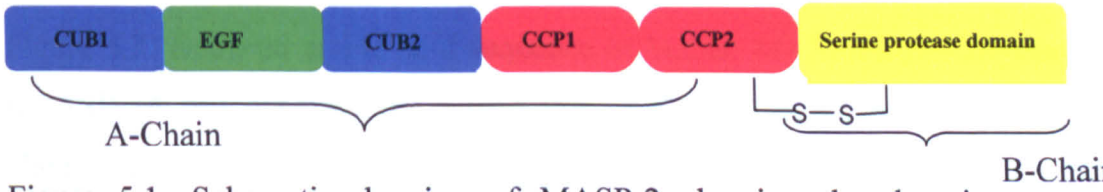


Figure 5.1: Schematic drawing of MASP-2 showing the domain structure and the disulphide bridge keeping the A and B chains together following autoactivation of MASP-2

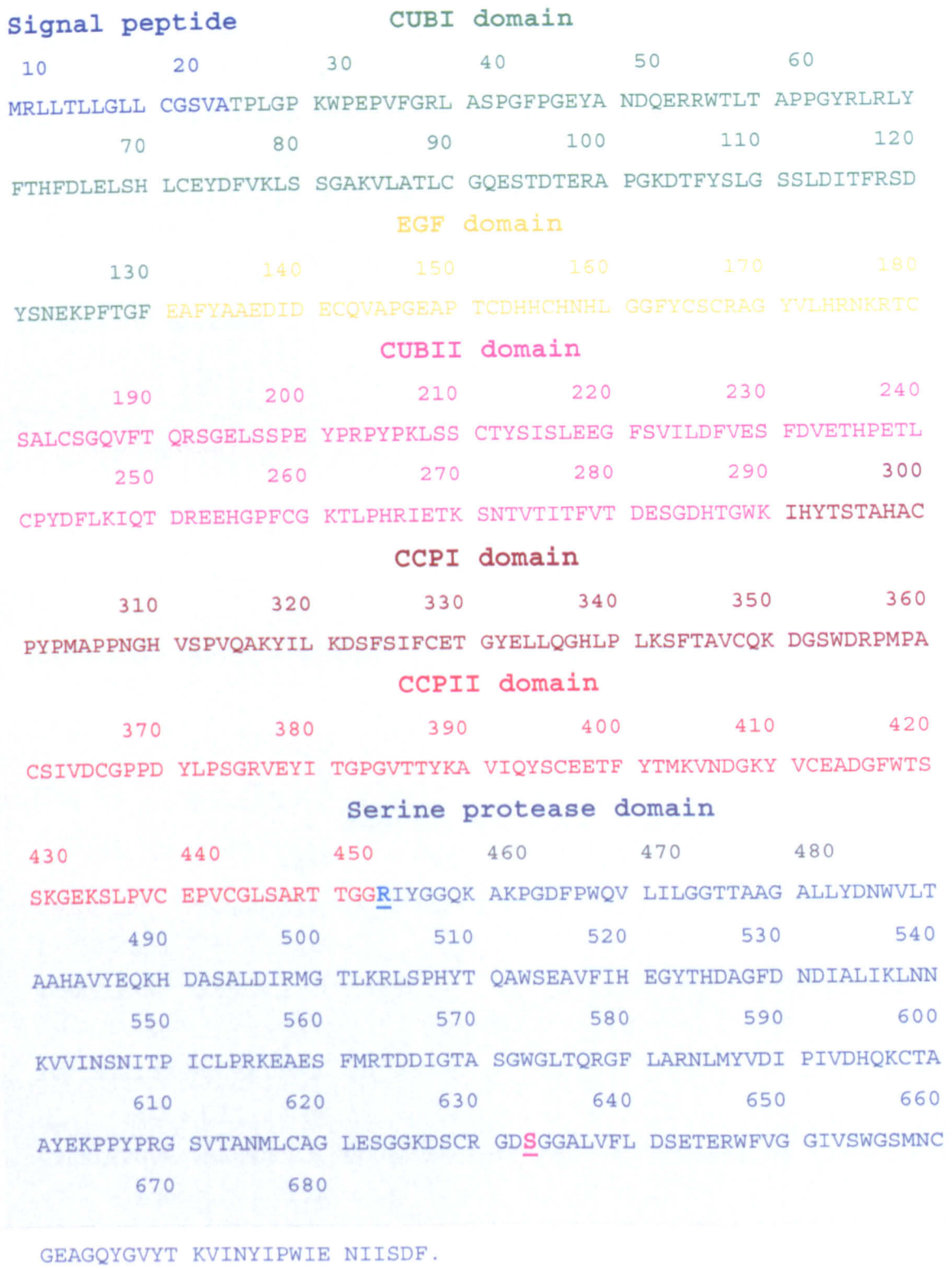


Figure 5.2: Deduced amino acid sequence of human MASP-2; the enzymatically inactive hMASP-2A was generated by substitution of a serine amino acid at position 633 with an alanine residue. A recombinant hMASP-2K with a reduced degree of autoactivation was engineered by substitution of an arginine residue at position 454 with a lysine residue.

Signal peptide CUBI domain

```
10         20         30         40         50         60
MRLLIFLGLL WSLVATLLGS KWPEPVFGRL VSPGFPEKYA DHQDRSWTLT APPGYRLRLY
        70         80         90        100        110        120
FTHFDLELSY RCEYDFVKLS SGTKVLATLC GQESTDTEQA PGNDTFYSLG PSLKVTFHSD
```

EGF domain

```
       130        140        150        160        170        180
YSNEKPFTGF EAFYAAEDVD ECRVSLGDSV PCDHYCHNYL GGYYCSCRAG YVLHQNKHTC
```

CUBII domain

```
       190        200        210        220        230        240
SALCSGQVFT GRSGYLSSPE YPQPYPKLSS CTYSIRLEDG FSVILDFVES FDVETHPEAQ
       250        260        270        280        290        300
CPYDSLKIQT DKGEHGPFCG KTLPPRIETD SHKVTITFAT DESGNHTGWK IHYTSTARPC
```

CCPI domain

```
       310        320        330        340        350        360
PDPTAPPNGS ISPVQAIYVL KDRFSVFCKT GFELLQGSVP LKSFTAVCQK DGSWDRPMPE
```

CCPII domain

```
       370        380        390        400        410        420
CSIIDCGPPD DLPNGHVDYI TGPEVTTYKA VIQYSCEETF YTMSSNGKYV CEADGFWTSS
```

Serine protease domain

```
       430        440        450        460        470        480
KGEKLPPVCE PVCGLSTHTI GGRIVGGQPA KPGDFPWQVL LLGQTTAAAG ALIHDNWVLT
       490        500        510        520        530        540
AAHAVYEKRM AASSLNIRMG ILKRLSPHYT QAWPEEIFIH EGYTHGAGFD NDIALIKLKN
       550        560        570        580        590        600
KVTINGSIMP VCLPRKEAAS LMRTDFTGTV AGWGLTQKGL LARNLMFVDI PIADHQKCTA
       610        620        630        640        650        660
VYEKLYPGVR VSANMLCAGL ETGGKDSCRG DSGGALVFLD NETQRWFVGG IVSWGSINCG
       670        680
```

```
AADQYGVYTK VINYIPWIEN IISNF.
```

Figure 5.3: Deduced amino acid sequence of mouse MASP-2; the enzymatically inactive mMASP-2A was generated by substitution of a serine amino acid at position 632 with an alanine residue. The partially active mMASP-2K was engineered by substitution of arginine residue at position 443 with a lysine residue.

5.1.2 Engineering of human and murine MASP-2A and MASP-2k constructs

Human MASP-2 is encoded by an open reading frame of 2058 base pairs coding 686 amino acid residues, while the open reading frame encoding mouse MASP-2 comprises of 2055 base pairs and encodes 685 amino acids. For the establishment of the expression constructs, hMASP-2 cDNA in (pBluescript vector) kindly provided by Prof. W. Schwaeble (University of Leicester) was used as a template for the generation of both hMASP-2A and hMASP-2K. To establish the mouse MASP-2 constructs, cDNA was prepared from mouse liver mRNA (see 2.2.4.1.4) and used as a template to engineer the coding sequences for mMASP-2A and mMASP-2K.

The mutated forms of MASP-2 i.e. hMASP-2K (Fig. 5.4), hMASP-2A (Fig. 5.5), mMASP-2K (Fig. 5.6) and mMASP-2A (Fig. 5.7) were generated as previously described (see 2.2.4.2.2). Each mutation set to generate the changes in the coding sequences was achieved by using two pairs of primers following a two step PCR protocol.

The PCR products were finally cloned into a pGEM-T easy vector (which is an open vector which facilitates subcloning of PCR products). The primers used to amplify the PCR fragments required to generate the full length encoding sequence for MASP-2A and MASP-2K were engineered to contain endonuclease restriction sites for BamH1 and Xho1. The mutated MASP-2 constructs were then excised from the pGEM-T easy vector by BamH1 and Xho1 restriction digestion and subsequently subcloned into the expression vector pSectag2/hygroB. The expression constructs were finally digested with BamH1 and HindIII to insert the coding sequence of a 6 histidine tag linker

which is required for the subsequent identification of the recombinant protein generated and its purification using nickel columns.

Before the transfection of the CHO-K1 cell line, all of the constructs were sequenced to confirm that all constructs were inserted in frame and to ensure that no other mutations were resulting from the PCR amplification steps.

