The molecular interactions between two activation pathways of complement are essential for a protective innate immune response to *Neisseria meningitidis* infection

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (The molecular interactions between two activation pathways of complement are essential for a protective innate immune response to *Neisseria meningitidis* infection) is based on work conducted by the author at the University of Leicester mainly during the period between March 2008 and March 2011.

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

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

Signed: Date:

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Abstract

The molecular interactions between two activation pathways of complement are essential for a protective innate immune response to *Neisseria meningitidis* infection.

Azam Hayat

The complement system forms a vital part of the immune system providing host defence against various pathogens, including *N. meningitidis*. The roles of the lectin and the alternative pathway of complement activation in meningococcal disease have been studied. Serum bactericidal assay against *N.meningitidis* suggested that both MBL and MASP-3 are essential for driving SBA on meningococci through a close association with the alternative pathway. A highly significant difference in survival between MASP-$2^{-/-}$ and MASP-$2^{+/+}$ wild-type mice was observed following *N. meningitidis* infection with a lethal intraperitoneal dose, showing that MASP-$2^{-/-}$ mice were significantly protected against meningococcal infection. MASP-$2^{-/-}$ mice also exhibited a significantly lower meningococcal burden in blood and different organs when compared to MASP-$2^{+/+}$ wild-type mice. The mRNA expression levels of inflammatory cytokines such as MIP-2, IFN-γ, IL-6 and IL-10 were significantly lower in different organs of MASP-$2^{-/-}$ mice compared to the MASP-$2^{+/+}$ control group. Therapeutic benefits of anti MASP-2 antibodies that inhibit the lectin pathway functional activity was also tested, and the findings showed that C57BL/6 wild-type mice treated with inhibitory MASP-2 antibody showed a significantly better survival when compared with wild mice treated with an irrelevant isotype control antibody following infection with a high dose of *N.meningitidis*. Furthermore, significantly reduced mortality rates were observed following application of recombinant properdin in a murine model of meningococcal infection. Finally, a combination therapy using inhibitory MASP-2 antibody and recombinant properdin was analysed in murine model of meningococcal infection using C57BL/6 wild-type mice. Following *N.meningitidis* infection, mouse groups which received both properdin and inhibitory MASP-2 antibody showed better survival with less disease severity scores when compared to infected untreated control mouse group, or mouse groups which received recombinant properdin or inhibitory MASP-2 antibody alone.

Abbreviations

α	Alpha
β	Beta
µ	Micro
AP	Alkaline phosphate
AP	Alternative pathway
bp	Base pair
BHI	Brain heart infusion
BSA	Bovine serum albumin
C1-INH	C1-inhibitor
C4bp	C4 binding protein
cDNA	Complementary deoxy-ribonucleic acid
CFP	Complement factor properdin
CP	Classical Pathway
CUB	C1r/C1s-Uegf-bone morphogenetic protein
DAF	Decay-accelerating factor
DEPC-H2O	Diethyl pyrocarbonate water
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate

E. Coli	*Escherichia coli*
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetra acetic acid
EGTA	Ethylene glycol tetraacetic acid
ES cells	Embryonic Stem cells
FCS	Foetal calf serum
FMS	Fulminant meningococcal sepsis
g	Grams
GAPDH	Glucose-6-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
HBSS	Hank's balanced salt solution
HI	Heat inactivated
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
kb	Kilobase
kDa	Kilodalton
KO	Knockout
LOS	Lipooligoccharides

LP	Lectin pathway
LPS	Lipopolysaccharide
mAbs	Monoclonal anitbodies
Map19	19 kDa mannose binding lectin associated protein
MASP	Mannose binding lectin serine protease
MAC	Membrane attack complex
MBL	Mannose binding lectin
min	Minutes
mRNA	messanger RNA
MW	Molecular weight
NeoR	Neomycin resistance cassette
NHS	Normal human serum
NKT	Natural killer T cells
OD	Optical density
OMP	Outer membrane protein
Opa	Outer membrane protein A
Opc	Outer membrane protein C
ORF	Open reading frame
PorA	Porin A
PorB	Porin B
PBS	Phosphate buffered saline

PBST	PBS with tween-20
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse Transcriptase- PCR
SBA	Serum bactericidal assay
SDS	Sodium dodecyl sulfate
sec	Seconds
TAE	Tris-acetate-EDTA
Tm	melting temperature of primer
WT	Wild-type

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Chapter 1. Introduction

1.1 The immune system

The most important function of the immune system is to provide protection to the body against invading microbial pathogens along with elimination of abnormal cells and cellular debris. The mammalian immune system is composed of various highly specialized immune cells and fluid phase effector systems and cellular receptors that allow communication between the cells of the immune system and the fluid phase effectors of immunity. There are two major branches of immune system; the innate immune system and the adaptive immune system, although both are highly interconnected. The innate immune response is composed of many specific and nonspecific resistance mechanisms providing a first and instant line of defence (Borghesi and Milcarek, 2007). The innate immune system represents the most ancient form of defence mechanisms. It reacts equally well to a variety of antigens and microbial pathogens with fast kinetics of activities. However, the innate immune system does not possess any immunological memory. It acts on several levels ranging from physiological and anatomical barriers (skin & surface of mucosal membranes up to ciliated epithelial); the chemical environment of the body (including pH) along with certain soluble and membrane-bound proteins and antimicrobial peptides. These recognition components often share conserved structural motifs for pathogen associated molecular patterns (PAMPS) recognition and facilitate phagocytosis and pathogen

elimination through an assortment of phagocytic cells (Goldsby *et al*., 2003; Hoffman *et al*., 1999; Medzhitov, 2007).

In contrast, the adaptive immune system responds relatively slow and requires time to launch a specific response against an invading pathogen following recognition of specific "non-self antigens'' during a process called antigen presentation. The adaptive immune system bears immunological memory against the encountered antigens and has the potential to produce a heightened and efficient state of immune response on subsequent exposure to the same antigens. The adaptive immune system can generate a vast diversity of antigen specific recognition molecules that are produced as a result of various genetic recombinations. There are two effector components of adaptive immunity: The cell-mediated and the humoral response (Schatz *et al*., 1992). The humoral adaptive immune response is illustrated by secreted antibodies produced by B lymphocytes following exposure to antigens where as cell-mediated immunity involves the activation of different immune cells (such as macrophages, natural killer cells (NK), antigen specific cytotoxic T-lymphocytes) and the release of various cytokines on recognition of an antigen (Holmskov *et al.,* 2003; Medzhitov, 2007).

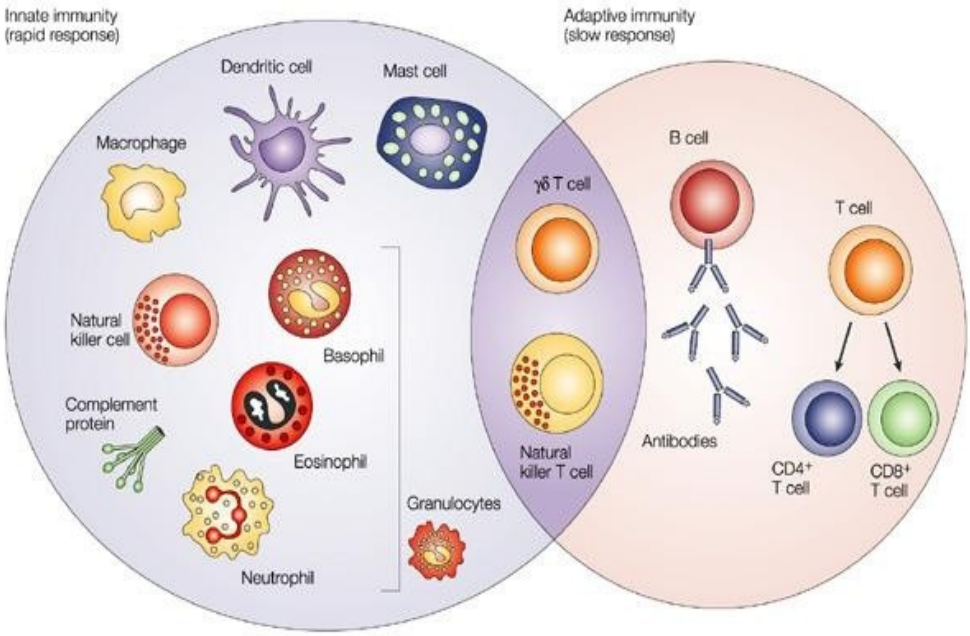

Figure 1.1: Innate and adaptive immune response as illustrated by Dranoff (2004).

1.1.1 The complement system

The complement system is an important and fundamental part of the immune system, providing protection against pathogens by innate as well as by antibody-mediated immunity (Fujita, 2002). Originally described as 'Alexion' by Jules Bordet, the complement was first identified as a heat-sensitive element in plasma showing antimicrobial activity which 'complemented' the ability of antibodies to kill bacteria (Murphy *et al.,* 2008). The term "Complement" was later coined to this heat sensitive component of the serum by Paul Ehrlich (Defranco *et al.,* 2007; Goldsby *et al.,* 2003).

The complement system is composed of more than 35 circulating and membrane-bound proteins, which are mostly present in their proenzymatic form. Once cleaved during the activation step, these zymogens are converted into their enzymatically active state to form part of a multimolecular enzyme complex that cleaves and activates other complement components, which then further continue to activate complement proteins through a cascade of sequential activation steps (Walport, 2001).

The complement system can be activated by three different pathways which include the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP) (Fujita, 2002; Schwaeble *et al*., 2002; Thiel, 2007). Although following different initiation steps, all the three pathways of complement system converge in the activation of C3 (via a C3 convertase). C3 activation is the central component of complement and leads to the formation of a bactericidal membrane attack complex. The classical pathway is initiated by the binding of multi-molecular C1 complex to antigen-antibody complexes whereas the lectin pathway is activated upon PAMPs sensing via a complex formed of carbohydrate recognition molecules associating with a serine protease called MASP-2 (for Mannan binding lectin associated serine protease-2). The alternative pathway is initiated by the binding of hydrolysed C3 to the surface of a pathogen. Subsequently, a C5 convertase is formed, which cleaves the C5 molecule into C5a and C5b. The biologically active C3a and C5a elicit a number of proinflammatory effects, such as degranulation of mast cells and basophils, chemotaxis of leukocytes, increased vascular permeability and smooth muscle contraction (Köhl, 2001). C5b is also the initial molecule in the formation of the terminal

C5b-9 complement complex (TCC) or the membrane attack complex, leading to the lysis of bacteria (Stover *et al.,* 1999; Schneider *et al.,* 2006).

1.1.1.1 The classical pathway

The classical pathway is activated by the binding of the multimolecular C1 complex to immune complexes. The C1 complex has a total molecular weight of approximately 790 kDa and consists of a recognition subcomponent C1q, along with two homodimers formed by the C1 zymogens C1r and C1s to form C1q:$C1r_2$:$C1s_2$ complex (Copper, 1985). It has been shown that C1q complex is sensitive to high salt concentrations, which dissociate the complex. A single C1q molecule is composed of six identical subunits joined together through their collageous stalks that end in globular head domains. Each subunit consists of three homologous polypeptide chains encoded by three homologous genes located within one gene locus (A, B and C) (Arlaud *et al*., 2002).

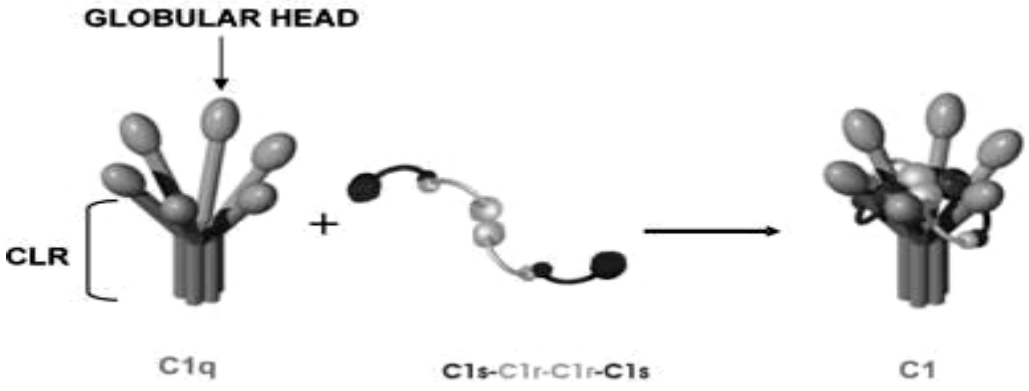

Figure 1.2: Structure of the C1 component of the classical pathway of complement showing homodimers of C1r and C1s with C1q molecule (Pflieger *et al*., 2010).

The classical activation pathway is initiated once the C1q either directly binds to bacterial surface components or indirectly binds to the Fc part on antigen-bound IgG and IgM antibodies (Boes *et al*., 1998). This binding of C1q to complement activators leads to a conformational change in the collagenous region of C1q leading to autoactivation of C1s–C1r–C1r–C1s tetramer, whereby C1r activates C1s that translates C1q activation into the cleavage of C4 and C4b bound C2. The C1s mediated cleavage of C4 splits C4 into C4a and C4b. The smaller peptide fragment C4a is released in the fluid phase as an anaphylatoxins whereas the larger fragment C4b binds covalently to the activator cell surface via its thioester group (Wallis *et al*., 2007). C2 will bind to C4b on the surface of the pathogen which is then cleaved by C1s into two parts: C2a and C2b. C2b will be released into the fluid phase, while C2a remains attached to C4b as the enzymatically active part of the classical pathway C3-convertase C4b2a. C4b2a proteolytically cleaves C3 into C3a and C3b. C3a is an anaphylatoxin and promotes pro inflammatory cellular response while C3b acts as an opsonin that binds to the pathogen surface and helps in phagocytosis. C3b bound to the C3 convertase will lead to the formation of C5 convertase ($C4b2a(C3b)_n$) that cleaves C5 into two fragments C5a and C5b. C5a is a potent fluid phase anaphylotoxin that mediates the recruitment of immune cells at the site of inflammation thus enabling the disposal of the invading pathogen. C5b will bind covalently to the pathogen surface thereby initiating the activation of the terminal complement activation cascade leading to the formation of the membrane attack complex which depending on the microorganism involved may directly lyse the pathogen (Vorup-Jensen *et al*., 2000; Arlaud *et al*., 2002).

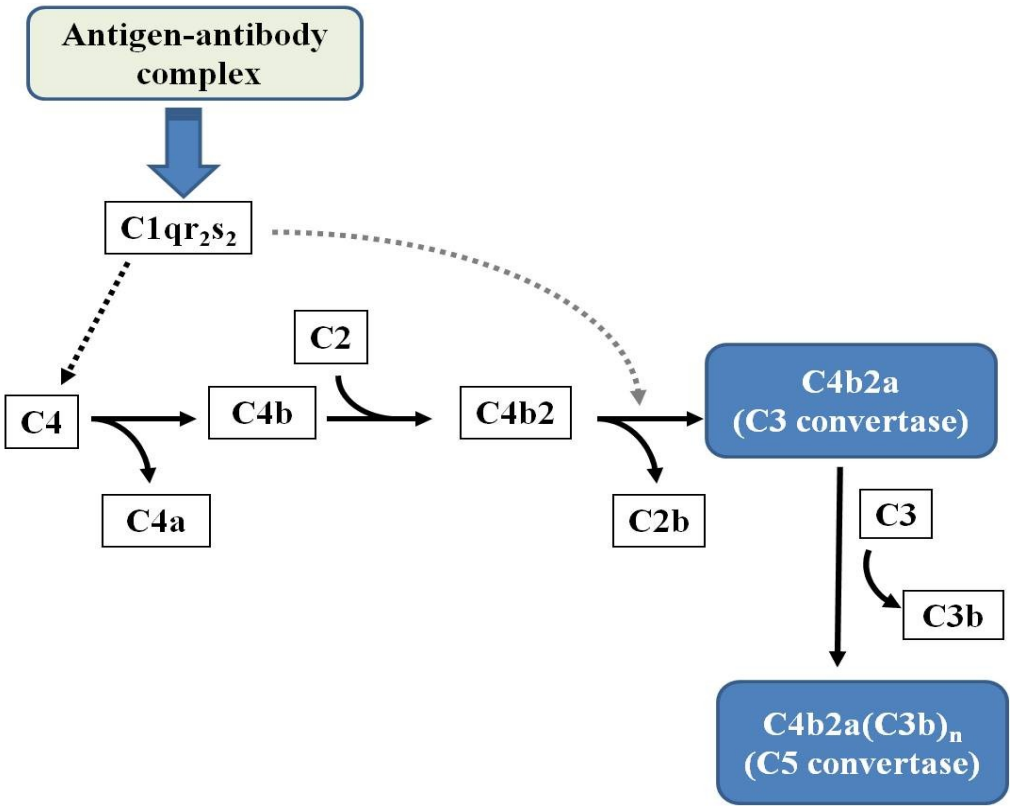

Figure 1.3: Classical pathway activation on the surface of immune complex.

1.1.1.2 The lectin pathway

The lectin pathway neutralizes the invading microorganisms through a rather selective and an antibody-independent mechanism (Turner, 1996). The lectin pathway of complement is initiated by the binding of a multimolecular activation complex (MBLs, CL-11 or Ficolins associated with MASP-2) to Pathogen-Associated Molecular Patterns (PAMPs) (Schwaeble *et al*., 2002; Thiel *et al*., 2000; Schwaeble *et al*., 2011). PAMPs are predominantly carbohydrate structures present on pathogens or oxygen-deprived cells. The binding of the respective carbohydrate recognition components leads to the activation of MBL-associated

serine proteases whereby MASP-2 cleaves complement component C4 into two fragments C4a and C4b. C4a is released while the C4b fragment remains attached to the cell surface. Activated MASP-2 also cleaves C4b bound C2 into two fragments C4b2a and C2b. C4b2a is the lectin pathway C3 convertase (C4b2a) and is identical to the classical pathway C3 convertase. C3 convertase activity eventually leads to the formation of a C5 convertase (C4b2a $(C3b)_n$), which initiates the formation of the terminal pathway as described before for the classical pathway (Schwaeble *et al*., 2002; Thiel *et al*., 2000).

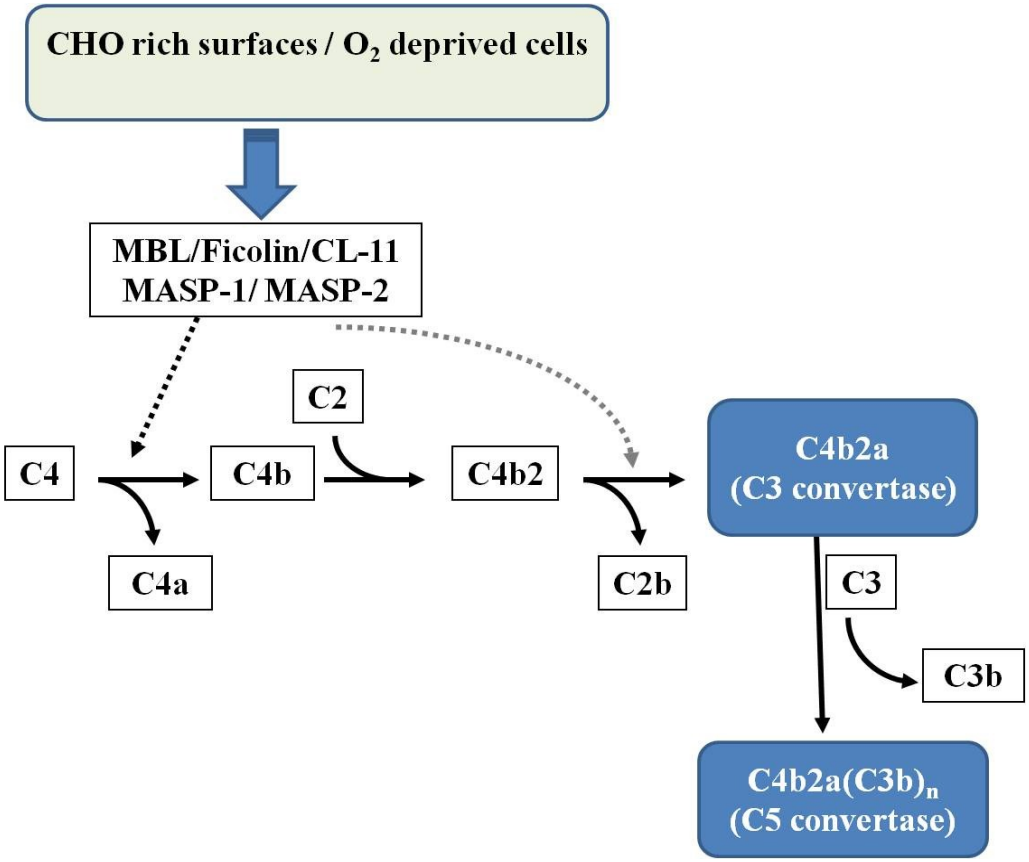

Figure 1.4: Lectin pathway activation on Pathogen-Associated Molecular Patterns (PAMPs).

1.1.1.2.1 Lectin pathway activation complexes

1.1.1.2.1.1 MBL and ficolins

The lectin activation pathway is much more complex and intricate as compared to the classical pathway and alternative pathway due to the interaction of several complex multiple components (Sorenson *et al.,* 2005). The lectin pathway activation complexes are composed of a multimeric carbohydrate recognition subcomponent (i.e. Mannan Binding Lectin (MBL) and ficolins) and the MBL-associated serine proteases MASP-1, MASP-2 and MASP-3, and a non-enzymatic 19kDa protein (called MAp19 or sMAP) (Stover *et al.,* 1999; Takahashi *et al.,* 1999).

MBL, a member of family of proteins known as 'Collectins', is mainly synthesized in the liver (Brouwer *et al.,* 2008). It circulates in the serum in the form of large oligomeric complexes (trimers, tetramers and hexamers). Each structural subunit of MBL is composed of multimers of three identical polypeptide chains (i.e. homotrimers), each of which is divided into a short N-terminal cysteine-rich domain, a collagen-like domain, a neck region and a globular head part representing the carbohydrate recognition domain (CRD). MASP-1, MASP-2, and MASP-3 interact with MBL through the collagen-like domain (Roos *et al.,* 2001). Mannose and *N*-acetyl-glucosamine (GlcNAc) serve as the prominent ligands for MBL, while other carbohydrates have shown a low or undetectable affinity for MBL. It has recently been reported that MBL can bind to IgM and is, therefore, able to activate the lectin pathway on IgM immune-complexes (McMullen *et al.,* 2006). Since MBL allows multiple interactions, the biological activity of MBL increases with the number of

oligomers (Drickamer, 1992; Wallis, 2005). Apart from activating the complement system, a role of MBL as a complement independent opsonin has been suggested by many recent reports **(**Kuhlman *et al*., 1989; Polotsky *et al*., 1997; Ogden *et al*., 2001; Jack *et al*., 2005**)**. A complement independent opsonophagocytic role of MBL against *N. meningitidis* has been proposed by Jack *et al.* (2001) and Jack *et al.* (2005) who provided evidence that MBL may serve as a dependent opsonin by accelerating the uptake and killing of meningococci by human professional phagocytes. Only one single MBL gene is expressed in human, whereas there are two genes for MBL in rodents: MBL-A and MBL-C (Sastry *et al.,* 1995).

Ficolins also act as carbohydrate recognition molecules of the lectin pathway of complement activation. There are three different types of ficolins present in human; L-ficolin, H-ficolin and M-ficolin. L-ficolin and H-ficolin are present in serum and activate the lectin pathway by direct recognition of the acetylated sugar moties on the surface of pathogens (Matsushita *et al.,* 2000). M-ficolin is a nonserum ficolin and is mainly found on the surface and in secretory granules of leukocytes (neutrophils and monocytes) and activate the lectin pathway by forming a complex with serine proteases MASP-1 and MASP-2 (Matsushita and Fujita, 2001; Liu *et al.,* 2005).

In mouse, there are only two types of ficolins present; ficolin A and ficolin B. Ficolin A is found in the serum and resembles L-ficolin in humans while ficolin B is found in bone marrow cells. Ficolin B shows 60 % structural similarity to Ficolin A, but unlike Ficolin A,

it doesn't bind to MASP-2 and therefore, cannot activate the lectin pathway of complement system (Endo *et al.*, 2005; Runza *et al*., 2008). Like MBL, ficolins show structural subunit composed of three identical polypeptide chains each of which is made up of a short N-terminal cysteine rich domain with one or two cysteine residues, a collagen like domain, a neck region and fibrinogen-like carbohydrate recognition domain (FBG) (Garred *et al*., 2009; Matsushita *et al.,* 2001). Primary ligands for ficolins include N-acetyl glucosamine sugars as well as lipoteichoic acids of gram-positive bacteria (Endo *et al.*, 2005; Lynch *et al.*, 2004).

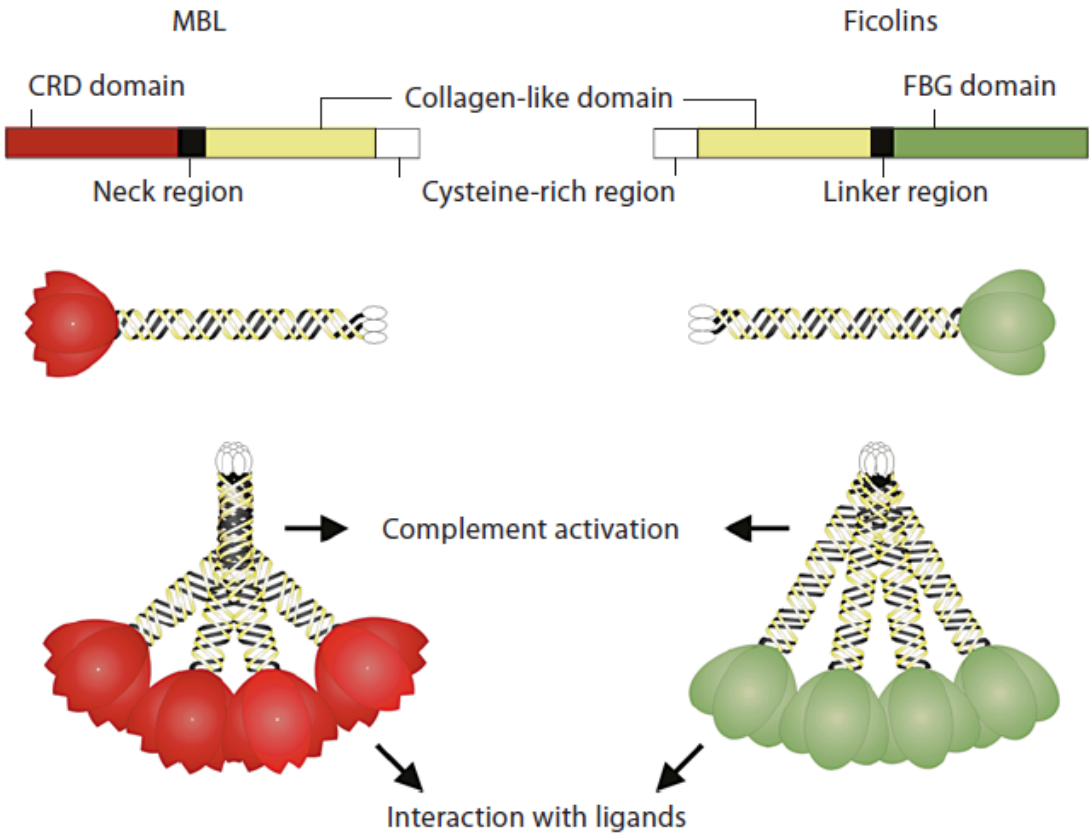

Figure 1.5: The domain structure and oligomerization of MBL and ficolins (Garred *et al*., 2009).

1.1.1.2.1.2 MBL associated serine proteases (MASPs)

MBL associated serine proteases (MASPs) are homologous to the classical pathway specific complement serine proteases and share the same domain organization as of C1r and C1s. However, MASPs are solely responsible for activation of lectin pathway of the complement system. There are three types of MASPs in mammals which include MASP-1, MASP-2 and MASP-3 along with a non-enzymatic MBL-associated protein of 19kDa (MAp19) (Schwaeble *et al*., 2002; Stover *et al*., 1999).