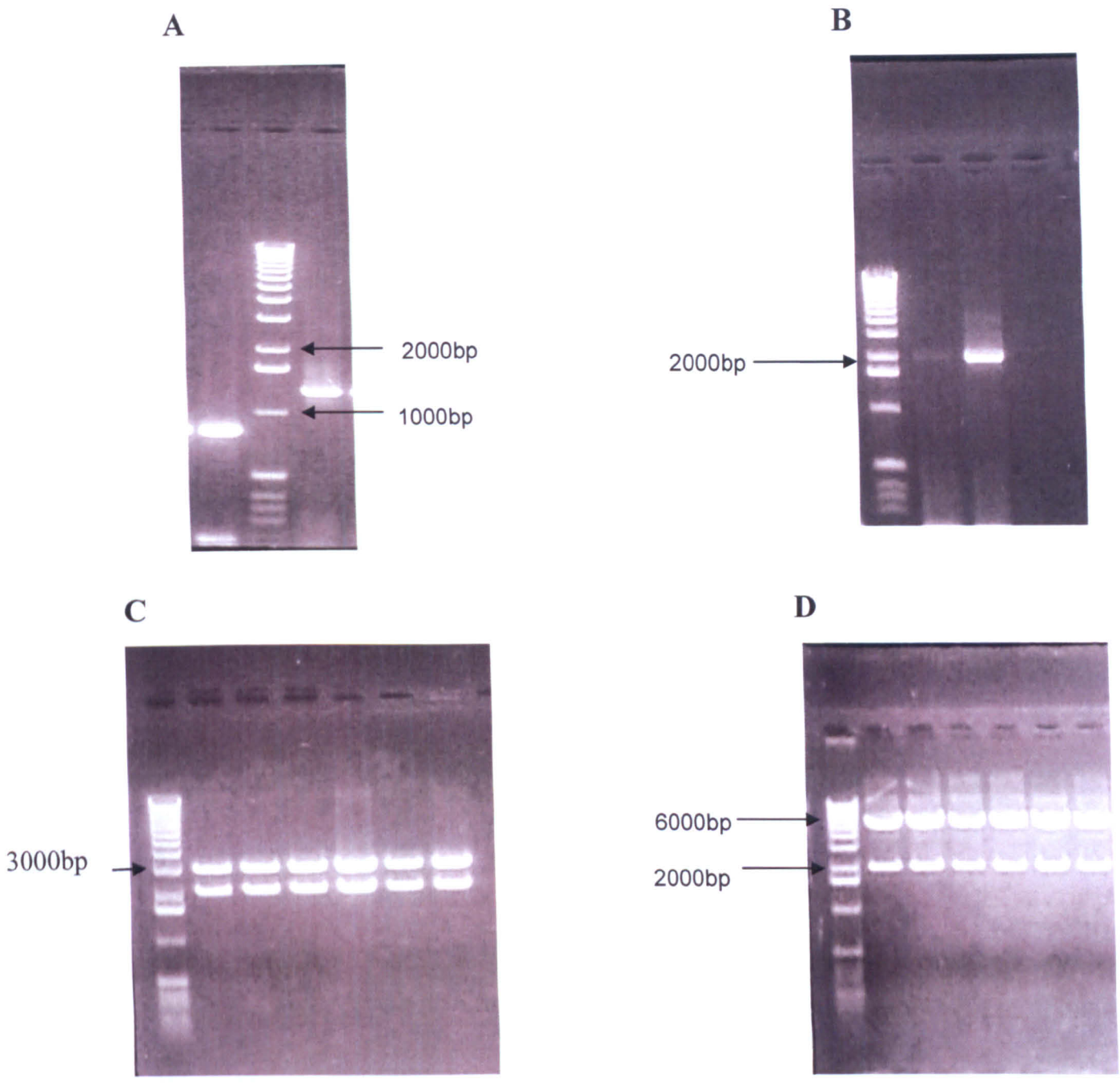


Figure 5.4: Generation of the two subfragments encoding hMASP-2k (800, 1400 bp) (A). Ligation of the two fragments with a second PCR to generate the full length coding sequence for hMASP-2k with an over all length of approximately 2000 bp (B). The full length coding sequence for hMASP-2k was cloned into the 3 kb PCR vector pGEM-T easy (C). hMASP-2k construct was digested with BamH1 and Xho1 and sub-cloned into the 5.2 kb expression vector Psectag2/HygroB (D).

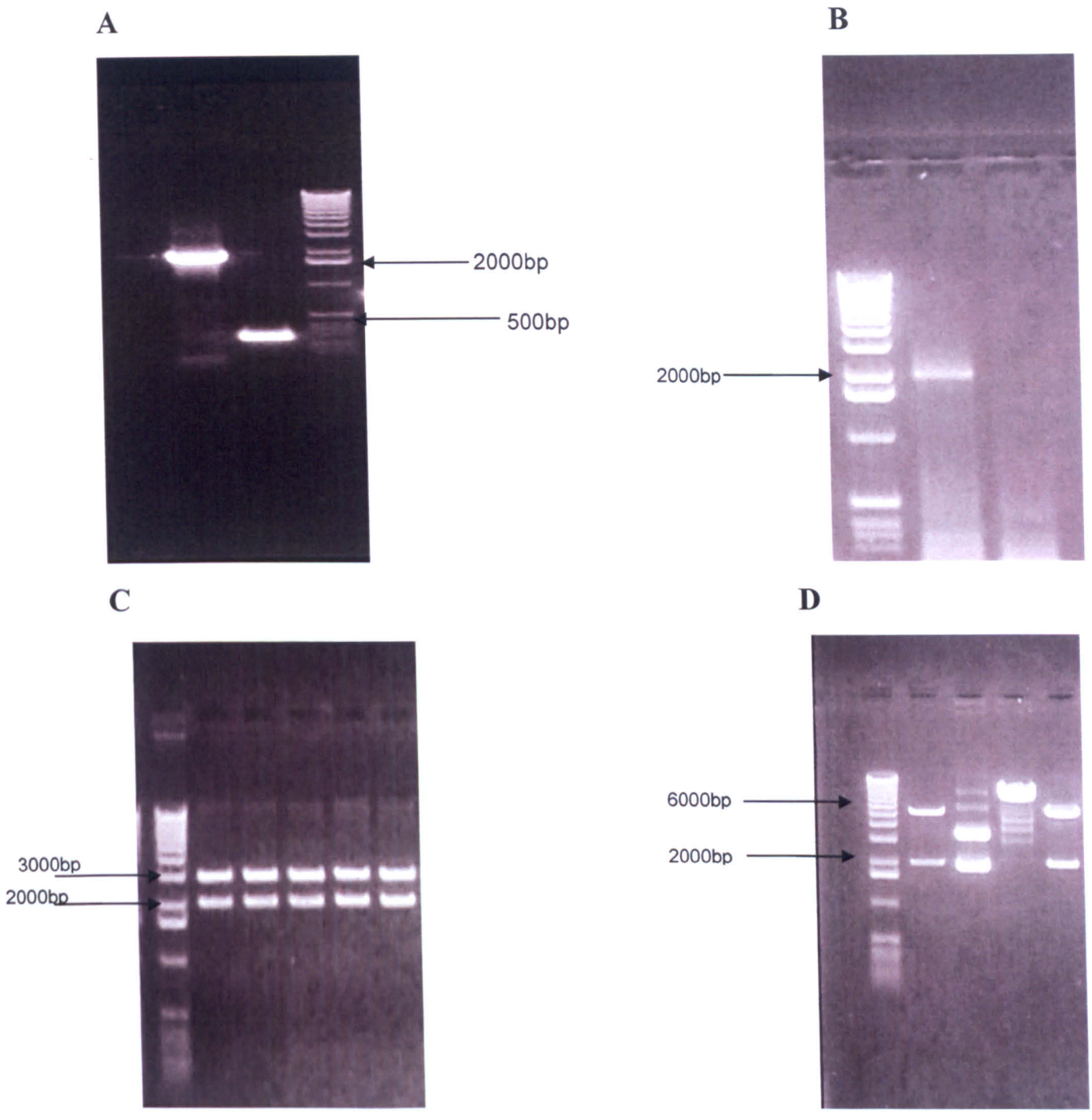


Figure 5.5: Generation of the two subfragments encoding hMASP-2A (250, 2000 bp) (A). Ligation of the two fragments with a second PCR to generate the full length coding sequence for hMASP-2A with an over all length of approximately 2000 bp (B). The full length coding sequence for hMASP-2A was cloned into the 3 kb PCR vector pGEM-T easy (C). hMASP-2A construct was digested with BamH1 and Xho1 and sub-cloned into the 5.2 kb expression vector Psectag2/HygroB (D).

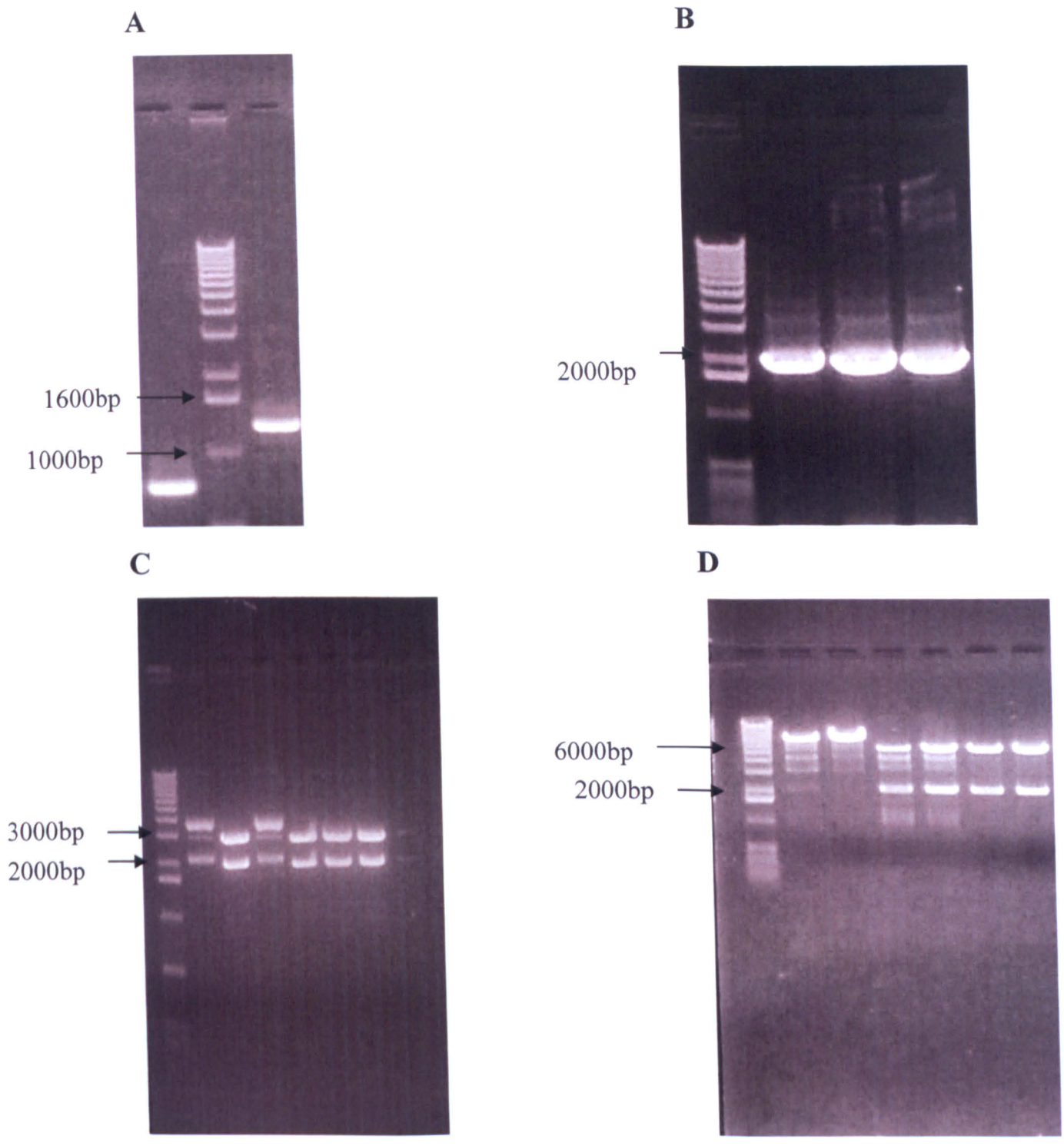


Figure 5.6: Generation of the two subfragments encoding mMASP-2k (800, 1400 bp) (A). Ligation of the two fragments with a second PCR to generate the full length coding sequence for mMASP-2k with an over all length of approximately 2000 bp (B). The full length coding sequence for mMASP-2k was cloned into the 3 kb PCR vector pGEM-T easy (C). mMASP-2k construct was digested with BamH1 and Xho1 and sub-cloned into the 5.2 kb expression vector Psectag2/HygroB (D).

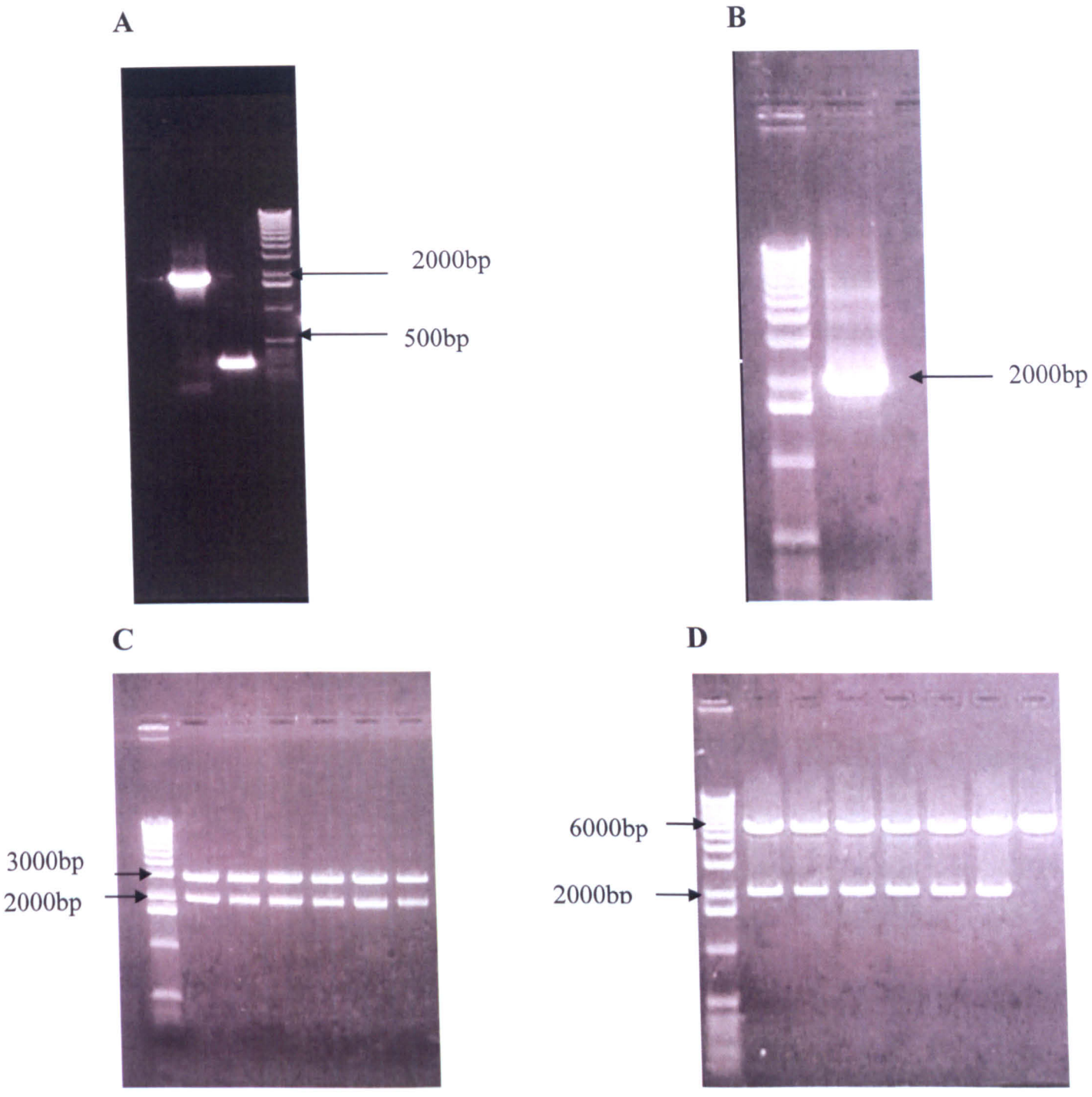


Figure 5.7: Generation of the two subfragments encoding mMASP-2A (250, 2000 bp) (A). Ligation of the two fragments with a second PCR to generate the full length coding sequence for mMASP-2A with an over all length of approximately 2000 bp (B). The full length coding sequence for mMASP-2A was cloned into the 3 kb PCR vector pGEM-T easy (C). mMASP-2A construct was digested with BamH1 and Xho1 and sub-cloned into the 5.2 kb expression vector Psectag2/HygroB (D).

5.1.3 Stable expression of human and murine MASP-2A and MASP-2K

CHO-K1 cells were transfected with pSectag2/hygroB expression vector Containing the constructs of human and murine MASP-2A and MASP-2K. Cells were maintained under hygromycin selection. After 14 days, samples were taken from the growing clones and positive clones expressing the mutated proteins were screened by dot blot technique using monoclonal anti-body against 6 Histidine tag. (Fig. 5.8). Supernatant from non transfected cells was used as a negative control.

Human and murine MASP-2A were successfully expressed using this system. Unfortunally, human and murine MASP-2K could not be expressed. Transfection of other cell lines as HeK293, Hela and COS7 cell lines also proved to be unsuccessful indicating that neither hMASP-2K nor mMASP-2K could be expressed.

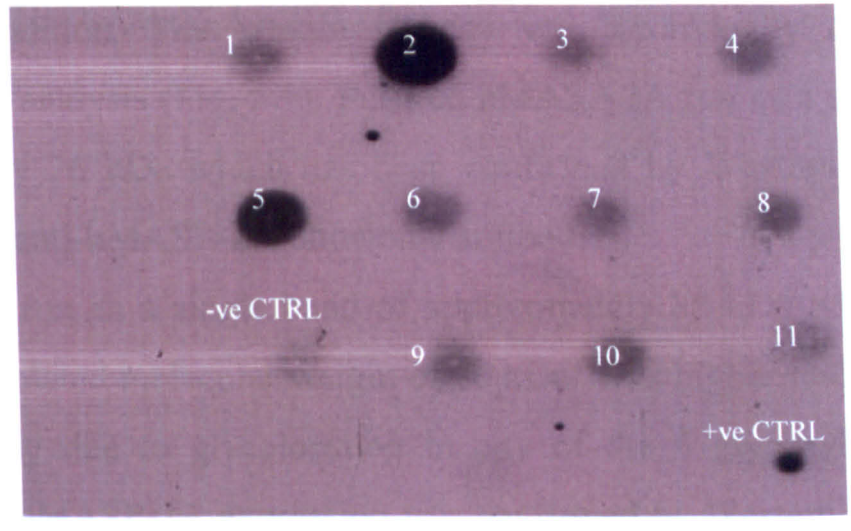


Figure 5.8: Screening of several clones for expression of hMASP-2A using monoclonal antibodies against 6 histidine tag. Recombinant MBL with poly-histidine linker was used as a positive control. Supernatant from non transfected cells was used as a negative control. Clones 2 and 5 were positive.

5.1.4 Purification of recombinant proteins

Expression of recombinant proteins furnished with histidine tag is a commonly used technique that is widely applied to facilitate purification of recombinant proteins by using immobilised Nickel affinity chromatography (IMAC) (Ford *et al.*, 1991). In addition, the histidine tag adds only a minor portion to the protein which is poorly immunogenic and so the recombinant protein can be used to generate monoclonal antibodies with out prior removal of the histidine tag.

5.1.4.1 Purification of hMASP-2A and mMASP-2A

His GraviTrap columns were used to purify the recombinant proteins as previously described (2.2.6.4.1).

Column load, flow through, column wash and elution fractions were collected and analysed on SDS-PAGE followed by Commassie stain under non reducing condition. The protein identity was additionally confirmed by Western blot analysis (Fig. 5.9). Purified hMASP-2A run as a single band of approximately 76 kDa which has been confirmed by Western blot analysis using mouse anti-hMASP-2 monoclonal antibodies.

mMASP-2A run as a single band of approximately 88 kDa which is higher than the calculated molecular weight of 76 kDa. This higher molecular weight is most likely due to glycolosation in any of the 5 glycosylation sites in mMASP-2. mMASP-2A expression was confirmed by Western blotting using mouse anti-histidine tag monoclonal antibodies (Fig. 5.10).

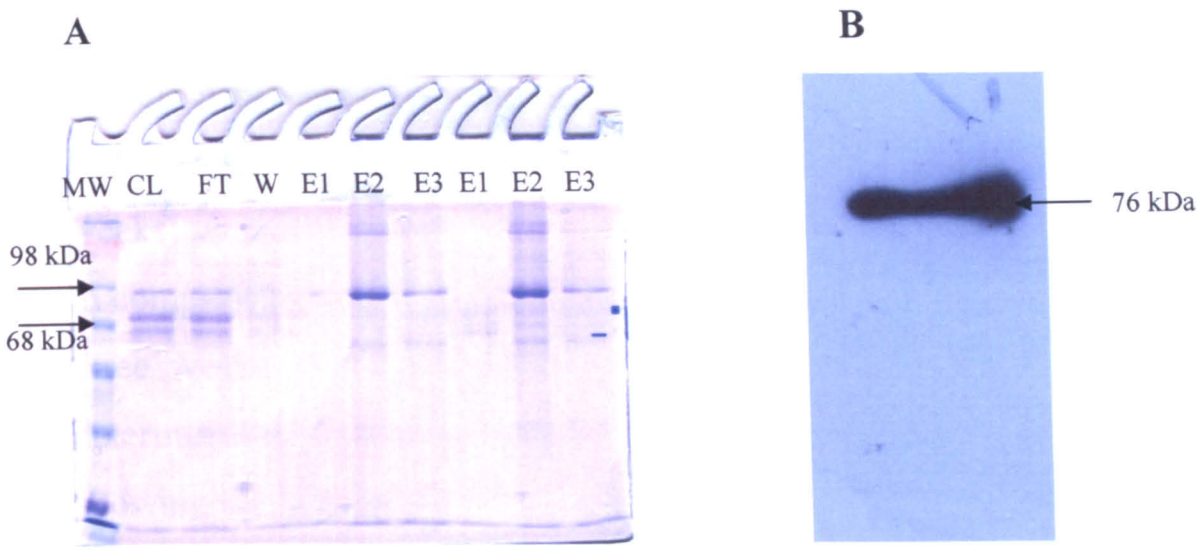


Figure 5.9: Purification of recombinant hMASP-2A using His GraviTrap column. (A) 12.5% SDS gel stained by coomassie stain during purification process where the purified protein showed a band of approximately 76 kDa. CL: column load, FT: flow through, W: column wash, E1-3: elution fractions, MW: molecular weight marker. (B) Western blot analysis using anti-hMASP-2 monoclonal antibodies was done to confirm that the purified protein is identical to hMASP-2.