MASP-1 and MASP-3 are alternative splicing products of a single structural *MASP1/3* gene on chromosome 3 in human (Dahl *et al*., 2001; Takada *et al*., 1995). The MBL-associated plasma protein (MAp19) and MASP-2 are encoded by two different mRNA species generated by alternative splicing from one structural *MASP2* gene mapped on chromosome 1 in human and chromosome 4 in mouse (Stover *et al*., 1999; Sorenson *et al*., 2005). All the three MASPs share the same domain organization. These domains are composed of an N-terminal CUB I (C1r/C1s/sea urchin Uegf/bone morphogenic protein) domain, followed by an epidermal growth factor (EGF)-like domain, another CUB domain (CUB II), two complement control protein domains; CCP1 and CCP2 (also called short consensus repeats (SCRs)) and a chymotrypsin-like serine protease domain (SP). MASP-1 and MASP-3 share a common N-terminal domain organization but differ in their serine protease domain (Thiel, 2007; Sorenson *et al.,* 2005).

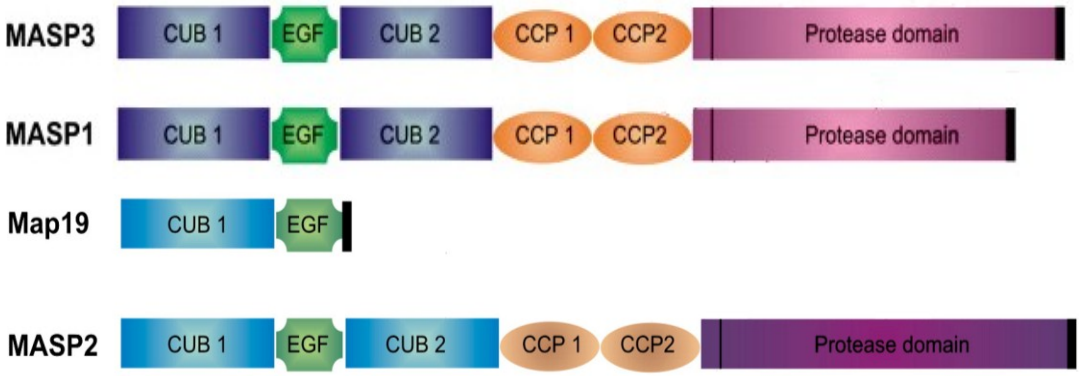

Figure 1.6: MASPs domain organization as described by Yongqing *et al.* (2012).

MASP-1, MASP-2, MASP-3 and MAp19 form homo-dimers through the corresponding CUBI-EGF domains in a Ca^{2+} dependant way. MASPs form complexes with MBL and ficolins by binding of their CUB I and EGF-like domains to the collagen-like regions of MBL and ficolins (Wallis *et al.,* 2004). MASPs are converted from their zymogen form to their enzymatically active form by the cleavage between the CCP-2 and the serine protease domain. This leads to the formation of a heavy chain (also called chain A; composed of N-terminal domains) and a light chain (chain B; representing the serine protease domain) both held together through a disulfide bridge (Matsushita and Fujita, 1995).

So far, only MASP-2 has an essential role for the activation of the lectin pathway of complement (Rossi *et al*., 2001). MASP-2 can cleave both C4 and C4-bound C2 to generate the LP C3 convertase C4b2a even without any participation of either MASP-1 or MASP-3 (Matsushita *et al.,* 2000; Vorup-Jensen *et al.*, 2000). A murine MASP-2 deficient model has been developed which is considered be the first model showing a very clear phenotype

with total lectin pathway deficiency. The analysis of sera and plasma of MASP-2 deficient mice clearly reveals that MASP-2 is the only enzyme required to activate complement via the lectin pathway and that neither MASP-1, nor MASP-3 can maintain or restore lectin pathway activity in MASP-2 deficiency (Schwaeble *et al.,* 2011). MASP-1 has been shown to possess thrombin-like activity by cleaving the complement component C2 (Matsushita and Fujita, 1992) but it is unable to cleave C4 (Chen & Wallis 2004). Another study has proposed the role of MASP-1 in lectin pathway activation but this contribution is mainly dependent on MASP-2 (Takahashi *et al*., 2008). An important role of MASP-1 in activation of the alternative pathway is also suggested in a recent study which shows that MASP-1 is required to convert zymogen form of factor D into its enzymatically active form (Takahashi *et al*., 2010).

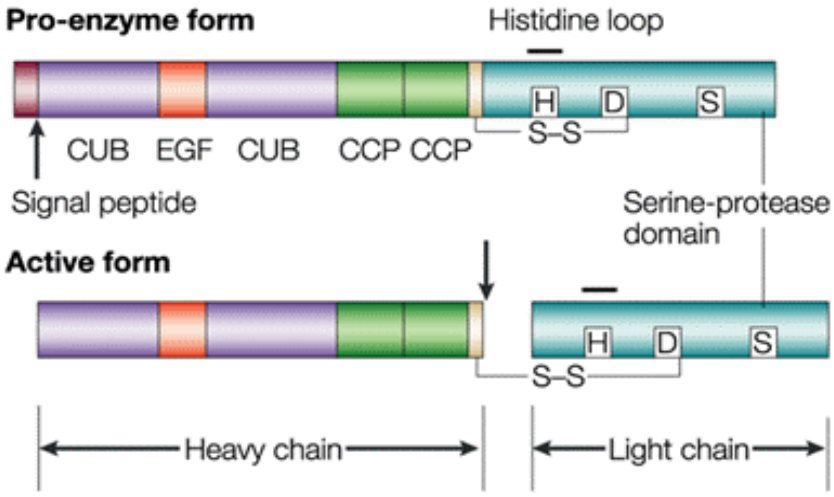

Figure 1.7: MASPs activation results in the formation of a heavy and a light chain held together through a disulfide bond (Fujita, 2002).

MASP-1 is mainly 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 as revealed by northern blot analysis and in-situ hybridization studies (Lynch *et al*., 2005). MASP-2 and MAp19 are exclusively synthesized in liver tissue with hepatocytes being the only source of biosynthesis (Endo *et al*., 2002; Schwaeble *et al*., 2002). The physiological substrates for MASP-3 have not yet been clearly defined, and MASP-3 is not thought to be involved in the cleavage of either C2 or C4 (Rossi *et al*., 2001; Thiel, 2007). However, an important role of MASP-3 in driving alternative pathway activation has recently been suggested. A very recent study by Iwaki *et al* (2011) shows that once incubated with bacterial targets *in vitro*, recombinant MASP-3 complexed with recombinant MBL can be converted into an active enzyme which initiates the activation of the alternative pathway of complement by cleaving C3b bound factor B (Iwaki *et al*., 2011).

Recently, a new collectin, collectin 11 (CL-11) was identified as a plasma component that associates with both MASP-1 and MASP-3 (keshi *et al*., 2006). CL-11 binds to D-mannose and L-fucose terminal saccharides of different microbes and may serve as a new recognition sub component of the lectin pathway (Hansen *et al*., 2010).

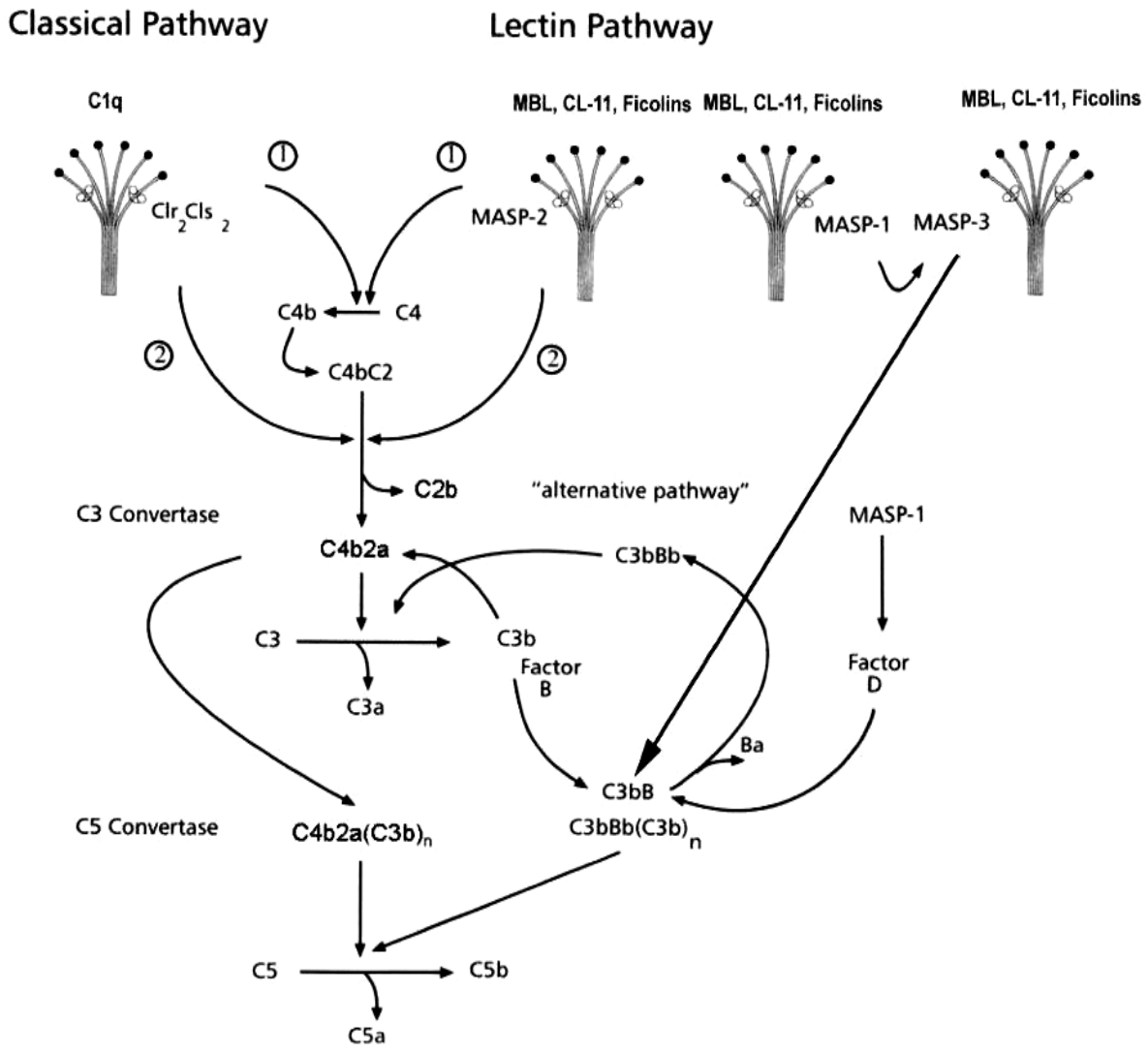

Figure 1.8: Lectin pathway activation complexes can also drive the alternative pathway of complement activation

(Figure courtesy of Professor W Schwaeble, University of Leicester UK).

1.1.1.3 The alternative pathway

Unlike the classical and the lectin pathways, the alternative pathway activation was thought to occur independent of a large multivalent recognition molecule. Until very recently it was perceived that alternative pathway activation relies on the unstable nature of thioester bearing the complement component C3. Factor B (fB), factor D and properdin (factor P) are specific components of the alternative pathway of complement activation (Matsumoto *et al.,* 1997). The present text books state that the alternative pathway is initiated by spontaneous low level hydrolysis of component C3 to form C3 (H_2O). The C3 (H_2O), in the presence of factor B, forms a C3 (H_2O)B complex in the presence of Mg^{2+} (Farries *et al*., 1988). Factor D cleaves C3 (H_2O)-bound factor B into the two fragments Ba and Bb. The Ba fragment is released from the complex, whilst Bb remains attached to the C3 (H_2O) complex to form the alternative pathway C3 convertase, i.e. C3 (H_2O) Bb. The alternative pathway C3 convertase cleaves C3 into C3a and C3b. The newly generated C3b will bind covalently 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; Muller-Eberhard, 1988). This binding is stabilized by properdin, which is a plasma protein and serves as a positive regulator of the alternative pathway of complement activation (Smith *et al*., 1984). The alternative pathway can also act as an amplification loop for classical and/or lectin pathway whereby C3b generated by either of the pathway binds to factor B, which is subsequently cleaved by factor D (Schwaeble and Reid, 1999). However, host cells often avoid complement activation on their surfaces by sequestration of the host complement

regulatory proteins (such as MCP, DAF, CR1 and factor H) on host tissues which prevent self-damage (Wallis *et al.,* 2007).

As seen with the classical and lectin pathway convertase before, further accumulation of C3b fragments in close proximity of the C3bBb complex (by the continuous activity of the C3 convertase C3bBb complex) shifts the specificity of this complex towards the cleavage of the substrate C5 and forms the alternative complement pathway C5 convertase complex C3Bb(C3b)n (Farries *et al*., 1988). The C5 convertase cleaves the C5 component of the complement and thereby releases C5a and initiates the formation of the membrane attack complex.