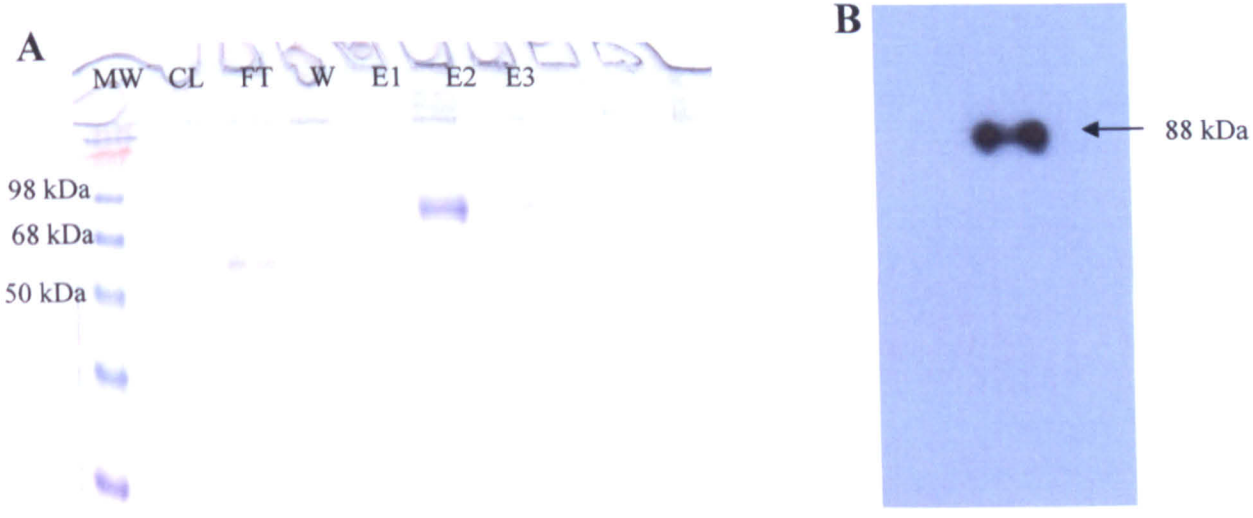


Figure 5.10: Purification of recombinant mMASP-2A using His GraviTrap column. (A) 12.5% SDS gel stained by coomassie stain during purification process where, the purified protein showing a band of approximately 88 kDa. CL: column load, FT: flow through, W: column wash, E1-3: elution fractions, MW: molecular weight marker. (B) Western blot analysis using mouse anti-histidine tag monoclonal antibodies was done to confirm the protein identity.

5.1.5 Generation of monoclonal antibodies

For the generation of monoclonal antibodies against hMASP-2A and mouse MASP-2A, four female MF1 mice (8 weeks) and two female Wistar rats (10 weeks) were immunised with hMASP-2A (25 μg) and mMASP-2A (100 μg) in complete Freund's adjuvant respectively. Animals were boosted weekly for three weeks. After the third booster dose, serum samples were taken for determination of the antibody titre by ELISA (Fig. 5.11 and 5.12). Animals showing the highest titter were boosted once more and three days after the last booster dose, mice and rats were sacrificed and spleens were removed. Splenocytes were isolated and fused with the NS0 myeloma cell line to generate hybridoma (James *et al.*, 1983). Antibody producing hybridomas were screened by ELISA. In this work, I isolated 5 different clones producing monoclonal antibodies against hMASP-2A. These clones were named; III 10.A(IgG1), III59(IgG2), III 44.E (IgM), III 48(IgM), III69(IgM). In addition, I generated another 5 clones producing rat anti-mMASP-2 monoclonal antibodies and these clones were named as III 1.7(IgG), III 4.5(IgG), III 23.3(IgG), III 11.1(IgG) and III 8 (IgG).

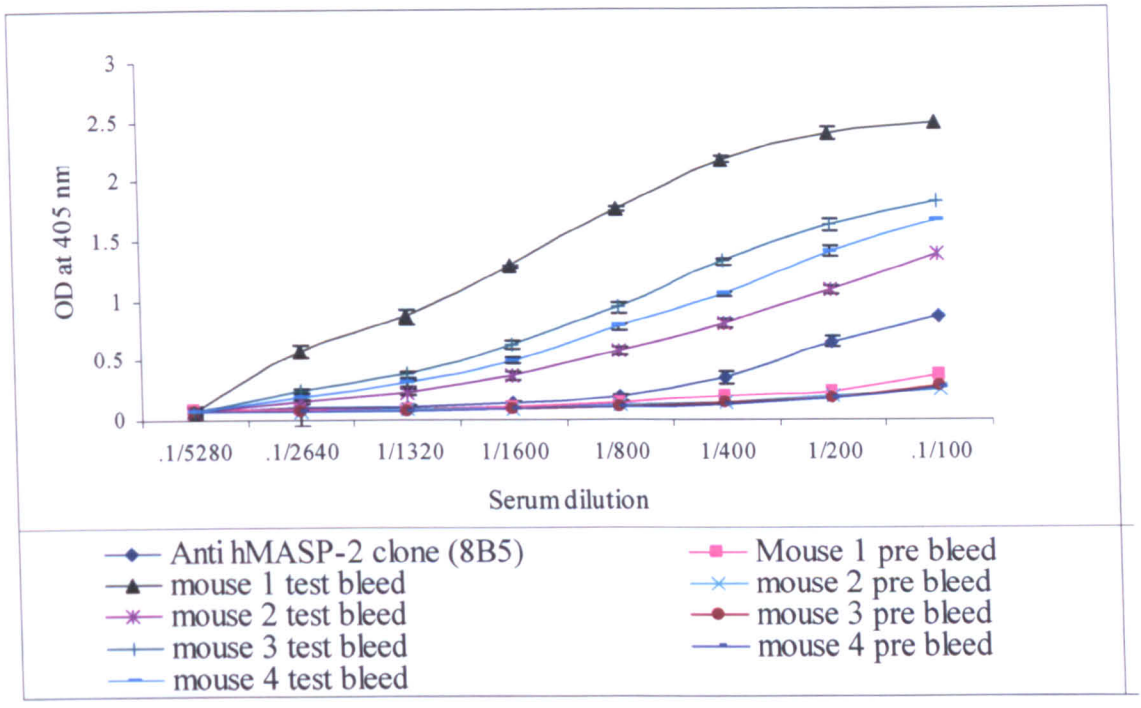


Figure 5.11: Antibodies titters in mouse sera after immunisation with hMASP-2A in comparison to sera of the same mice taken before immunisation. Anti-hMASP-2 clone (85B) was used as a positive control. Results are means (±SD) of duplicate.

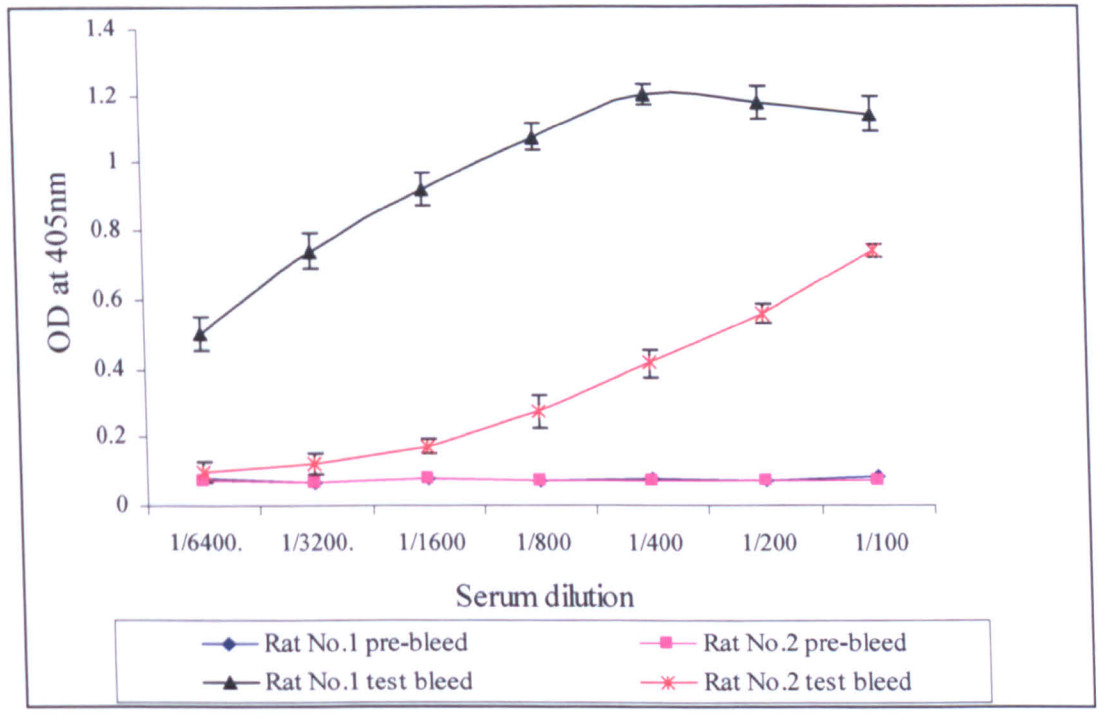


Figure 5.12: Antibodies titers in the sera of Wistar rats after immunisation with mMASP-2A in comparison to sera of the same rats prior to immunisation. Results are means (±SD) of duplicate.

5.1.6 Purification of monoclonal antibodies

Protein G sepharose column chromatography is the method of choice for purification of IgG monoclonal antibodies due to the high binding affinity of protein G sepharose to mouse and rat IgG. In this work, 2 clones producing IgG monoclonal antibodies against hMASP-2A and 5 clones producing IgG anti-mMASP-2A were generated. Protein G sepharose columns were used successfully to purify these antibodies from the hybridoma supernatant (Fig. 5.13, 5.14). Elution fractions containing antibodies were pooled together and dialysed against water, then concentrated to a final concentration of 0.5 mg/ml in PBS. Purified IgG antibodies were analysed by 12.5 SDS-PAGE under reducing conditions where it gives one band at 75 kDa corresponding to the IgG heavy chain and another band at 25 kDa corresponding to the IgG light chain. On the other hand IgG give a band at 150 kDa corresponding to the full length IgG under non reducing conditions.

IgM however, has no binding affinity to protein G columns. To overcome this problem, IgM antibodies were partially purified by centrifugation using spin columns with a cut-off of 100,000 kDa. This type of columns remove all the BSA (65 kDa) contaminations and allows to concentrate the IgM antibodies (960,000 kDa) to 0.5 mg/ml in PBS.

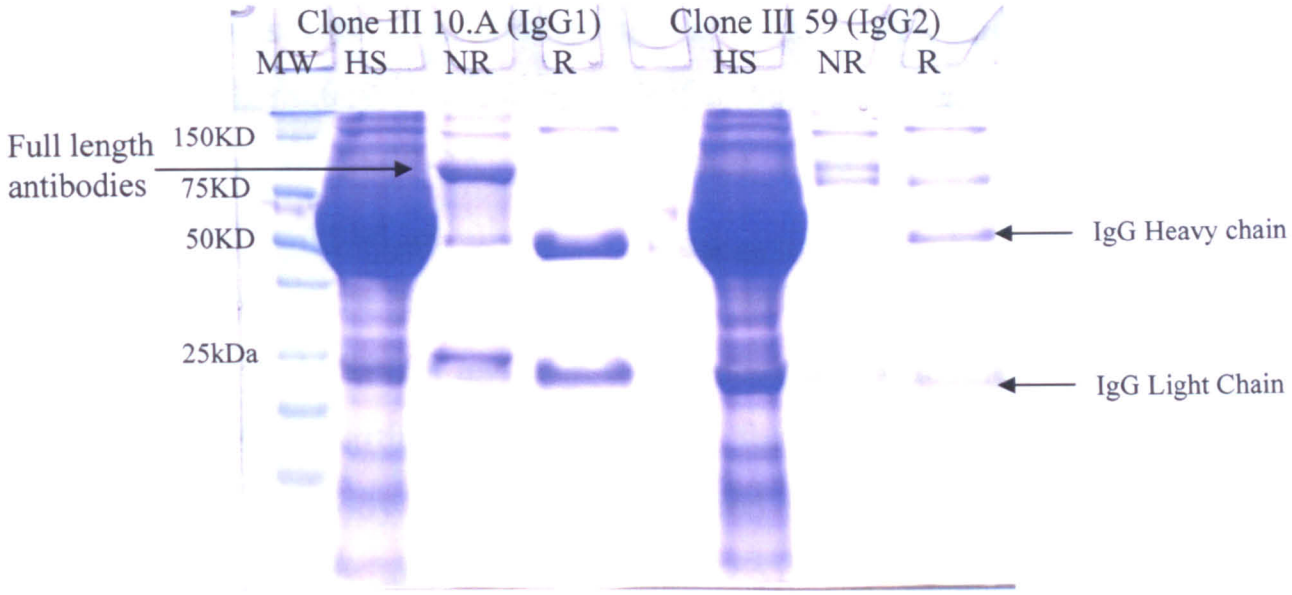


Figure 5.13: Purification of anti-hMASP-2A monoclonal antibodies (Clones III 10.A & III 59) using affinity chromatography. Hybridoma supernatant was loaded on protein G sepharose column and purified protein was eluted and analysed by 12.5% SDS-PAGE and visualised using commassie stain. HS: hybridoma supernatant, NR: running of antibodies under non reducing conditions, R: running of antibodies under reducing conditions, MW: molecular weight marker.

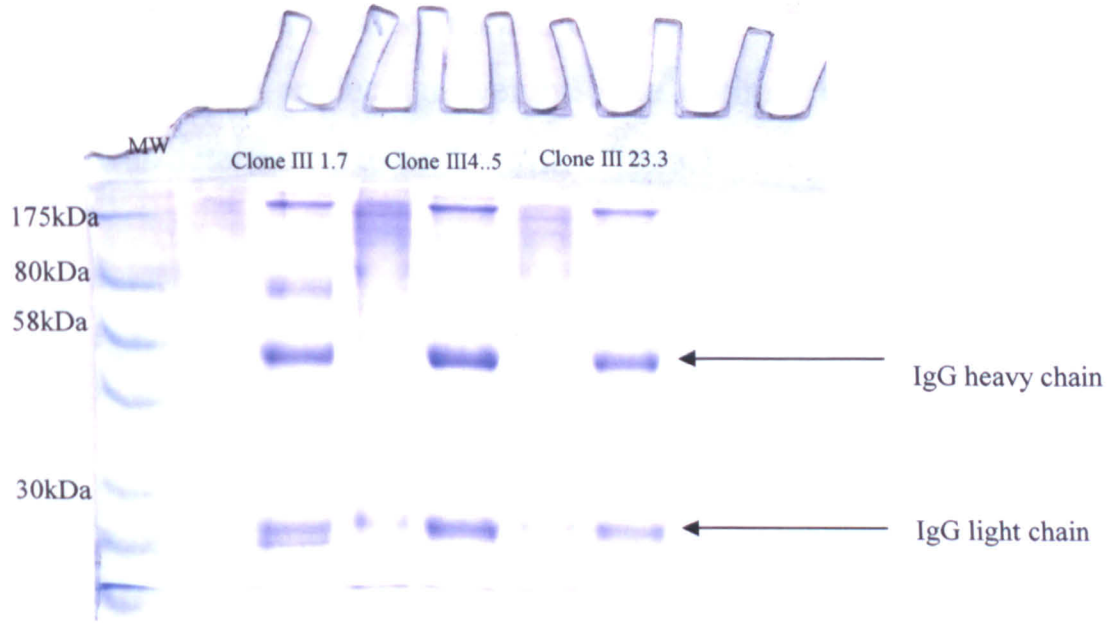


Figure 5.14: Purification of anti-mMASP-2A monoclonal antibodies (Clones III 1.7 & III 4.5 and III 23.3) using affinity chromatography. Purified mAbs were analysed by 12.5% SDS-PAGE under non reducing conditions and stained with commassie stain.

5.1.7 Characterisation of antibodies against hMASP-2A and mMASP-2A

In order to characterise the monospecific antibodies established against hMASP-2A and mMASP-2A, the antibodies were tested for their use in ELISA and Western blot techniques.

For ELISA, microtitre plates were coated with 10 µg/ml of recombinant hMASP-2A or mMASP-2A and then the monoclonal antibodies were added at different dilutions starting with 0.5 µg/ml for IgM and 5 µg/ml for IgG antibodies and antibody binding was detected using the corresponding AP-conjugated secondary antibody (see 2.2.1.2.5). The concentration of 0.25 µg/ml of clone III 44 was found to be optimal for the detection of hMASP-2A while 0.5 µg/ml was determined to be optimal for clones III 48 and III 69 (Fig. 5.15). Concentrations of 5 µg/ml of clones III 10.A and III 59 were found to be optimal to detect hMASP-2A (Fig. 5.16). For Western blot analysis, the monoclonal IgG antibodies generated against hMASP-2A and mMASP-2A bound with high affinity to the native human or the mouse proteins staining a band at the corresponding molecular weight. The optimal concentration of these antibodies was determined to be 5 µg/ml (Fig. 5.17).

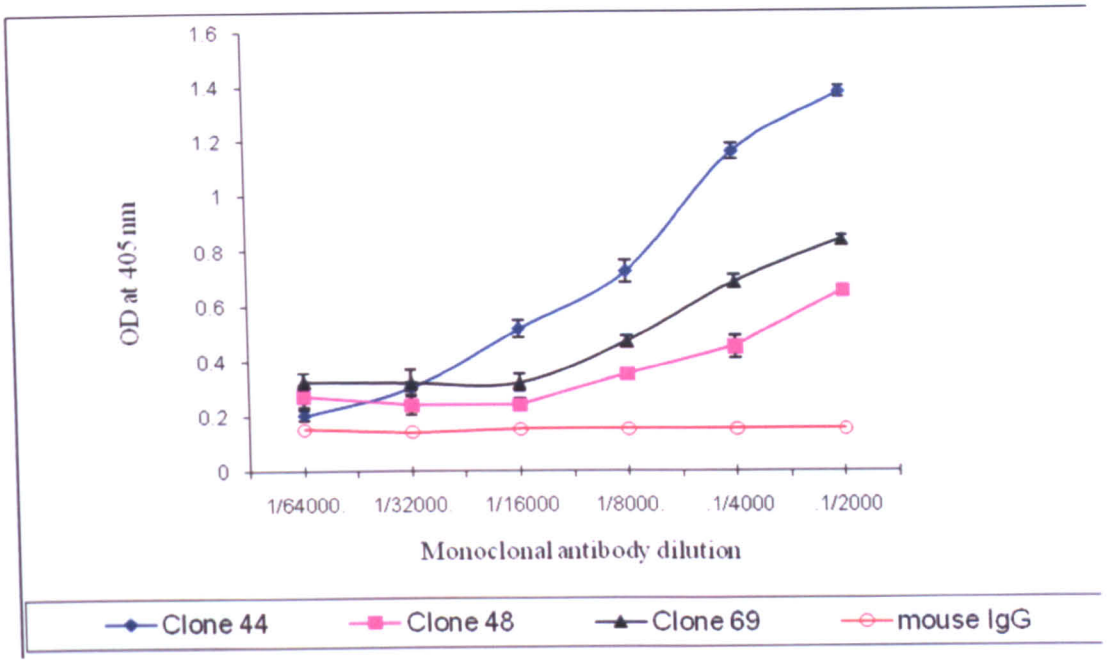


Figure 5.15: Identification of hMASP-2A with purified anti-hMASP-2A using different clones of IgM antibodies using ELISA technique. An irrelevant mouse IgG was used as an isotype. Results are means (±SD).

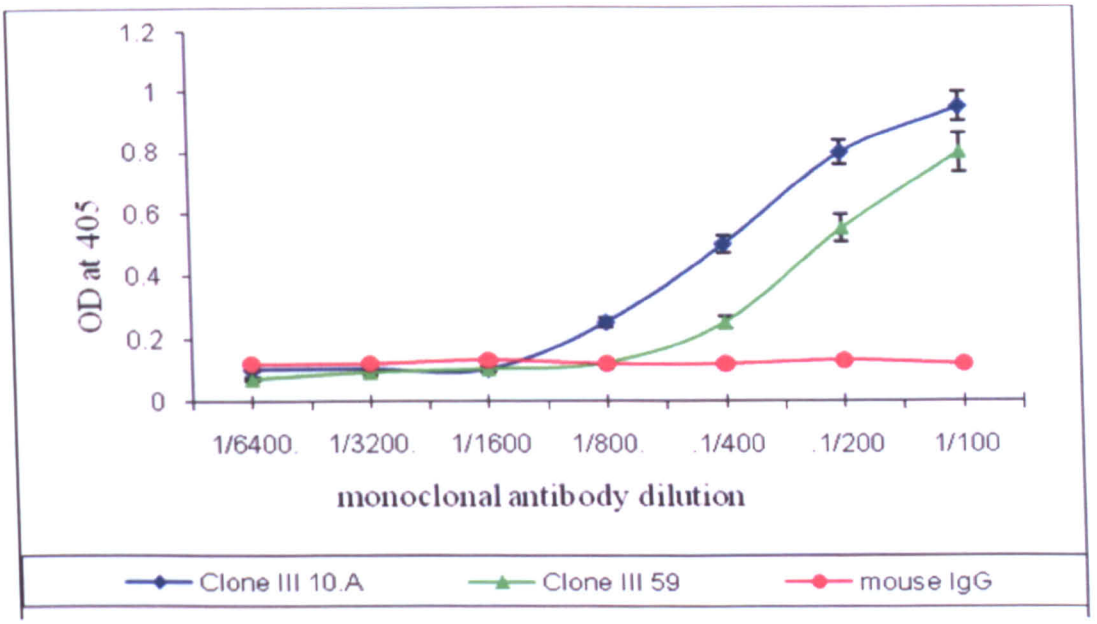


Figure 5.16: Identification of hMASP-2A with purified anti-hMASP-2A using different clones of IgG antibodies using ELISA technique. An irrelevant mouse IgG was used as an isotype. Results are means (±SD).