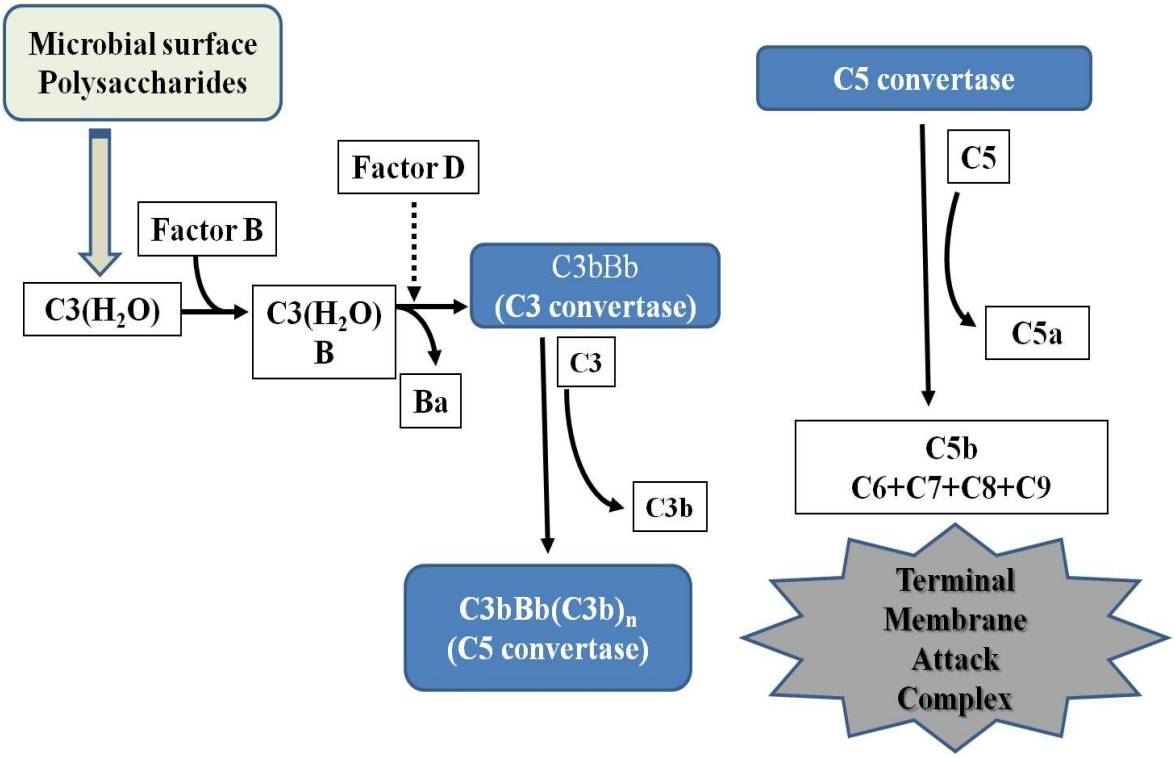

Figure 1.9: Alternative pathway activation of complement system

1.1.1.4 Membrane attack complex (MAC)

Lysis of the invading pathogen through the formation of a membrane attack complex is one of the key bactericidal functions of the complement system. C5 convertase complexes formed through LP/CP (C4b2a (C3b) n) or through AP (C3bBb (C3b) n), cleave C5 into C5b and C5a. C5b then binds covalently to the pathogen surface and reacts with C6, C7 and C8 to form a C5b-8 complex that leads to polymerisation of 10-16 molecules of C9, which composes a pore forming structure. The channelled pore forming complex then inserts into the lipid bilayer of the bacterial cell membrane leading to the disruption of membrane functions and allowing a water influx into the cell which finally leads to cell lysis (Podack *et al*., 1982).

1.1.1.5 Biologic activities of complement system

Complement once activated leads to a multitude of biological activities, including opsonisation of the invading pathogens, initiation of a proinflammatory response, direct lysis of pathogens via the membrane attack complex and finally immune complex clearance (Walport, 2001; Walport, 2001b).

The complement system protects the body against infection via three main mechanisms;

i) Opsonisation of pathogens is one of the key activities of complement system. It facilitates the uptake of pathogens by phagocytic cells. Opsonisation of an invading pathogen is primarily mediated by the binding of C3b or iC3b (the haemolytically

inactive cleavage product of C3b) and C4b to a lesser extent (Mevorach *et al*, 1998). C3b binds covalently to the pathogen surface and enhances phagocytosis of complement opsonized particles by leukocyte via binding to complement receptor type 1(CR1) and type 3 (CR3) (Aoyagi *et al*., 2005). It has also been reported that ficolins and MBL may initiate phagocytosis directly by binding to pathogens and stimulate phagocytosis by binding to collectin receptors on the surface of the phagocytes (Jack *et al*., 2001). Complement also plays a critical role in the clearance of cellular debris in addition to immune complexes. C1q binds to the surface of apoptotic cells and facilitates the uptake of this complex by macrophages (Taylor *et al*., 2000). In addition, deposition of iC3b on the surface of apoptotic cells leads to recognition of these cells by CR3 and CR4 receptors on the surface of phagocytes with subsequent elimination of these cells (Mevorach *et al*., 1998). Deposition of C3b on immune complexes facilitates their clearance by erythrocytes (displaying CR1) which transfer them into the liver, and the spleen where they are cleared from the circulation by the resident macrophages (Manderson *et al*., 2004).

ii) Complement also plays an important role in chemotaxis of immune cells to the sites of infection and inflammation. During complement activation, proinflammatory cleavage products such as C4a, C3a and C5a are released. Amongst these, C3a and C5a are potent anaphylatoxins that enhance the recruitment of inflammatory mediators and inflammatory cells to the site of infection by increasing the vascular permeability and formation of inflammatory exudates. These anaphylatoxins trigger

degranulation and histamine release by mast cells (Nataf *et al.,* 1999). Increased vascular permeability leads to extravasations of leukocyte to the site of inflammation, which helps in clearing invading pathogens. Anaphylatoxins, especially 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 are able to induce gene expression and production of monocyte chemotactant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) from mouse endothelial cells (Laudes *et al*., 2002). C5a also reacts with the C5a receptor on granulocytes and monocytes. This binding then induces the upregulation of CR3, which is one of the receptors involved in the phagocytosis of C3b-coated bacteria (Sprong *et al.,* 2004).

iii) One of the major functions of complement cascade is direct killing of invading pathogen, (especially gram-negative bacteria) through the formation of the membrane attack complex formation (Nauta *et al*., 2004). Each complement activation pathway culminates in the formation of a C5b molecule that binds covalently to the pathogen surface and reacts with C6, C7 and C8 to form a C5b-8 complex that catalyses the polymerisation of 10-16 molecules of C9 which then form transmembrane channels in the lipid bilayer of the bacterial plasma membrane. This leads to the disruption of membrane functions and allows the passage of ions and water influx, effectively creating an osmotic grandient which finally leads to cell lysis (Podack *et al*., 1982).

1.1.1.6 Regulation of complement system

The effect of excessive complement activation can be detrimental to host tissues, so there are several complement regulatory proteins that tightly regulate the activation of complement. These proteins include fluid-phase regulatory proteins and membrane-bound regulatory proteins (Kirschfink and Mollens, 2003).

1.1.1.6.1 Fluid phase regulators

A number of regulatory proteins present in plasma control the activity of complement. C4 binding protein (C4bp), an acute phase serum protein, is a main fluid-phase regulator of the complement system (Blom *et al.,* 2004). C4bp binds with C4b and inhibits its binding with C2a, which blocks the formation of C3-convertase C4b2a. C4bp also accelerates the natural decay of the formed C3-convertase through similar mechanism. Furthermore, C4bp acts as a cofactor in the factor I mediated conversion of C4b into inactive fragments (iC4b, C4e and C4d) that have no further role in complement activation cascade (Jurianz *et al*., 1999).
C1 inhibitor (C1-INH) or serpin is a physiological serine protease inhibitor that blocks the spontaneous activation of the proenzymes C1r and C1s by binding to these serine proteases in the C1 complex (Harpel and Cooper, 1975). C1-INH can also block the CP by displacing activated $C1r_2$-$C1s_2$ complexes from C1 complex on pathogen surfaces therefore, blocking any further downstream activation (Chen and Boackle, 1998; Prada *et al.,* 1999).

Factor H is the main negative fluid-phase regulator of the alternative pathway. It is a 150-kDa soluble protein that is present in all the body fluids, including the secretions in the nasopharynx (Zipfel *et al.,* 1999). It controls the alternative pathway activation at the C3 convertase (C3bBb) and C5 convertase (C3bBb $(C3b)_n$) level by binding to C3b (Pangburn *et al*., 2000). It acts on C3b on two levels; i) it accelerates the decay of the formed C3 convertase by binding to C3b and removing it from the complex (C3bBb) and ii) it acts as a cofactor in the factor I mediated cleavage of C3b to iC3b, C3c and C3dg (Turnberg and Botto, 2003).

Vitronectin (S-protein) and clusterin are regulators for the terminal activation cascade of complement. They inhibit the MAC formation in the host cells by binding to C5b-7 complex and preventing the insertion of C8 and C9 in host cell membranes therefore, preventing the host cells lysis' (Jenne and Tschopp, 1989).

Serum Carboxypeptidase N (SCPN) is a member of the family of carboxypeptidases, which regulates complement activation-mediated anaphylotoxin responses by enzymatically cleaving and removing the terminal basic amino acids from inflammatory mediators, including C3a and C5a, therefore, rendering them inactive (Bokisch and Muller-Eberhard, 1970; Campbell *et al.,* 2002).

1.1.1.6.2 Membrane-bound regulators

Complement activity is also controlled by membrane-bound proteins or receptors, which are expressed on the surface of host cells. These include; complement receptor 1 (CR1 or CD35), protectin (CD59), membrane cofactors of factor I mediated proteolysis such as membrane cofactor protein (MCP) or CD46 and decay accelerating factor (DAF).

CR1 (CD35) is expressed by many cells, including erythrocytes and lymphocytes. It prevents the formation of C3 convertase by binding to C3b (alternative pathway) and C4b (classical and lectin pathway). Like factor H, it serves as a cofactor for factor I mediated cleavage of C3b to smaller inactive fragments and dissociates C3b out of its complexes (decay accelerating function). MCP binds to C3b and acts as a cofactor for factor I and facilitates the inactivation of C3b (Whaley and Schwaeble, 1997). DAF is attached to cell membranes and controls the complement activity by dissociating the formed C3 and C5 convertases (Medof *et al*., 1984). CD59 is a regulator of the terminal complement complex present on the surface of different type of cells. It binds to C8 and C9 (MAC) components and inhibits polymerization of C9 molecules on C5b-8, which subsequently prevents the formation of MAC (Rollins *et al*., 1991).

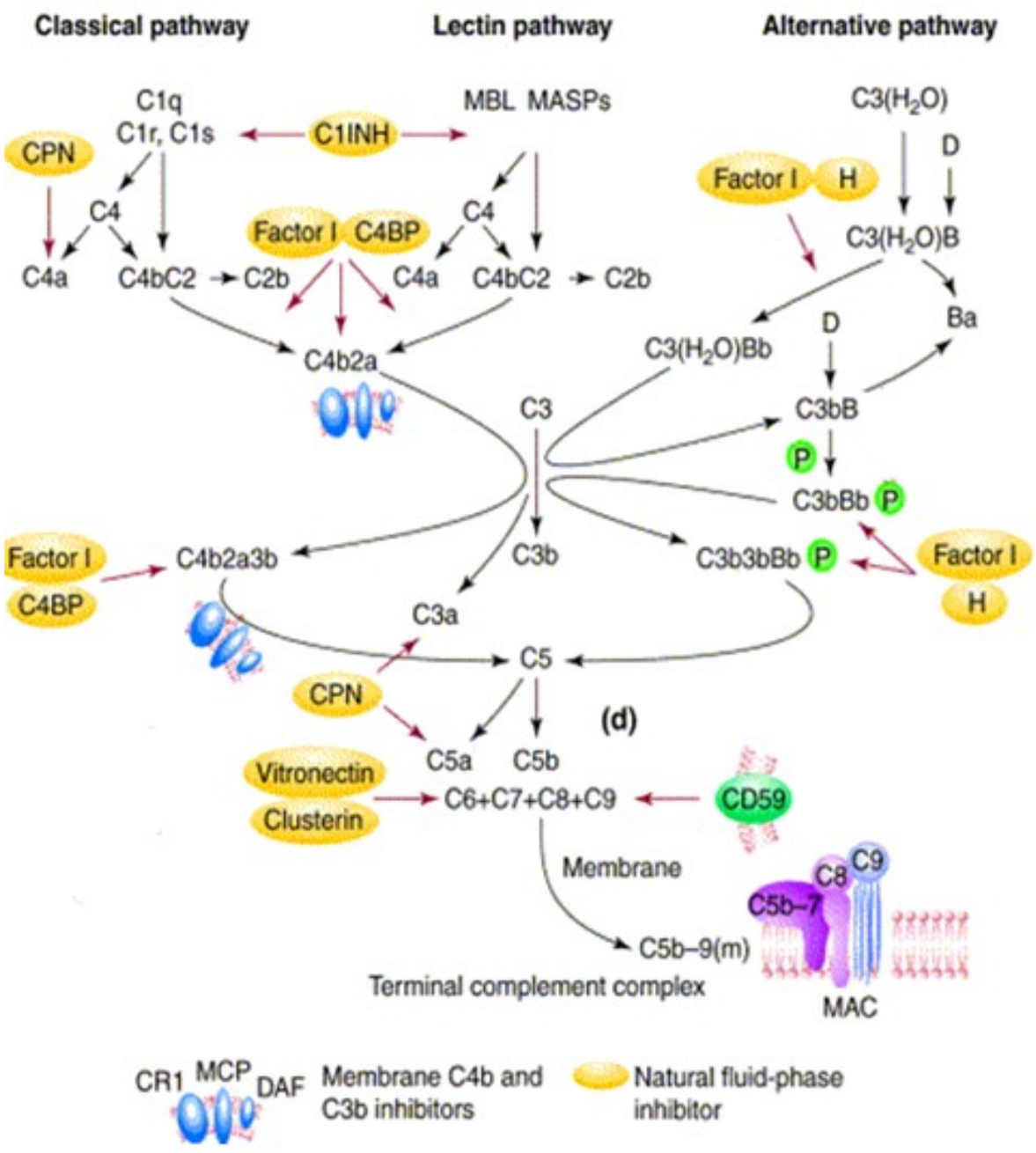

Figure 1.10: Regulation of complement activation by membrane bound and fluid phase regulators as described by Mollnes *et al.* (2002).

1.1.1.7 Complement deficiencies

Complement system plays a major role in the protection against invading pathogens as well as in maintaining the integrity of the body by supporting tissue regeneration and repair (Laufer *et al.,* 2001). Deficiencies in one or more complement components are associated with recurrent invasive bacterial infections and development of autoimmune diseases (Figueroa and Densen, 1991).

1.1.1.7.1 Classical pathway deficiencies

A deficiency in any component of the classical pathway is associated with high incidence of immune complex diseases. Deficiency of early classical pathway components (C1q and C1s) results in systemic lupus erythematosis (SLE) like syndrome, which develops mainly due to the impaired function of the complement system in clearing immune complexes and apoptotic cells (Amano *et al*., 2008; Carroll, 2004). The deficiency in classical pathway components also increases the risk of recurrent invasive bacterial infections and polymicrobial peritonitis (Brown *et al*., 2002; Celik *et al*., 2001). C2 deficiency was found as a predisposing factor for atherosclerosis and rheumatic disorders (Jönsson *et al*., 2005). C2 deficiency is also associated with invasive infections such as septicaemia and meningitis caused by *S.pneumoniae.* Children deficient in C2 are susceptible to recurrent pneumococcal infections (Jönsson *et al*., 2005). C3 deficiency is considered to be the most serious complement defect and is prominent in individuals with complete C3 deficiency. Although homozygous C3 deficiency is rare, these individuals are highly susceptible to

recurrent severe infections caused by virulent encapsulated bacteria such as meningococci, pneumococci and *Haemophilus influenza* (Ross and Densen, 1984).