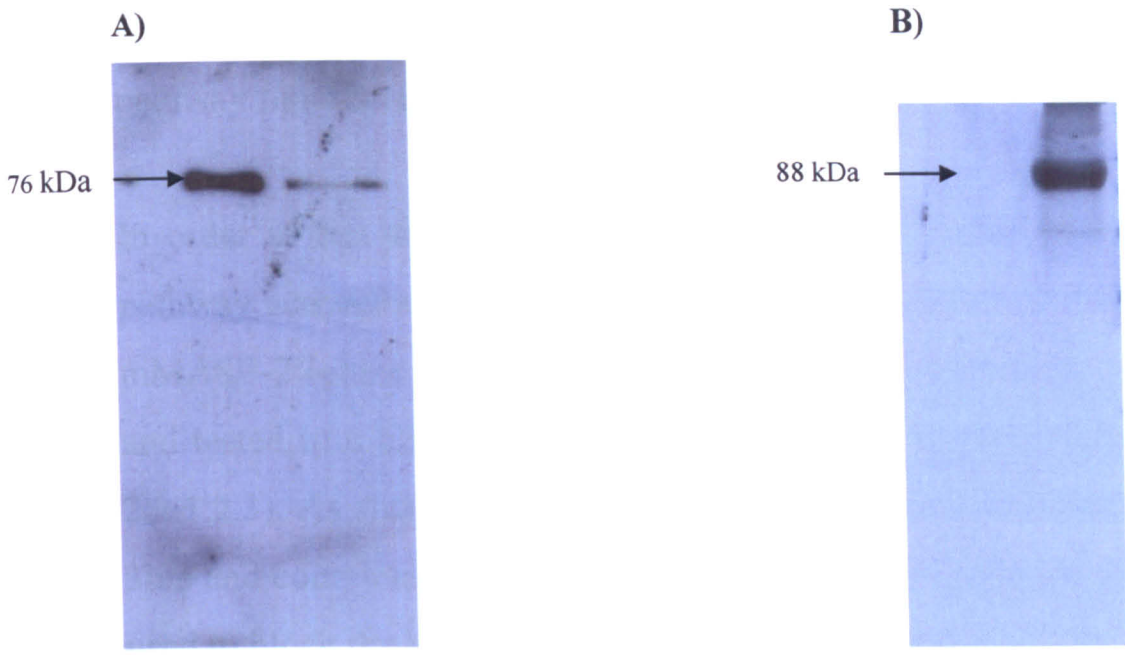


Figure 5.17: Western blot analysis staining under non reducing conditions of (A) recombinant human MASP-2A (76 kDa) with mAb clone III 10.A (mouse anti-hMASP-2). (B) recombinant mouse MASP-2A (88 kDa) with mAb clone III1.7 (rat-anti mouse MASP-2A). Both antibodies are of IgG isotype.

5.1.8 Depletion of human and murine lectin pathway functional activity using monoclonal antibodies against human and murine MASP-2

In order to see whether lectin pathway dependent activation of C3 can be inhibited with monoclonal antibodies against MASP-2, I tested the potential of each of the monoclonal antibodies to bind to native MASP-2 in plasma or serum and dissociate MASP-2 from lectin pathway activation complexes.

Anti-hMASP-2 mAbs or anti-mMASP-2 mAbs were added to either human or mouse serum dilution (1:80) at a final concentration of approximately 0.4 µg/ml and 0.6 µg/ml respectively. Antibodies were added 5 minutes prior to adding the sera to the Mannan coated ELISA plates (see 2.2.1.2.3). As shown in figure 5.18, four out of the five mAbs that I established against hMASP-2 efficiently blocked lectin pathway mediated activation and deposition of complement C3, while clone III 48 (IgM) had no inhibitory activity. Likewise,

all of the five anti-mMASP-2 mAbs (all IgG) effectively inhibited lectin pathway functional activity in mouse serum (Fig. 5.19).

In order to test the ability of the anti-mMASP-2 mAbs to block the lectin pathway activation *in vivo*, 4 MF1 mice were injected i.p. with 25µg of anti-mMASP-2 (clone III 1.7). Serum samples were taken after 3 hrs and 12 hrs and tested in a C3 deposition assay on Mannan as previously described (see 2.2.1.2.3). As shown in figure 5.20, C3 deposition was reduced by 50% after 3hrs and completely inhibited after 12hrs indicating that the antibodies can be used to block the lectin pathway activation *in vivo* (Fig. 5.19).

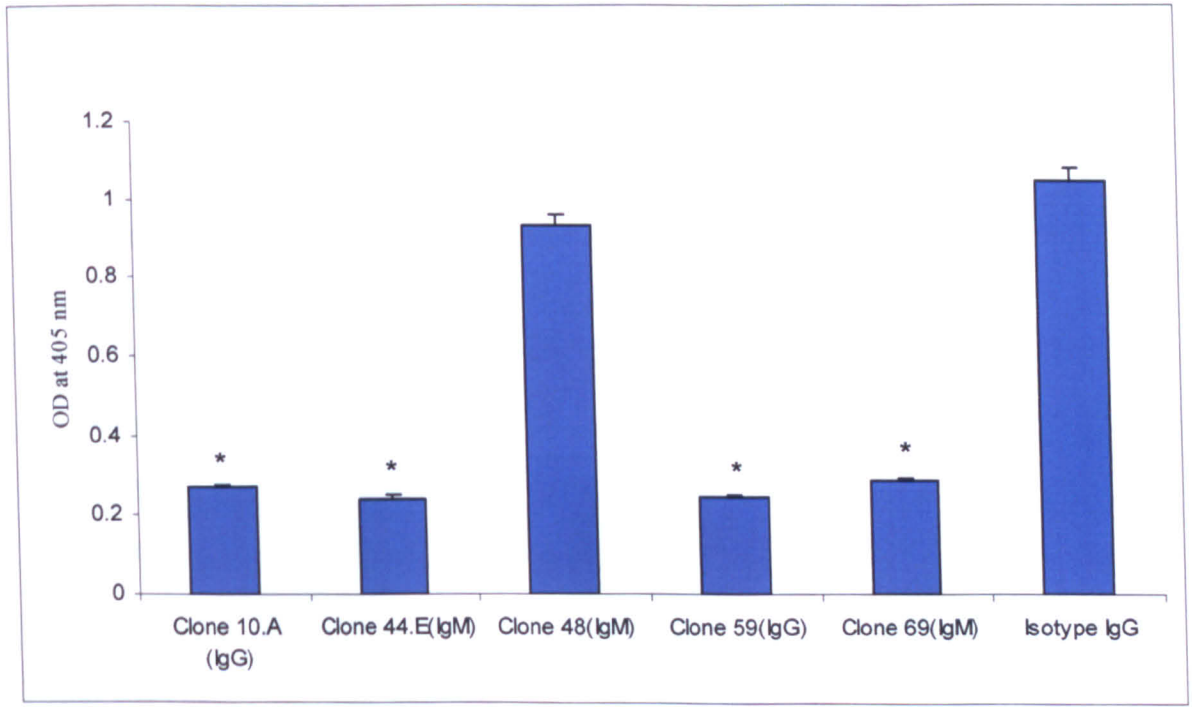


Figure 5.18: C3 deposition inhibition assay on Mannan using different clones of antibodies against hMASP-2. Isotype IgG was used as a negative control. *p< 0.001.
Results are means (±SEM) of 3 different experiments.

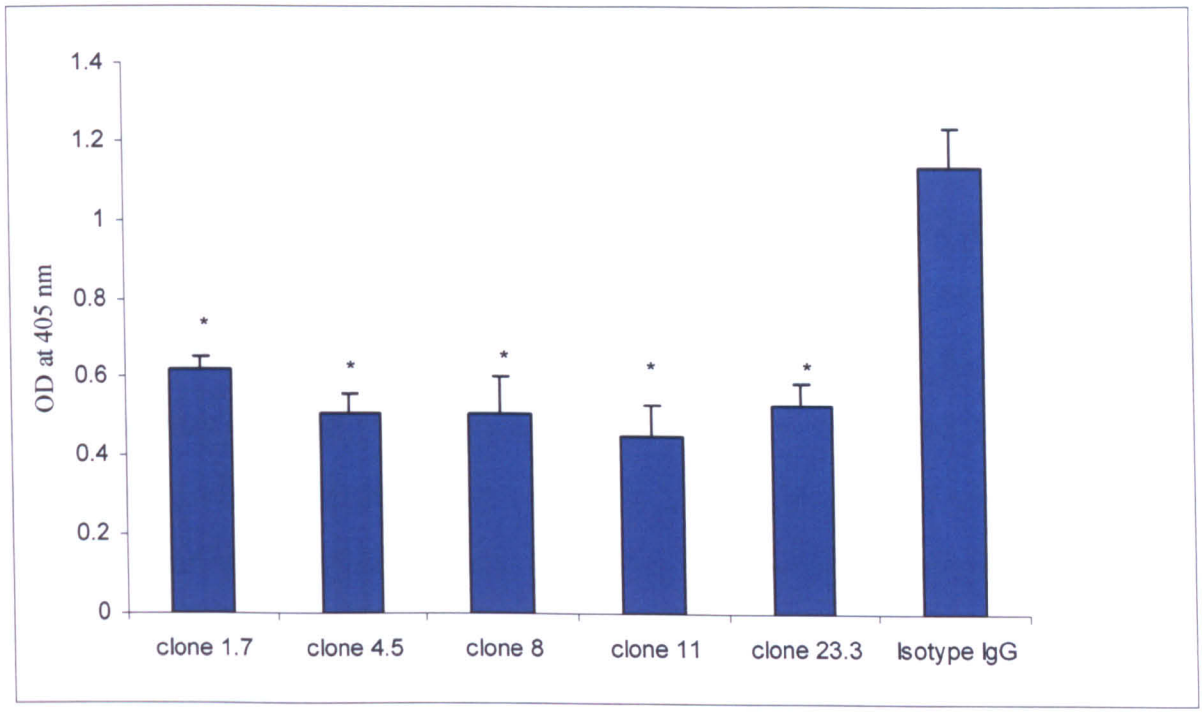


Figure 5.19: C3 deposition inhibition assay on Mannan using different clones of antibodies against mMASP-2. Isotype IgG was used as a negative control. *p< 0.01.

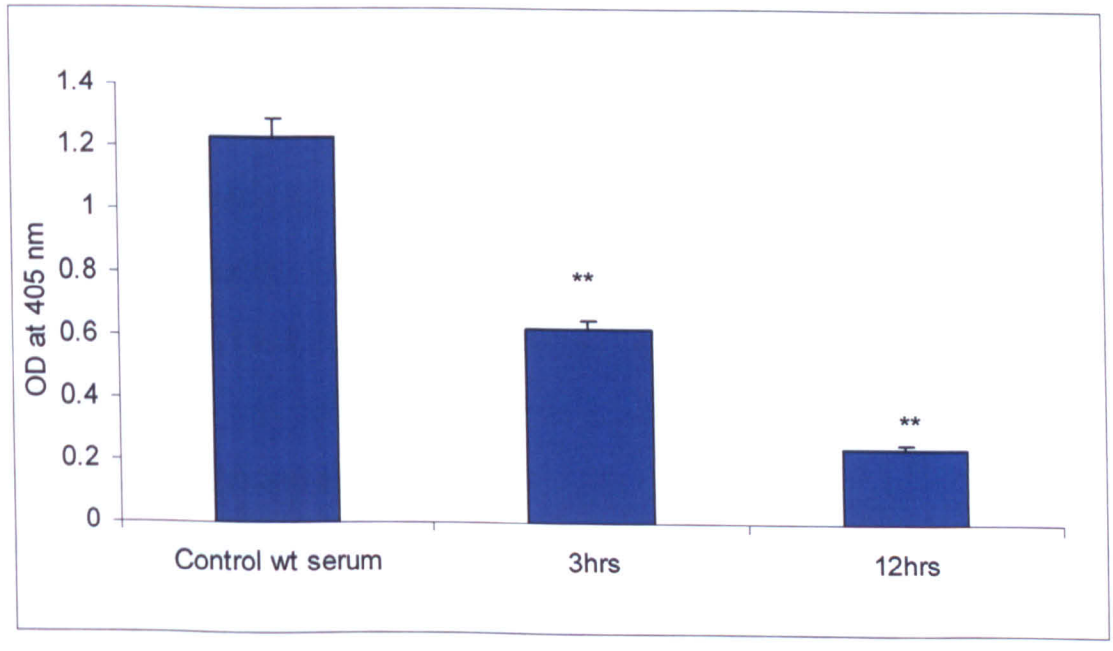


Figure 5.20: C3 inhibition assay of serum samples taken 3 and 12 hours after i.p. injection of 4 MF1 mice with 25 µg of anti-mMASP-2 clone III 1.17 in 100 µl PBS.
Results are means (±SEM). **p< 0.01.

Discussion

The aim of this part of my work was to express recombinant human and mouse MASP-2 devoid of enzymatic activity in order to produce highly specific monoclonal antibodies against native hMASP-2 and mMASP-2 to be used as inhibitors of lectin pathway functional activity in human and mouse plasma. These antibodies would provide valuable tools establish whether lectin pathway inhibitors targeting MASP-2 in order to modulate lectin pathway mediated inflammatory response could be applied as a new therapy to reduce human morbidity and mortality following myocardial infarction, transplantation and septic shock.

The mammalian expression vector pSectag2/HygroB was used as a vector to express all the above mentioned proteins in the Chinese hamster ovary cell line CHO-K1. Presence of the early human cytomegalovirus (CMV) promoter in this vector allows high levels of protein expression (Boshart *et al.*, 1985).

Extracellular expression of hMASP-2A and mMASP-2A was successfully achieved using stably transfected CHO-K1 cell lines with high levels of protein expression and a relatively short time (48 hrs). The amount of the protein expressed was relatively high (0.2 μg/ml and 0.35 μg/ml for hMASP-2A and mMASP-2A respectively).

The use of serum free medium to express the protein and the advantage of my system to express the protein extra-cellularly helped to purify hMASP-2A and mMASP-2A to homogeneity following a one step affinity chromatography protocol using Sepharose columns charged with Nickel. My results showed not only the high efficiency of protein expression but also the simplicity, the high yield and the high purity of the expressed proteins. My thesis described

for the first time the abundant expression of hMASP-2 or mMASP-2A using a transfected mammalian cell line. Until now, the only available published data were on the expression of wild type hMASP-2 using a Baculovirus/insect cell system where the protein could not be purified to homogeneity due to a very low efficiency of protein recovery (Rossi *et al.*, 2001).Expression of mMASP-2 was also succeeded using Baculovirus/insect cell system but with a low protein recovery (Iwaki and Fujita, 2005).

My work has produced strong evidence of the important role of the lectin pathway of complement in mediating a strong inflammatory response during bacterial infection and in septic peritonitis in mouse. The principle benefits of a therapeutic inhibition of complement activation in order to reduce the overshooting inflammatory response contributing to post ischaemic injury in myocardial infarction have been shown using an approach of rather limited targeted specificity by using C1-inhibitor (C1-INH), a rather broad serine esterase inhibitor which targeted C1s, C1r and also MASP-2 (Caliezi *et al.*, 2000). The therapeutic benefit of C1-inhibitor in experimental models of myocardial ischemia/reperfusion injury was shown in cats (Buerke *et al.*, 1995); rats (Murohara *et al.*, 1995) and in pigs (Horstick *et al.*, 1997). In a clinical trial, the use of continues infusion of C1-INH in patient with myocardial infarction showed a significant inhibition of the complement system and complement mediated inflammation and so C1-INH may reduce the myocardial injury (Zwaan *et al.*, 2002).

In addition, the use of nafamostat (a potent synthetic serine protease inhibitor) also showed a significant level of protection in a rabbit model of myocardial ischemia/reperfusion injury when compared to the control rabbits (Schwertz *et al.*, 2008). A recent work carried out by my supervisor Professor

W. Schwaeble with the KCL cardiologist Professor M. Marber demonstrated specifically that mice deficient of MASP-2 showed a significant and high level of protection in a model of myocardial ischemia/reperfusion injury when compared to their wild type controls (W. Schwaeble *et al*, unpublished data). The therapeutic benefits of complement inhibition have been also observed during sepsis. In a baboon model of septic shock, administration of C1-INH improves the survival (Caliezi *et al.*, 2000).

All these observations prompted us to generate monospecific inhibitory antibodies against hMASP-2 and mMASP-2 to assess the therapeutic use of MASP-2 inhibition in treatment of complement mediated inflammation during ischemia/reperfusion injury and sepsis.

This part of my work resulted in the generation of monoclonal antibodies against hMASP-2 and mMASP-2 after immunisation of 4 MF1 mice and 2 Wister rats with enzymatically inactive form of hMASP-2. Splenocytes from mice with highest antibodies titer were fused NS0 myeloma cells for generation of antibodies producing hybridoma. The screening procedures used for the identification of monoclonal antibodies were based on an ELISA in which the hybridoma supernatants were tested for their ability to bind recombinant hMASP-2A. Anti-mouse IgG antibodies (which cross-reacted with mouse IgM) were used to identify the antibodies producing hybridoma against hMASP-2. This screening procedure allowed screening allows me to isolate both IgG and IgM producing hybridoma.

Five different hybridoma producing anti-hMASP-2 were successfully isolated. Anti-hMASP-2 IgG was purified to homogeneity using a one step protein G

sepharose column chromatography. Protein G is the bacterial cell wall protein isolated from group G streptococci that can bind to IgG and albumin. For such protein G columns, a recombinant form of protein G is used where the albumin binding sites were completely eliminated to reduce the non specific binding of albumin and allowing purifying IgG to homogeneity (Eliasson *et al.*, 1988). However, as anti-hMASP-2 IgM shows no binding affinity to protein G, IgM antibodies were partially purified using spin columns with a cut-off 100,000 kDa to eliminate albumin contaminations and concentrate the IgM antibodies.

All IgG anti-hMASP-2 identified hMASP-2 on Western blots and worked perfectly in ELISA while the IgM clones identified hMASP-2 in ELISA only and could not be used in Western blot analysis. Interestingly, anti-hMASP-2 (IgM) clones showed to be excellent antibodies to recognise for h-MASP-2 in ELISA. This may be explained by the fact that IgM antibodies are pentameric and each monomer has 2 antigen binding sites, so the pentameric IgM has 10 antigen binding sites. The increased numbers of binding sites in IgM increase the binding affinity of anti-hMASP-2 and so may give a stronger and faster signal than IgG in the ELISA assay.

Immunisation of rats with recombinant mMASP-2A resulted in establishment of 5 clones producing IgG monospecific antibodies against mMASP-2. In this experiment, no IgM antibodies were isolated. This is most likely due to lack of the cross activity of the anti-rat IgG antibodies used for the screening with IgM antibodies. The isolated anti-mMASP-2 identified recombinant mMASP-2 *in vitro* by ELISA and Western blot.

The *in vitro* inhibition assays showed that 4 different clones (2 IgG and 2 IgM) against hMASP-2 completely inhibited the lectin pathway functional activity *in vitro*. All of the anti-mMASP-2 generated showed a significant inhibition of mMASP-2 functional activity *in vitro*. The inhibition of lectin pathway function activity *in vitro* may be due to steric interference by the relatively large antibody molecules with the binding site of MASP-2 on its substrates C2 and C4 (Wallis *et al.*, 2007). Inhibition of lectin pathway functional activity by these antibodies could be based on a steric hindrance preventing the binding between the N-terminal MASP-2 domains (CUB1–EGF–CUB2) and MASP-2 binding sites on the collagenous domain of MBL or ficolins (Wallis, 2007). Another possibility would be that these antibodies dissociate MASP-2 from the lectin pathway activation complexes and depletes MASP-2 in plasma rendering the lectin pathway activation complexes unable to activate complement.

Interestingly, the antibodies generated against mMASP-2 also successfully blocked the lectin pathway functional activity *in vivo* after i.p. injection of mice with 25 μg of anti-mMASP-2 (clone III 1.7) after 12 hrs.

For testing the inhibitory activity of the mouse anti-hMASP-2 antibodies *in vivo* a novel transgenic mouse line has been established by Dr. Nicholas Lynch in our laboratory where the deficiency in MASP-2 in the $MASP2^{-/-}$ mouse line has been replaced by a human Minigene construct of MASP-2 which transgenically expresses human MASP-2 and reconstitutes the defective murine lectin pathway activation in $MASP2^{-/-}$ mice. This line was generated through microinjection of the human Minigene construct into eggs harvested after superoculation of $MASP2^{-/-}$ mice. The resulting positive offspring were backcrossed to F10 generation against C57BL/6 background. At the mean

time Dr. Nicholas Lynch and Professor W. Schwaeble (University of Leicester) have already generated this transgenic mouse line. At the time of the completion of my work there were no enough mice available for me to use.