1.1.1.7.2 Lectin pathway deficiencies

Lectin pathway activation is mediated by mannan binding lectin (MBL), ficolins (L-Ficolin, H-ficolin and M-ficolin) and CL-11 forming complexes with MBL associated serine proteases (MASPs). Lectin pathway deficiency is associated with a high risk of recurrent bacterial infections and autoimmune disorders (Sorensen *et al*., 2005). The most frequent cause of lectin pathway deficiency in humans is due to MBL deficiency while deficiencies in L-ficolins and MASP-2 are very rare. Low levels of MBL in humans are caused by one of three allelic variants with frequently occurring structural single point mutations found within exon 1 of the *mbl2* gene located on human chromosome 10. These nucleotide polymorphisms cause a single amino acid substitution at codons 52 (substitution of arginine with cysteine), 54 (substitution of glycine with aspartic acid) and 57 (substitution of glycine with glutamic acid) resulting in the loss of intact protein as these mutations interfere with the formation of MBL polymers, therefore, lowering the MBL levels (Wallis and Cheng, 1999).

Most individuals with these inherited MBL deficiencies remain healthy and show no increased predispositions for recurrent infections or morbidity when compared with MBL sufficient control populations (Dahl *et al*., 2004). However, under certain conditions

patients with MBL deficiency show increased susceptibility for severe bacterial infections especially when associated with other immunocompromised conditions (HIV infection, cancer, transplant, post chemotherapy) (Super *et al.,* 1989; Peterslund *et al*., 2001; Turner, 2003). MBL deficiency plays a significant role in the development of cardiovascular disorders and arterial thrombosis (Ohlenschlaeger *et al*., 2004). MBL deficiency is also associated with high risk for the development of rheumatoid arthritis and persistent inflammatory conditions (Garred *et al*., 2000). Associations between low MBL levels and meningococcal disease have also been suggested by different case reports (Bax *et al*., 1999; Hamblin *et al*., 2004). Low plasma levels of L-ficolin are linked with recurrent respiratory tract infections in children (Atkinson *et al*., 2004).

MASP-2 deficiency is not very common among different populations. However, recently it has been shown that a single nucleotide polymorphism in the MASP-2/MAp19 gene on chromosome 1p36.2–3 results in decreased levels of serum MASP-2. This alteration (SNP) causes a substitution of aspartic acid by glycine residue at position 105 (D105G) on mature protein (equivalent to position 120 on the proenzyme) leading to loss of one Ca^{2+} binding site in CUB1 domain of MASP-2, which subsequently interferes with the interaction of MASP-2 with MBL and ficolins leading to dysfunctional LP initiation complex. Homozygous deficiency of MASP-2 is linked with several autoimmune manifestations and multiple infections (Stengaard-Pedersen *et al.,* 2003).

1.1.1.7.3 Alternative pathway deficiencies

A deficiency of the alternative pathway is manifested by either the deficiency of properdin or factor B or factor D. Properdin deficiency is the most common cause of alternative pathway deficiencies. Properdin deficiency in humans is associated with a high risk of meningococcal infections (Sprong *et al.,* 2006). In a mouse model of polymicrobial peritonitis, it was found that properdin deficient mice were more susceptible to polymicrobial peritonitis with significantly higher mortality and severity when compared to their wild-type littermates (Stover *et al*., 2008).

Factor B deficiency is associated with increased risk of *Pseudomonas aeruginosa* infections (Mueller-Ortiz *et al*., 2004). In contrast, the deficiency of complement factor D is shown to provide a significant protective effect on the development of proliferative renal disease in a murine model of systemic lupus erythematosus (Elliott *et al*., 2004).

1.1.1.7.4 Terminal complements components deficiencies

Lysis of bacteria by terminal complement components is one of the most important functions of complement system. Therefore, deficiencies in any of the terminal complement components (C5 through C9) can lead to poor bactericidal function especially against meningococci. People lacking one or more of the terminal complement components are highly susceptible to recurrent meningitis (Fernie *et al.,* 1996). There is a 7,000- to 10,000-fold-higher risk of developing meningococcal disease in people with terminal complement

deficiencies (Figueroa and Densen, 1991). Studies have shown that incidence of hereditary deficiencies of terminal complement components varies with ethnic groups. Several families in the Western Cape region of South Africa are diagnosed with C6 deficiency (Zhu *et al.,* 1998). It has previously been reported that the incidence of hereditary C6 deficiency among African-Americans (1 in 1600) is much higher than Caucasians. C9 deficiency has mostly been reported in Japanese population (Hayam *et al.,* 1989).

1.1.1.7.5 Deficiencies of complement regulatory components

Deficiency of C1 inhibitor is associated with inherited or acquired angioedema. The angioedema associated with C1 inhibitor deficiency is not due to a dysregulated complement system but due to an impairment of the regulation of the contact system as C1-INH, in addition to its role as a complement regulator, is the major inhibitor of factor XIIa and kallikrein of the contact system. This impairment results in excessive uncontrolled generation of bradykinin, which subsequently leads to an increased vascular permeability resulting in the development of angioedema (Cugno *et al.,* 2009; Karmer *et al*., 1991). Complement factor H and factor I are very important fluid phase regulators of the alternative pathway. Deficiencies of factor H and factor I are usually associated with atypical haemolytic-uremic syndrome (aHUS) which is characterized by renal failure due to thrombocytopenia, microangiopathic haemolytic anaemia and glomerular thrombotic microangiopathy (Thompson and Winterborn, 1981; Thurman and Holers, 2006).

Complete deficiency of either factor H or factor I is linked to secondary C3 deficiency due to constant consumption of plasma C3 (Pickering and Cook, 2008). Deficiency of complement factor I has also been associated with renal diseases (Genel *et al.,* 2005). Deficiency of decay accelerating factor (DAF) and CD59 is associated with paroxysmal nocturnal haemoglobinuria due to the absence of these phosphatidyl-inositol anchored receptors from the surface of erythrocytes making them susceptible to autologous complement-mediated lysis with consequent haemolytic anaemia (Nicholson-Weller *et al.*, 1985; Yamashina *et al.,* 1990).

1.2 *Neisseria meningitidis*

Amongst the 12 species of the genus *Neisseria* isolated from humans, only two species, *Neisseria gonorrhoeae* (the gonococcus) and *Neisseria meningitidis* (the meningococcus), are frequently pathogenic (Barrett and Sneath, 1994). Human *Neisseria* species are mainly divided into two major groups based on colony characteristics and morphology. The first group of *Neisseria* species includes *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria flavescens*, *Neisseria cinerea* and *Neisseria polysaccharea*. Except for *N. flavescens*, (which is yellow-pigmented), members of this group generally grow as non-pigmented, translucent colonies. The second group of *Neisseria* species includes *Neisseria subflava* biovars *perflava*, *Neisseria subflava* biovars *flava*, *Neisseria mucosa* and *Neisseria sicca*. Colonies of these species are generally opaque and yellow-pigmented (Knapp, 1988).

Neisseria meningitidis infections are the leading cause of bacterial meningitis and sepsis with significant mortality rates worldwide (Rosenstein *et al.,* 2003; Emonts *et al*., 2003). Despite the fact that great progress has been achieved in recent years in the diagnosis, vaccination and therapy against meningitis, meningococcal infections remain a prevalent disease with a mortality of up to 10% (Connolly and Noah, 1999). It is an obligate human commensal and is not adapted for survival outside of the host. Meningococcus is primarily transmitted between hosts by an aerosol spread. This gram-negative diplococcal bacterium is usually present in the upper respiratory tract in 10-40% population, where it is responsible for only marginal systemic diseases (Taha and Alonso, 2008). However, it is still not clear why some meningococci remain as commensals in one individual but cause invasive infections in others leading to severe disease (van Deuren *et al.*, 2000; Yazdankhah *et al*., 2004). Children under 5 years of age are mostly at risk of contacting the meningococcal disease due to their waning maternal passive immunity (Edwards and Baker**,** 1981).

The classification of the meningococcus was originally based on the antigens expressed on the bacterial surface. *Neisseria meningitidis* is surrounded by an outer membrane composed of lipids, outer membrane proteins (OMPs) and lipooligoccharides (LOS) (Rosenstein *et al.*, 2001). Meningococci show higher genetic diversity than most other pathogenic bacteria causing human diseases, mainly because of horizontal intraspecies recombination from closely related *Neisseria* species. In addition, the genes encoding the capsule, OMPs and LOS antigens are highly variable. Pathogenic meningococci also have a polysaccharide capsule covering the outer membrane (van Deuren *et al.*, 2000). About 13 serogroups of *N.*

meningitidis (A, B, C, E-29, H, I, K, L, M, W-135, X, Y and Z) are identified based on the immunological specificities of *N. meningitidis* capsule (Choudhury *et al.,* 2008; Stephens *et al.*, 2007). Amongst these, five serogroups (A, B, C, Y, and W135) are responsible for most of the diseases worldwide (Zhu *et al.*, 2005; Spinosa *et al.,* 2007). Besides capsular serotyping, meningococcal strains are also characterized into 20 serotypes (identifying class 2/3 or PorB outer membrane proteins) and 10 subtypes (identifying class 1 or PorA outer membrane proteins) (Frasch *et al.*, 1985). Meningococci are distinguished into 13 immunotypes on the basis of the antigenic properties of lipooligosaccharide (LOS) (Mandrell and Zollinger, 1977). The most recent classification of meningococci is based on genetic characteristics of different strains and according to variations in enzymes with metabolic functions. This technique, called as multilocus sequence typing (MLST) is used to characterise different clinical isolates of *N. meningitidis* by using the sequences of internal fragments (450-500 bp) of seven house-keeping genes distributed across the genome. The different sequences present within a bacterial species are assigned as distinct alleles for each house-keeping gene. Whereas for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). Sequence types are grouped into clonal complexes based on their similarity. Currently, 37 clonal complexes have been identified in *N. meningitidis* (Caugant, 2008; Maiden *et al.*, 1998).

Meningococcal disease occurs throughout the world as endemic infections. Cases of meningococcal disease tend to occur at a rate of about 1 case per 100,000 people worldwide (Achtman, 1995; Raymond *et al.*, 1997; Schwartz *et al.*, 1989). Serogroups B and C mostly

cause infections in industrialized countries whereas serogroups A and C are responsible for meningococcal infections in third-world countries (Achtman, 1995; Caugant, 1998; Takahashi *et al*., 2008). Sub-Saharan Africa is affected by yearly recurrent waves of meningococcal disease (by serogroup A) and is designated as the meningitis belt. The disease incidence may approach 1,000/100,000 inhabitants during epidemic peaks (Moore, 1992; Riedo *et al.,* 1995). Outbreaks of meningococcal disease have also been reported in Asia, which were caused by two clones of serogroup A that originated in northern China, and eventually spread throughout the world (Wang *et al.*, 1992). Most cases of meningococcal disease in the United States are due to serogroup Y (Racoosin *et al.*, 1998). Since the mid-1970s, serogroups B strains have shown a high degree of virulence in northwestern Europe (England, Norway, Iceland, and The Netherlands), with an attack rate of 4 to 50/100,000 (Caugant, 1998; van Deuren *et al.,* 2000). *N. meningitidis* affects over 2,000 people annually in the UK alone, where the mortality rate is approximately 10%. Patients who recover may suffer permanent damage from meningococcal infection, including partial paralysis or deafness and mental retardation (Baraff *et al.,* 1993).

Capsular based vaccines for *N. meningitidis* serogroups A, C, Y, and W135 have been developed, which have proven to be effective in controlling outbreaks in communities, still no effective vaccine is available for serogroup B as the capsular polysaccharide of serogroup B shows a structural similarity to the human neural cell adhesion molecules (N-CAM) (Zhu *et al*., 2005). This antigenic mimicry elicits a weak immune response thus

making capsular polysaccharide a poor candidate for serogroup B vaccine (Wyle *et al*., 1972; Caugant, 1998).

1.2.1 Virulence factors associated with *N. meningitidis* and their role in immune system evasion

1.2.1.1 Capsule and lipooligosaccharide (LOS)

Polysaccharide capsule is one of the most important virulence factors of *N.meningitidis*. Meningococcal capsule is very essential for the survival of bacteria in the blood stream as it protects the bacteria from complement-mediated killing and phagocytosis by neutrophils, Kupffer cells, and spleen macrophages (Takahashi *et al.,* 2008; Klein *et al*., 1996). Serogroups B, C, X, Y, and W-135 meningococci express their polysaccharides capsules, which are almost entirely composed of sialic acid linked to glucose or galactose (Frosch *et al.,* 1989). The sialic acid in the capsules of serogroup C strains is α2-9 linked, while serogroup B capsule is α2-8 linked (Edwards *et al.,* 1994). The serogroup B polysaccharide shows structural similarity to the serogroup C polysaccharide (Bhattacharjee, 1975). *N.meningitidis* serogroup B and C have the ability to endogenously sialylated their LOS up to variable degrees (Mandrell *et al*., 1991). The capsule of *N. meningitidis* group A is composed of α1-6-linked N-acetyl-D-mannosamine-1-phosphate polymers (Swartley *et al*., 1997).

The meningococcal capsule-synthesis (*cps*) gene cluster is composed of five regions. Region A comprises the genes that are involved in polysaccharide synthesis and are

serogroup specific with variants of the *siaD* gene required for synthesis of the sialic acid-containing capsules (B, C, Y and W-135) and the *myn* genes necessary for the expression of a serogroup A capsule (Claus *et al*., 2002; Edwards *et al*., 1994). The region B harbors genes that are responsible for lipid modification (Frosch and Müller, 1993). *Ctr* gene in region C is responsible for polysaccharide transport and is conserved in most meningococcal isolates from patients (Frosch *et al*., 1992; Guiver and Borrow, 2001). Region D in meningococcal capsule-synthesis (*cps*) gene cluster is involved in lipopolysaccharide synthesis where as the function of the *tex* gene homologue present in region E is still not known (Claus *et al*., 2002; Vogel *et al*., 2004).

Studies have proven that a considerable proportion of uncapsulated meningococci (16%) can persist in nasopharyngeal transmission systems of human even without a capsule. Such neisserial strains lack the genetic material necessary for capsule synthesis and instead bear a *cnl* (capsule null locus) sequence in place of the capsule region of chromosome. Such *cnl* sequence is present in at least four genetically distinct groups of meningococci. Previous studies have suggested that either several *cnl* sequences have been retained independently in the meningococcal population since speciation or these *cnl* sequences have arisen in meningococci as an outcome of horizontal genetic exchange from *N. lactamica* (Claus *et al*., 2002). It is now well established that meningococcal capsular expression is phase variable and is mostly expressed upon invasion of bloodstream or cerebrospinal fluid. On the other hand, the unencapsulated strains of *N.meningitidis* show enhanced capability to adhere to the mucosal epithelium (Dolan-Livengood *et al.,* 2003). Studies using capsule-

deficient meningococcal strains have suggested that capsule provides significant protection against serum lytic activity and mutants, which are deficient in capsular gene show increased susceptibility to killing by human serum when compared to wild-type strains (Vogel e*t al*., 1996).

Meningococcal LPS, also called as lipooligosaccharide (LOS) is another major virulence factor of *N.meningitidis* (Jonsson *et al*., 1998). Meningococcal LOS is required for resistance to serum bactericidal activity besides being involved in the colonization and invasion of epithelial cells. The lipid A portion of LOS mediates the endotoxic shock which leads to the activation of macrophages and production of a wide range of cytokines and chemokines (Albiger *et al*., 2003; Rosenstein *et al*., 2001). The severity of the meningococcal disease and the clinical syndromes produced have been correlated to high levels of circulating endotoxin and the resultant proinflammatory response (Brandtzaeg *et al*., 1992). Meningococcal LOS, an amphipathic glycolipid of approximately 4.8 kD, is composed of a short polysaccharide chain containing two to five sugar residues attached to the meningococcal LOS inner core (and O-acetyl groups) via two 2-keto-3-deoxyoctulosonic acid (KDO) molecules (Gamian *et al.*, 1992). Meningococcal LOS is heterogeneous and highly variable due to the presence and location of phosphoethanolamine (PEA) and O-acetyl groups as well as due to the presence and absence of sugar residues (DiFabio *et al.*, 1990; Michon *et al.*, 1990). Meningococcal LOSs have been serologically divided into at least 12 immunotypes (L1-L12) determined by the groups on the α and β chains added to the inner core region (Mandrell and Zollinger, 1977).

However, most neisserial strains often express more than one immunotype-specific epitope on their LOS, which leads to the classification of strains as L3,7,9, L2,4, or L1,8 (Tsai *et al.*, 1983). Meningococcal isolates expressing immunotype L3, 7, 9 are most commonly associated with disease (Griffiss *et al.*, 2000). Studies have shown that the LOS-deficient mutants are unable to adhere to host epithelial cells and also showed a significantly reduced competence for DNA transformation despite the expression of pili and pilus associated protein C (PilC) (Albiger *et al*., 2003; Jonsson *et al.*, 1998). Recently, Sprong *et al* (2004) investigated the role of meningococcal LPS in complement activation and in complement-dependent inflammation using wild-type *Neisseria meningitidis* H44/76 (LPS^+) and LPS-deficient *N. meningitidis* H44/76*lpxA* (LPS^-) in a whole blood model of *N. meningitidis*-induced complement activation and inflammation. It was observed that both LPS^+ and LPS^- *N. meningitidis* strains were able to activate complement effectively. Production of Interleukin-1ß (IL-1ß), tumor necrosis factor-alpha (TNF-α), and macrophage inflammatory protein-1α (MIP-1α) was significantly higher in LPS^+ *N. meningitidis* when compared to LPS^- *N. meningitidis* strains. However, LPS^- *N. meningitidis* strains were able to induce a marked IL-8 production. Upregulation of CR3 and induction of oxidative burst in granulocytes was found to be independent of LPS expression (Sprong *et al.,* 2004). Similar results were shown by other *in vitro* studies suggesting that components of *N. meningitidis* other than LPS contribute to the inflammatory response of the host (Pridmore *al.,* 2001; Sprong *et al.,* 2003). A negligible role of lipopolysaccharide sialylation was suggested by Vogel *et al* (1998) in infant rat model of systemic meningococcal infection.

1.2.1.2 Pili and Pilus subunits

Type IV pili (Tfp) and pili subunits are among many factors that play an essential role in the ability of meningococci to interact with host cells in order to establish a productive infection (Ieva *et al.,* 2005). Tfp are surface-associated long hair-like structures which are mainly involved in the initiation of infection following attachment to membrane cofactor protein or CD46 receptors (expressed on all human cells except erythrocytes) on the host cells (Johansson *et al.,* 2003). The type IV pili of pathogenic *Neisseria* are composed of thousands of major pilus subunits, PilE proteins and a few copies of pilus-associated proteins, such as PilC and PilV of which PilC proteins play a crucial role (Albiger *et al.*, 2003). The PilC proteins, PilC1 and PilC2, are two highly homologous proteins and key elements in the formation of pili.

The expression of PilC1 is upregulated briefly during the initial level of interaction which modulates adhesiveness between the bacteria and epithelial mucosa. Besides, the expression of capsule in *N. meningitidis* is down-regulated upon contact with host cells. Meningococcal transcriptional factor CrgA (contact-regulated protein A) is a regulatory protein mainly involved in the intimate bacterial adhesion to human epithelial cells **(**Deghmane *et al.,* 2000). Once pathogenic *Neisseriae* are attached to the host cells then subsequent intimate attachment is induced by another important pilus component, PilT which promotes pilus retraction required for twitching motility (Pujol *et al*., 1999).

1.2.1.3 Outer membrane proteins

Besides pili, and pilus subunits, another important group of invasion-associated proteins are the outer membrane proteins (OMPs) (Dehio *et al*., 1998). Previously, outer membrane proteins were classified into five structural classes (Class 1–5 proteins) based on relative mobility on SDS-polyacrylamide gel electrophoresis (Tsai *et al.*, 1981). However, subsequent studies revealed the class 2 and 3 proteins as a product of a single *porB* gene, therefore designated as PorB. The Class 1, 4 and 5 proteins are now designated as PorA, Rmp and Opa/Opc respectively (Hitchcock, 1989).

Meningococcal porins (PorA and PorB) are the most abundant and represented proteins present on the bacterial surface. Assembled as trimers within the outer membrane, porins serve as membrane pores through which small hydrophilic nutrients can diffuse into the cell (Tommassen *et al*., 1990). Meningococcal PorA (formerly called as class1 porins) varies in molecular mass (44–47 kDa) amongst different neisserial strains whereas meningococcal PorB is present as PorB2 (formerly Class 2 protein, typically 40–42 kDa) and PorB3 (Class 3, 37– 39 kDa) (Frasch *et al*., 1985). As the major and most abundant proteins on the neisserial surface, the porins are also the most immunogenic and are therefore used for the serological classification of meningococci. The PorB protein is responsible for serotype specificity while PorA is responsible for serosubtype specificity (Massari *et al*., 2003). PorB has the ability to induce proliferation of B cells as well as it enhances the interactions between B and T cells (Burke *et al*., 2007). Similarly PorA exhibits immunomodulating properties by activating T cells by dendritic cell and also by directing T-cell differentiation

towards the Th2 type response required for generating the antibody responses (Mackinnon *et al*., 1999). The opacity proteins comprise two families of neisserial outer membrane proteins; Opa and Opc. Opa proteins are more abundant than Opc, which were so named due to their homology to Opa. Meningococcal opacity proteins mediate intimate adhesion to host epithelial cells via heparin sulphate proteoglycan (HSPG) receptors, and also interact with the CEACAM receptors expressed by phagocytic host cells (De Vries *et al*., 1998).

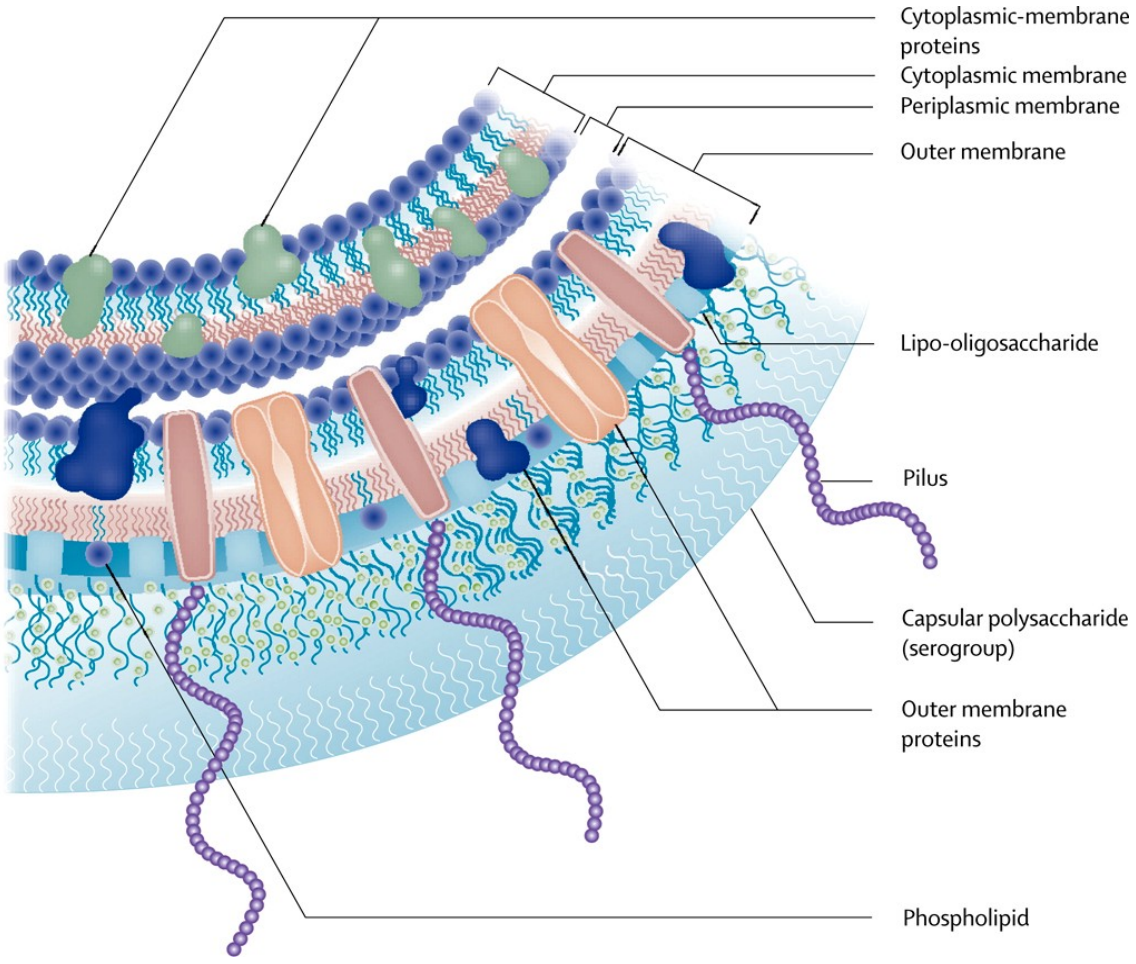

Figure 1.11: Cross-sectional view of the meningococcal cell membrane showing different virulence factors (Stephens *et al.*, 2007).

1.2.2 Colonization and invasion of *N. meningitidis*

N. meningitidis is a strict human commensal, and the sole natural habitat for *N. meningitidis* is the human nasopharyngeal mucosa. *N. meningitidis* is transmitted by aerosol or contact with infected secretions. In most of the individuals, *N. meningitidis* colonizes the human upper respiratory mucosal surface as a commensal without affecting the host, a phenomenon known as carriage (Broome, 1986). The asymptomatic carrier state is observed in almost 10% of the population in nonepidemic settings. There are several factors which are important for the development of invasive disease. These include virulence of the meningococcal strain, susceptibility of host, environmental influences, preceding or concomitant viral infections and geographical location of disease prevalence (Anderson *et al*., 1998). The rates of meningococcal carriage and transmission increase in closed and semi-closed populations (such as military recruits, university students and in the household contacts) where neisserial carriage rate can be as high as 40% (Ala'Aldeen *et al*., 2000; Caugant *et al*., 1992). Long-term carriage of the meningococcus in the nasopharynx can lead to loss of capsular expression rendering serologically nongroupable (NG) strains (Ala'Aldeen *et al*., 2000).

Colonization by meningococci takes place both at the exterior surface of the mucosal cell and intra- or sub-epithelially. Occasionally, *N. meningitidis* strains will penetrate the mucosal membrane via phagocytic vacuoles as a result of endocytosis, enter the bloodstream and cause a range of different clinical presentations ranging from sepsis and mild systemic meningococcal infection to fulminant meningococcal septicemia and distinct

meningitis (Van Deuren *et al.*, 2000). A low level bacteremia sometimes leads to a short febrile flu-like episode and is even cleared out spontaneously (Gedde-Dahl *et al.,* 1990). On the other hand, clinically overt disease develops if the bacteremia is not cleared and a very high degree of bacterial replication occurs in the systemic circulation (levels can reach up to 10^6 colony-forming units per ml) which is mainly accounted for the substantial vascular and tissue destruction by the meningococci (Brandtzaeg *et al.*, 1995). The outer membrane of *N. meningitidis* is rich in lipooligosaccharides (LOS). During bacteremic phase of disease, endotoxins are continuously released by meningococci in the form of vesicular outer membrane structures (blebs) which mainly consist of LOS along with OMPs, lipids, and capsular polysaccharides. This further stimulates the production of inflammatory mediators into the blood stream (Hackett *et al*., 2001). Therefore, invasive *N. meningitidis* infection leads to a potent inflammatory response which is responsible for some of the severe symptoms typical of meningococcal disease (Hong *et al.*, 2008). The beginning of the bacteremic phase is marked by the onset of chills, acute fever, low-back pain, thigh pain and/or generalized muscle aches (Louria *et al*., 1985).

Very rarely, meningococcus may cause other diseases such as septic arthritis, conjunctivitis, otitis, sinusitis, pneumonia, purulent pericarditis and urethritis (Ross and Densen, 1984; Tzeng and Stephens, 2000). Meningococcal polysaccharide capsule is considered to be the most essential bacterial virulence factor for survival in the bloodstream which protects against complement mediated bacteriolysis and phagocytosis by neutrophils, Kupffer cells, and macrophages (Mackinnon *et al*., 1993; Spinosa *et al*., 2007). From the

blood, *N. meningitidis* crosses the blood-brain barrier and gains access to cerebrospinal fluid (CSF).