The availability of antibodies generated against either hMASP-2 or mMASP-2 added a valuable tool for studying the physiological role of MASP-2 in models of infection or models of ischaemia/reperfusion injury.

Chapter 6

Summary & Bibliography

Summary

The complement system is an essential component of the innate immune response and plays a major role in protection against bacterial infection. Complement is activated via three distinctive pathways; the classical, the alternative and the lectin pathway (Schwaeble *et al.*, 2002). Deficiency in any of the complement components is often associated with recurrent bacterial infections or predisposes to develop an auto-immune disease (Mollnes *et al.*, 2007). Several previous studies reported the important role of an intact complement system in the protection against bacterial infections with either single pathogen such as *Streptococcus pneumoniae* (Brown *et al.*, 2002) or *Pseudomona aeruginosa* infection (Muller-Ortiz *et al.*, 2004) or a mixture of commensials which may cause diseases when invade parts of the body which are usually sterile, such as in poly-microbial peritonitis (Stover *et al.*, 2008; Celik *et al.*, 2001).

Streptococcus pneumoniae is a Gram-positive bacterium that grows as diplococci and forms short chains. *S. pneumoniae* infection is the major cause of pneumonia, otitis media, septicemia and meningitis in the UK and it causes substantial morbidity and mortality, especially in young children and elderly patients (> 65 years old) (Kyaw *et al.*, 2003; Miller *et al.*, 2000).

Despite the fact that many of the previous studies have looked at the role of complement activation during *S. pneumoniae* infection (Brown *et al.*, 2002; Yuste *et al* 2005) my work is novel and unique as it has defined for the first time the role of lectin pathway of complement activation in protection against *S. pneumoniae* infections. In this work, I used a unique strain of mice with a

gene targeted deficiency of MASP-2. At the present, these mice provide the only available mouse model of complete lectin pathway deficiency without any residual lectin pathway activity.

The first part of my work defined the binding of carbohydrate recognition molecules of the lectin pathway in mouse i.e. (MBL-A, MBL-C and ficolin-A) to *S. pneumoniae*. The results clearly demonstrated that *S. pneumoniae* is recognised and complement is activated via lectin pathway through the lectin pathway recognition molecule ficolin A. In contrast, I observed a fairly week binding affinity of MBL-C and no binding affinity of MBL-A to *S. pneumoniae*. The binding of ficolin-A to carbohydrate structures on the surface of *S. pneumoniae* was shown to result in complement C3 deposition leading to a critical degree of opsonisation and the clearance of *S. pneumoniae* through phagocytes.

Interestingly, no C4 deposition could be determined on the surface of *S. pneumoniae* while a strong lectin pathway dependent C3 deposition was observed. The inhibition of C4 deposition is most likely due to the presence of the pneumococcal surface protein A (PspA) which is a potent virulence factor of *S. pneumoniae*. It has been described recently that PspA effectively prevents C4 deposition on the outer polysaccharides capsule of *S. pneumoniae* (Li *et al.*, 2007). Nevertheless, a surprising and novel finding of my work was that the strong C3 deposition on *S. pneumoniae* is lectin pathway dependent in absence of C4. I further investigated the possible composition of this lectin pathway specific C4-bypass activation role of C3 by measuring the C3 deposition on *S. pneumoniae* using sera from different mouse strains with targeted complement deficiencies including ($C1q^{-/-}$, $fB^{-/-}$ and $C4^{-/-}$, $MASP2^{-/-}$,

MASP1/3$^{-/-}$). The results showed that lectin pathway C3 deposition was observed in all of these sera except of MASP-2$^{-/-}$ serum which showed no C3 deposition indicating that C3 deposition on *S. pneumoniae* is MASP-2 dependent and C4 deposition could be by-passed via MASP-2 dependent C3 cleavage.

In vitro opsonophagocytosis of *S. pneumoniae* by human neutrophils showed that phagocytosis of *S. pneumoniae* opsonised with MASP-2 deficient serum was greatly impaired when compared with bacteria opsonised with MASP-2 sufficient serum. This observation confirmed the previous finding that C3 deposition on *S. pneumoniae* is MASP-2 dependent.

In vivo studies demonstrated that intranasal challenge of MASP-2 deficient mice showed a dramatically increased rate of mortality after 72 hrs of infection (85%) when compared to wild type littermate controls at the same time point (25%). In addition, MASP-2 deficient mice showed a higher bacterial load in blood and in lung tissues after intranasal challenge in comparison to the wild type controls during the course of infection. The inability of MASP-2 deficient mice to clear the infection is mainly due to the lack of C3b deposition on *S. pneumoniae* that impaired the opsonophagocytosis which is the main mechanism for clearing of *S. pneumoniae* from blood and lung tissues (Ren *et al.*, 2003).

Studying the mRNA expression profiles for the proinflammatory mediators during the course of *S. pneumoniae* infection showed that wild type mice expressed higher levels IL-1β in lung tissues when compared to MASP-2 deficient mice. High levels of IL-1β were reported in several other studies to

be responsible for protection against *S. pneumoniae* infection (Rupprecht *et al.*, 2007; Kerr *et al.*, 2002). Elevated levels of mRNA expression of TNF-α and Macrophage inflammatory protein 2 (MIP-2) were observed in MASP-2 sufficient mice 12 hrs after intranasal infection in comparison to $MASP2^{-/-}$ mice. The early expression of TNF-α and MIP-2 enhances the recruitment of leukocyte into the site of infection and accelerates bacterial clearance (Neumann *et al.*, 1996). At the late stage of the infection, the majority of $MASP2^{-/-}$ mice died. mRNA expression levels of TNF-α and MIP-2 peaked in $MASP2^{-/-}$ mice at the terminal stage of *S .pneumoniae* infection.

The high bacterial load in the lung tissues of MASP-2 deficient mice appears to enhance the recruitment of PMNs into the lung tissues. Excessive TNF-α and MIP-2 production may effect neutrophil activation, which may critically contribute to lung injury (Windsor *et al.*, 1993; Linden *et al.*, 2005). Again, the level of mRNA expression of IL-6 in lung tissues was significantly higher in $MASP2^{-/-}$ mice when compared to wild type controls at the late stage of infection. The high level of IL-6 was associated with acceleration of tissue injury and lung damage (Dallaire *et al.*, 2001).

The *in vitro* and *in vivo* results clearly demonstrated that the lectin pathway of complement activation plays a major role in the protection against *S. pneumoniae* infection and it was postulated that the lack of C3 deposition on *S. pneumoniae* in MASP-2 deficient mice is the molecular reason for the inability of these mice to clear the infection via phagocytosis in contrast to the wild type controls that efficiently clear the infection due to an intact lectin pathway. My results indicate that the lectin pathway specific C4-bypass activation of C3 provides a critical clearance mechanism responsible for the opsonisation of *S. pneumoniae* with C3 and subsequent clearance of the

pathogen through phagocytes. In addition, the delayed inflammatory response in MASP-2 deficient mice may play a significant role leading to the severe course of *S. pneumoniae* infection in lectin pathway deficient mice.

An established model of poly-microbial peritonitis induced by CLP (Cecal ligation and puncture) was used to test the role of the lectin pathway of complement activation during poly-microbial sepsis. This experimental model of bacterial peritonitis resembles the clinical picture of bacterial peritonitis in humans which is characterised by hyper-inflammatory phase at the early stage followed by hypo-inflammatory phase during the later stage of infection where the mice are more susceptible to bacterial super-infection (Echtenacher *et al.*, 1996).

My results showed that both $MASP2^{-/-}$ and $MASP2^{+/+}$ mice have the same rate of mortality (approximately 75% 3 days after CLP). Although both groups showed the same rate of mortality, $MASP2^{-/-}$ mice showed a significantly higher bacterial load in peritoneal lavage when compared to wild type mice. The impaired C3 deposition in the MASP-2 deficient mice as well as the deficiency in MAC formation (Podack *et al.*, 1982) may be the main reason for the high bacterial load in peritoneal lavage which finally leads to septicemia and death. The surprising high rate of mortality amongst the MASP-2 sufficient mice even with low bacterial load could be due to the sever inflammatory response that leads to multi-organ failure and death.

Analysis of mRNA expression profiles following CLP demonstrated that MASP-2 sufficient mice have significantly higher mRNA expression levels of TNF-α, IL-1β and MIP-2 when compared to their MASP-2 deficient littermate

controls. The serum level of TNF-α was also significantly higher in wild type mice. The elevated level of TNF-α could be the main reason for the high rate of mortality in wild type mice due to TNF-α induced tissue destruction and multi-organ failure (Takahashi *et al.*, 2002).

The conclusion of this work is that inhibition of MASP-2 could be used to reduce the inflammatory immune response during sepsis and inhibition of the lectin pathway may be used as therapeutic adjuvant to protect against multi-organ failure during sepsis.

Taken together, my results using the models of *S. pneumoniae* infection and CLP (as a model for poly-microbial peritonitis) suggest that a deficiency of MASP-2 is responsible for a significant delay of the inflammatory cytokine storm during infection. This delay could be beneficial in case of poly-microbial peritonitis where it protects the mice form death due to sever inflammatory response leading to multi-organ failure.

To establish MASP-2 specific inhibitors of lectin pathway functional activity, I generated monospecific antibodies against human and murine MASP-2 that can be used to deplete the low abundant enzyme MASP-2 and transiently block the lectin pathway functional activity *in vivo*.

In order to produce stable antigens, catalytically inactive forms of human and mouse MASP-2 were generated by substitution of the serine residue in the catalytic domain with an alanine residue to generate MASP-2A which is enzymatically inactive. This mutation also decreases the cytotoxic effects of the active form of MASP-2 towards the transfected CHO-k1 cell line used for eukaryotic expression. It addition, the lack of proteolytic activity of MASP-2A

prevents activation and the decay of the protein during the expression and purification process. Unfortunately, generation of recombinant hMASP-2K and mMASP-2K was unsuccessful, most likely due to a high degree of autoactivation of the proenzyme generated which proofed toxic to the expression cell lines (Chen and Wallis, 2001).

Monoclonal antibodies (mAbs) against human and murine MASP-2 were successfully generated. Some of these mAbs I established showed to be effective inhibitors of the lectin pathway function activity *in vitro* and *in vivo* (anti-mMASP-2). The production of inhibitory monoclonal antibodies provides a new window of opportunities to study the use of MASP-2 inhibition as a therapeutic approach in the treatment of septic shock or ischemia/reperfusion injury.

In conclusion, my results strongly underline and clearly demonstrate the important role of lectin pathway of complement activation during *S. pneumonia* infection. In contrast, inhibition of MASP-2 activity during septic shock could provide a therapeutic approach in the near future as it may limit the inflammatory response in septic peritonitis considerably, thus leading to a relative reduction of the inflammation driven mortality during sepsis through multi-organ failure.

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