In CSF, bacterium proliferates in an uncontrolled manner which is followed by an acute inflammatory response due to the liberation of major proinflammatory cytokines and chemokines (interleukin (IL)-1b, IL-6, IL-8 and tumour necrosis factor (TNF)-α) (Brandtzaeg, 1995). This leads to a severe form of infection in the form of fulminant septicemia or meningitis (van Deuren *et al*., 2000). Acute meningitis is often characterized with a sudden onset of headache, fever, stiff neck, accompanied by nausea, vomiting, photophobia and an altered mental status (Rosenstein *et al.,* 2001). Approximately 55% of patients developing a systemic meningococcal infection present with clinical symptoms of distinct meningitis (Brandtzaeg *et al*., 1983). Meningococcal LPS activates the coagulation system through upregulation of tissue factor. An excessive stimulation of the coagulation system leads to disseminated intravascular coagulation (DIC). Fulminant meningococcal sepsis in humans is characterized by shock and DIC (Hardaway, 1982). An exaggerated immune response in the form of meningococcal septic shock leads to hypotension, organ failure, and finally death (Kahler and Stephens, 1998).

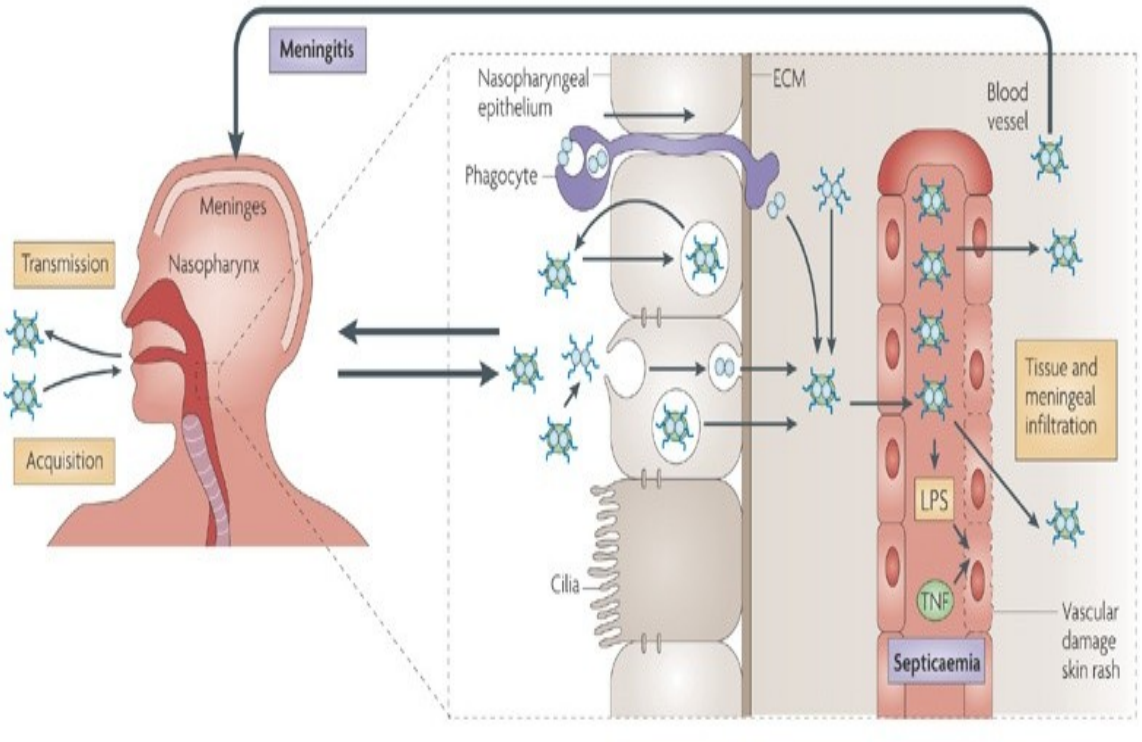

Figure 1.12 Different stages in the pathogenesis of *N. meningitidis* (Virji, 2009)

1.2.2.1 Iron acquisition by meningococci

It is now well established that replication of *N. meningitidis* requires the uptake of iron in various environments within the host (including the oropharynx, bloodstream, and cerebrospinal fluid) which contain various forms and concentrations of free and complexed iron. In the mammalian host, the majority of iron in the body is stored intracellularly in ferritin and haemoglobin rendering it inaccessible for the microorganisms. Extracellular iron found in body fluids is complexed with the major iron-binding proteins, such as transferrin (in serum) and lactoferrin (in milk and on mucosal surfaces) (Holbein, 1980).

Within the mammalian host, the cerebrospinal fluid has the highest content of free iron, at 2.2 μmol/liter (Evans *et al*., 1998). Many pathogens produce low molecular weight compounds known as siderophores, which have high affinity for iron and are capable of scavenging iron from transferrin and lactoferrin (Crosa, 1984). The meningococcus does not produce siderophores by itself yet it is able to utilise siderophores produced by other bacteria (Rutz *et al*., 1991). Meningococci express an array of receptors that show specific binding to host iron-containing proteins, including lactoferrin and transferrin (Mickelsen and Sparling**,** 1981). Acquisition of iron from these sources is mediated by removing the metal ion from these carrier proteins utilizing a number of OMPs, the expression of which is regulated by iron availability (Pettersson *et al.*, 1993). One such iron acquisition receptor in meningococci is Transferrin receptor (Tf). The Tf is comprised of two iron-regulated outer membrane proteins; the Transferrin binding protein A (TbpA) and B (TbpB), which are expressed at the bacterial surface and work antagonistically to bind human transferring (Irwin *et al.*, 1993). The lactoferrin receptor of *N. meningitidis* has a similar composition and is composed of two protein components; lactoferrin-binding protein A (LbpA) and lactoferrin-binding protein B (LbpB) (Schryvers and Morris, 1988).

N. meningitidis is also capable of utilizing a variety of different haeme-containing proteins as a source of iron for growth, including haeme, haemoglobin and haemoglobin–haptoglobin complexes (Schryvers and Stojiljkovic, 1999). Two independent haeme transport systems have been identified in *N. Meningitidis,* which recognize haeme-containing proteins, Hpu (haemoglobin–haptoglobin utilization) and HmbR (haemoglobin

receptor) (Dyer *et al.*, 1987; Perkins-Balding *et al.*, 2004). Iron acquisition is also shown to be a crucial factor in developing meningococcal infection in animal models (Holbein, 1980; Holbein, 1981). Holbein *et al* (1979) injected iron compounds (iron dextran or human transferrin) into mice before challenging them with *N. meningitidis* in order to develop a lethal infection. In contrast, mice injected with the same doses of *N. meningitidis* in the absence of iron addition developed a transient short lived bacteraemia but cleared the infection.

1.2.3 The role of immune system in *N. meningitidis* infections

Meningococcal infection usually triggers an immune response aimed at eliminating the pathogen. *N. meningitidis* is restricted by various innate and specific host factors, which include the complement system, cationic antimicrobial peptides (CAMPs), reactive nitrogen and oxygen radicals and a number of components of the inflammatory response. Although the adaptive immune system in form of antibodies provides a more targeted approach for killing bacteria, the time it takes for this specific immunity to develop after first contact requires a strong first-line defence. Therefore, innate immune response is considered to be more essential to mount a fast response against *N. meningitidis* (van Deuren *et al.,* 2000; Hermaszewski and Webster, 1993).

1.2.3.1 The interaction of immune cells with *N. meningitidis*

Neisseria meningitidis is an obligate human pathogen whose primary ecological niche is on the mucosal surface of the human nasopharynx. Although a considerable percentage of healthy humans (10-40%) are colonized by meningococci in their nasopharynx, the vast majority of these colonisations will not proceed to an invasive infectious disease (Cartwright, 1995). During the course of disease, *N. meningitidis* encounters a range of immune cells, including phagocytic cells, or leukocytes, that represent the cellular arm of the innate immune response. Granular leukocytes are characterised by the presence of granules in the cytoplasm and include neutrophils, basophils and eosinophils. Agranular leukocytes include monocytes, macrophages and lymphocytes.

The mucosal surfaces provide the main points of entry for *N. meningitidis,* and are protected by the subtly regulated mucosal immunity (Stephens *et al.*, 1983). Originally programmed to induce tolerance against any encountered antigens (including food antigens in the intestine and inhaled antigens in the respiratory tract), mucosal immunity is capable of rapidly switching from the generation of tolerance to the induction of protective immunity once invaded by foreign pathogens. This complex function is maintained by a variety of immune cells amongst which dendritic cells play a crucial role (McGee *et al.*, 1983). Dendritic cell (DCs) precursors originate from the haematopoietic stem cell system in bone marrow from where they are dispersed in almost all human tissues. Along with macrophages and granulocytes, dendritic precursor cells phagocytose and degrade the bacterial antigens. Bacterial peptides are then presented on the surface of these professional

antigen-presenting cells (APC) by the major-histocompatibility complex (MHC) (Roake, 1995). This process leads to the maturation of DCs which then migrate to the lymphoid tissues and present the MHC bearing antigens, in combination with co-stimulatory molecules on the cell surface to T cell and B cells (Lipscomb and Masten, 2002).

Expression of the polysialic acid capsule, elongation of the LPS α-chain, and sialylation of the LPS are the major factors which influence the uptake of meningococci by dendritic cells and macrophages (Kurzai *et al.*, 2005; Unkmeir *et al.*, 2002). Several studies have shown that elimination of *N. meningitidis* in human is efficiently carried out by dendritic cells following opsonisation-independent phagocytosis (Al-Bader *et al.*, 2003; Kolb-Maurer *et al.*, 2001; Peiser *et al.*, 2002; Unkmeir *et al.*, 2002). Scavenger receptor A (SRA) is the single most important uptake receptor on the surface of dendritic cells responsible for phagocytosis of meningococci (Peiser *et al.*, 2002). Human dendritic cells control neisserial infections mainly by their bactericidal activity and efficiently present neisserial antigens to T-cell, and it is phagocytosis and not antibody mediated opsonisation that are considered important for the uptake of meningococci by dendritic cells (Al-Bader *et al.*, 2003; Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002). A somewhat negligible influence of human plasma on the adherence and phagocytosis of the bacteria was reported during these studies (Unkmeir *et al.*, 2002).

Macrophages are the other type of important immune cells in the nasopharyngeal epithelium. Along with neutrophils, macrophages are the predominant type of cells found in the cerebrospinal fluid of patients suffering from meningococcal meningitis. Irrespective

of the presence or absence of a polysialic capsule, *N. meningitidis* are internalised by resident macrophages and are rapidly inactivated and killed in the cells' phagolysosomal compartments. However, recent work with meningococcus has demonstrated that the initial recruitment of endosomal and lysosomal markers to the phagolysosome is more efficient when using unencapsulated strains as compared to capsulated neisserial strains (Read *et al.*, 1996). Macrophages produce nitric oxide (NO) which has direct antimicrobial activity and contributes to the mammalian host defence as a signaling molecule of innate immune responses. It has been shown recently that the genome of *N. meningitidis* contains certain genes (such as *norB* encoding nitric oxide reductase and *cycP* encoding cytochrome *c′*) which are able to detoxify the nitric oxide (NO) produced by the macrophages and therefore, enhance the intracellular survival (Stevanin *et al.*, 2005). The NO detoxification mechanism plays a crucial role in modifying the release of nitric oxide-regulated cytokines and chemokines resulting in decreased production of TNF-α, IL-12 and CXCL8 and an increase in the production of IL-10 and CCL5. This also suggests that cytokine production by phagocytic cells is also manipulated by meningococci (Stevanin *et al.*, 2005; Tunbridge *et al.*, 2006). In addition, *N. meningitidis* produces superoxide dismutase which provides protection to opsonised bacteria against phagocytosis by human macrophages (Dunn *et al.*, 2003). Similar to dendritic cells, the phagocytosis by macrophages is independent of antibody opsonisation (Dunn *et al.*, 2003; Jack *et al.*, 2005).

Polymorphonuclear leukocytes (PMN) or neutrophils are the leukocytes with the highest proportion (50–70% of total leukocytes) in human peripheral blood (Borregaard, 1984).

Differentiated from myelopoietic stem cells in the bone marrow, neutrophils circulate in the blood until they are recruited to the site of infection by the release of chemotactic factors and cytokines (such as C5a, IL-8 and CXC-type cytokines) released by infected epithelial and endothelial cells. PMNs are the first type of immune cells to be recruited at the site of infection. PMNs tend to rapidly internalise invading bacteria and inactivate them in the phagolysosomal compartment (Hampton *et al.*, 1998; Lee *et al.*, 2003; Thomas *et al.*, 1998). Following opsonisation of *N. meningitidis* by C3b or IgG, PMNs rapidly engulf the meningococci via CR3 and Fcγ receptors, respectively (Fijen *et al.*, 2000). In case of individuals with complement defects, non-opsonic phagocytosis of the meningococcus is carried out by PMNs which is primarily mediated by direct interaction between neisserial Opa proteins and CD66 (or CEACAM) receptors expressed by PMNs (Estabrook *et al.*, 1998; Virji *et al.*, 1996). Meningococcal porins play an important role in inhibiting the uptake of *N. meningitidis* by neutrophils by impairing the expressional regulation of Fc receptors and complement receptors. Besides, neisserial surface porins (in their purified form) are also able to prevent the responsiveness of neutrophils towards chemoattractants and their degranulation (Bjerknes *et al.*, 1995). The oxidative burst is one of the most prominent effector mechanisms in neutrophils by which following phagocytosis, the internalised pathogen is degraded by the rapid release of reactive oxygen species (superoxide radical and hydrogen peroxide). However, *N. meningitidis* has evolved mechanisms to withstand the reactive oxygen species by the production of a number of enzymes such as catalase and superoxide dismutase (Dunn *et al.*, 2003). The important role of PMNs in meningococcal disease was also demonstrated during *in vivo* experiments using

a rat model by Perez *et al* (2001) where depletion of neutrophils led to mortality rates of 100% after challenging with *N. meningitidis* even after vaccination.

B and T lymphocytes form the cellular repertoire of adaptive immunity. Once derived from precursor cells in the bone marrow, T cell precursors migrate to the thymus for maturation while B lymphocytes undergo most of their developments in the bone marrow. Once encountered by the specific antigen, immature B cells (combined with the activity of helper T cells) are activated resulting in rapid production of specific antibodies. The humoral immune response against *N. meningitidis* mainly relies on the production of antibodies by B lymphocytes as they are also the cell type responsible for recognition of the meningococcal capsule polysaccharides during the course of infection (Goldschneider *et al.*, 1969a; Guttormsen *et al.*, 1993). Opsonisation of meningococci by antigen-specific antibodies leads to phagocytosis and/or complement-mediated lysis. Membranes in the human nasopharynael mucosa are protected against pathogens by the presence of Immunoglobulin A (IgA) antibody which is the most abundant immunoglobulin isotype produced in the body. However, *N. meningitidis* is able to evade this defence by secreting IgA1 proteases that destroy these antibodies (Kilian *et al.*, 1996). Studies have shown that antibody-mediated immunity against *N. meningitidis* develops over time, and it varies between individuals mainly depending on the age of the individual (Caugant *et al.*, 1992; Reller *et al.*, 1973). Newborn infants receive anti-meningococcal antibodies by trans-placental transfer of immunoglobulins. The low incidence of disease in infants is explained by the fact that sera from 50% of newborns show anti-meningococcal bactericidal activity

(Goldschneider *et al.*, 1969a). However, the maternal immunity does not last for long and the children aged between 6 to 12 months show highest susceptibility to meningococcal infection. (Goldschneider *et al.*, 1969b). Carriage or repeated transient colonisation of a meningococcal strain not only results in the induction of bactericidal antibodies specifically directed against this particular strain, but also confer a degree of cross-reactive protection against other meningococcal isolates (Reller *et al.*, 1973). In addition, infection with certain bacterial species such as *N. lactamica* (which express antigens related to the meningococcal outer surface antigens) also results in development of some protective immunity (Hoff and Hoiby, 1978). Some bacteria such as *Bacillus pumilus* and *Escherichia coli* K1 show antigenic mimicry to the meningococcal capsule and therefore, induce cross-reacting antibodies (Grados and Ewing, 1970). There is evidence that IgA antibodies bind and block the antigenic epitopes of meningococci. These blocked epitopes are not recognized by IgG and IgM antibodies, which further prevent classical or lectin pathway mediated complement-killing of the bacteria (Griffis, 1995).

T cells play a crucial role against meningogocci by regulating the Ab class-switching, affinity maturation, and the immunological memory. T cells express the T-cell receptor (TCR) and are classified into subsets of helper, cytotoxic, regulatory and memory T cells (Gatenby *et al.*, 1982). At the mucosal surface, the encounter between *N. meningitidis* and T cells elicit proinflammatory Th1-type response or an Ab-associated Th2 response (Pollard *et al.*, 1999). Elevated levels of inflammatory cytokines such as interleukin-12 (IL-12) and gamma interferon (IFN-γ) were measured in the serum and cerebrospinal fluid of

children with meningitis (Kornelisse *et al.*, 1997). Studies conducted on human monocytic cells stimulated by meningococci with a deficiency in LPS, showed that even non-LPS components of meningococci induced a considerable production of the tumor necrosis factor-α (TNF-α) and interleukin -1ß (IL-1ß) and almost all types of interferons (Sprong *et al.,*2001).

Immune cells express certain receptors that recognize conserved structures found on a wide range of pathogens. These receptors are often referred to as pattern recognition receptors (PRRs). Different classes of PRRs have been identified, which include Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin like receptors (CTLRs) and RIG-I like receptors (RLRs) (Iwasaki and Medzhitov, 2004). The TLR family was the first and the most studied class of pattern recognition receptors. Activation of all TLRs eventually leads to activation of the immune system. Similar to the complement system, TLRs are able to recognize their specific pathogen-associated molecular patterns (PAMPs) on the pathogen surface followed by the activation of various intracellular signaling cascades leading to the activation of several pathways that end up with cytokine and inflammatory mediator release as well as expression of effector molecules (Faure *et al.* 2001). In this way, signaling by the TLRs allows the host to efficiently combat microbial infections by bridging the innate and adaptive immune response (Medzhitov, 2007). To date, about eleven members of human TLR family have been characterized and their ability to recognize different structures derived from microbes has been studied (Jin and Lee, 2008). Activation of immune cells by meningococcal LPS has been shown to be mediated by TLR4. TLR4 recognizes the lipid A

moiety of the LPS molecule (Manicassamy and Pulendran, 2009). Besides, the host immune response against neisserial porin PorB is mediated by TLR2 (Massari *et al.* 2002). TLR activation is a key event in meningeal inflammation and, even more interestingly, a pivotal factor for meningitis-associated tissue damage (Koedel, 2009). The covalent linkage of a keto-deoxy-D-manno-octulosonic acid to lipid A is necessary for efficient activation and further biological activity of the TLR4 (Zughaier *et al.* 2004). It is shown that the liberation of the proinflammatory cytokine TNF-alpha and release of nitric oxide and reactive oxygen species are severely diminished in meningococcal LPS with KDO-deficiency (Zughaier *et al.* 2005).

Bacterial invasion leads to the expression of various antimicrobial substances within the human host. One of such antimicrobial peptide is cationic antimicrobial peptide (CAMP). CAMPs constitute an important effector component of the innate immune system. These are mainly produced by phagocytic cells and are also expressed by epithelial cells after the bacterial invasion (Hancock, 2001). *N. meningitidis* generally has high resistance to CAMPs by various mechanisms, which include expression of capsule, lipid A modification and the type IV pilin secretion system (Takahashi *et al.,* 2008; Tzeng *et al.,* 2005).

1.2.3.2 The interaction of complement system with *N. meningitidis*

The complement system provides one of the major effector mechanisms of the innate immune response. The complement system comprises more than 30 different fluid-phase

and membrane-bound proteins and can be activated via three different pathways: the classical, the lectin, and the alternative pathway. Complement activation leads to formation of C3 convertase, which acts and cleaves complement component C3 to produce C3b (Walport, 2001b; Walport, 2001a). Binding of C3b to the bacterial surface results in phagocytosis by cells of the innate immune response, or it can lead to the lysis by formation of the membrane attack complex (MAC) (Walport, 2001b; Walport, 2001a).

The importance of the complement system in the prevention of meningococcal disease is highlighted by different studies, which show that deficiencies in complement components such as C3, C4 and most strikingly, elements of the MAC result in increased susceptibility to neisserial infections (Rosa *et al.*, 2004; Spath *et al.*, 1999; Rossi *et al.*, 2001; Fine *et al.*, 1983). However, evidence is provided that complement deficiency in human is associated with lower mortality rates with less severe meningococcal disease and lower levels of circulating endotoxins when compared to immunocompetent subjects. This is highlighted in a case report where a C6-deficient person with meningococcal sepsis showed a dramatic increase in endotoxin levels following infusion with fresh frozen plasma (Lehner *et al.*, 1992).

The polysaccharide capsule can play an essential role in avoiding complement-mediated killing of *N. meningitidis*. Un-encapsulated strains or the strains which express truncated LPS isoforms are highly susceptible to lysis by the complement system, although the basis for this is not fully understood (Schneider *et al*., 2006; Geoffroy *et al*., 2003). Vogel *et al*

(1996) conducted a study where they inactivated the gene responsible for capsule expression and then compared the survival of *N. meningitidis* serogroup B strains with and without capsule in human serum. It was shown that the acapsulate meningocci were significantly impaired in their survival in human serum when compared to wild-type strains. However, no difference in C3 deposition on the surface of the wild-type strain and its unencapsulated mutant was observed in the study which indicates that the capsule does not affect steps leading to the cleavage of C3 on menigococcal surface (Vogel *et al.,* 1996). On the contrary, it is shown that even in the presence of capsule, extensive truncation of the LPS molecule renders the meningococcus highly sensitive to complement (Estabrook *et al.*, 1997).

Meningococcal lipopolysaccharides consist of two chains (each having up to ten external sugar residues) linked to the conserved lipid A portion of the LPS backbone embedded in the outer membrane (Brandtzaeg *et al.*, 1995). This unique structure confers substantial resistance to *N. meningitidis* against complement killing. *N.meningitidis* serogroup B and C strains can add sialic acid onto the lacto-N-neotetraose (LNnt) moiety of their LOS. The sialylation of LOS by *N. meningitidis* is considered as one of the main mechanisms adapted by the bacteria to avoid lysis by the complement system (especially the alternative pathway) and is often associated with the increased serum resistance of meningococcal strains (Estabrook *et al.*, 1997). This sialylation occurs gradually and to various degrees through endogenously synthesised sources of sialic acid, or through exogenous CMP-

NANA (Cytidine-5′-monophospho-N-acetylneuraminic acid) in the surrounding environment (Estabrook *et al.*, 1992; Exley *et al.*, 2005; Tsai *et al.*, 1983).

Neisserial Opc has the capacity to modulate host immune responses by binding to vitronectin which is one of major regulators for the terminal complement activation. This binding also confers increased serum resistance to many meninogococcal isolates (Virji *et al.,* 2008).

In the naive host, the classical pathway (CP) is considered to initiate complement activation via natural antibodies of the immunoglobulin class IgM during meningococcal infection. Specific Abs raised during infections and cross-reactive Abs may also develop as a result of colonization with meningococci or against colonising apathogenic neisserial species (Finne *et al.*, 1987; Goldschneider *et al.*, 1969a; Hoff and Høiby, 1978; Liu *et al.,* 2008). It was previously shown that *N.meningitidis* binds one of the major fluid-phase complement regulator of the classical and lectin pathways; C4 binding protein (C4bp). The physiological role of C4bp in immune evasion is not clear since the interaction only occurs at non-physiological sodium concentrations. However, it is reported that expression of meningococcal porin PorA is essential for this binding (Jarva *et al.,* 2005).

Factor H is a regulatory component of the alternative pathway, which acts as a cofactor for the factor I mediated cleavage of C3b into its enzymatically inactive decay fragment (i.e. iC3b) (Pangburn *et al.*, 2000). Previously, it was suggested that *N.meningitidis* recruits fH

to its surface via a specific factor H-binding protein (fHbp; also known as LP2086 or Genome-derived Neisserial Antigen (GNA)) thereby limiting complement activation on the pathogen surface (Pangburn *et al.*, 2000; Schneider *et al.*, 2006). However, more recent studies have shown that factor H binds to meningococci through neisserial surface protein A (NspA) which is a protein of approximately 17 kDa. It was shown that NspA is able to confer serum resistance to meningococci even in the absence of the previously characterized fHbp (Lewis *et al.*, 2010). Furthermore, it was reported that terminal sialic acid residues in the *N.meningitidis* serogroups B and C increase the affinity of factor H to C3b. This binding activity of factor H to factor B or Bb bound C3b decays already formed C3 convertase complexes leading to the inhibition of complement activation via the alternative pathway (van Deuren *et al.,* 2000).

Complement factor D is an alternative pathway specific serine protease which is very essential in the formation of intrinsic alternative pathway initiation and amplification of C3 convertases (Choy and Spiegelman, 1996; Matsumoto *et al.,* 1997). Sprong *et al* (2006) studied a family with frequent meningococcal disease and identified a defective factor D allele leading in homocygosity to factor D deficiency. It is reported that factor D deficiency predisposes to invasive meningococcal disease mainly due to reduced alternative pathway–dependent complement activation (Sprong *et al.,* 2006).

Properdin is a serum glycoprotein which is considered to be the only positive regulator of the alternative pathway of complement activation. The human properdin gene designated as *CFP* (Complement Factor Properdin) is composed of 10 exons, which span over 6 kb of the

genome (Nolan *et al.,* 1992; Pangburn *et al.,* 1989). Each properdin monomer is a 53 kDa molecule which circulates in serum as a cyclic dimer, trimer or tetramer form (Smith *et al*., 1984). Properdin is present in human serum at a concentration of 22 to 25μg/ml (de Paula *et al*., 2003). Unlike most of the components of the complement system which are produced mainly in liver, properdin is synthesized in spleen, liver, adipose tissue, sinonasal tissue and mammary gland (Avery *et al.*, 1993; Choy and Spiegelman, 1996; Maves and Weiler, 1992; Nakamura *et al.,* 2006). At a cellular level, properdin synthesis is observed in many cell types including macrophages, monocytes, T cells, astrocytes, hepatoma cells, neutrophils, bronchial epithelial cells and dendritic cells. (Avery *et al.*, 1993; Reis *et al.,* 2006; Maves and Weiler, 1992; Nakamura *et al.,* 2006; Schwaeble *et al*., 1993; Schwaeble *et al*., 1994; Wirthmueller *et al.*, 1997).

At first, properdin was considered to be an initiator of the alternative pathway (Pillemer *et al.*, 1954). Later on, a more widely accepted role of properdin was proposed according to which properdin stabilises the labile C3 convertase (C3bBb) of the alternative pathway by preventing the dissociation of Bb from C3b and the inactivation of the C3 convertase by factor I and co-factors (Fearon and Austen, 1975). However, a relatively recent publication by Spitzer *et al.* (2007) implied that properdin can bind directly to AP activator surfaces (such as zymosan and rabbit erythrocytes) where it initiates the formation of the C3 convertase. Spitzer and collaborators also claimed that properdin bound to a bacterial surface (*N. gonorrhoeae* and a LPS mutant of *Escherichia coli* K12) was capable of enhancing C3 deposition on these bacteria following addition of properdin-deficient serum

(Spitzer *et al*., 2007). These data have important implications because pronounced properdin deficiency in human is associated with increased susceptibility to severe invasive meningococcal infections often with a higher mortality than normal individuals (Braconier *et al*., 1983; Densen *et al*., 1987; Morgan and Walport, 1991; Fijen *et al.*, 1995; Spath *et al.*, 1999). A recent study by Agarwal *et al* (2010) has suggested that native properdin does not bind directly to *N. meningitidis,* however, unfractionated properdin following preincubation with *N.meningitidis* leads to high levels of C3 deposition after addition of properdin-depleted serum. In contrast, a significantly low C3 deposition on meningococci was observed following incubation with properdin-depleted serum alone (Agarwal *et al.,* 2010). A protective role of properdin was suggested in an *in vivo* model of polymicrobial septic peritonitis. Following sublethal cecal ligation and puncture (CLP), properdin deficient mice showed significant impairment in their survival when compared to their wild-type littermates (Stover *et al*., 2008).

The role of the lectin pathway in relation to meningococcal infection is controversial. A study conducted by Garred *et al* (1993) shows that low MBL concentrations do not appear to predispose to meningococcal disease with serogroup B or C. On the contrary, two independent studies (one hospital-based and one community-based) conducted by the Department of Paediatrics, Imperial College School of Medicine at St Mary's, UK have provided some evidence that children with genetic variants of Mannose-Binding Lectin (MBL) are more susceptible to meningococcal disease (Hibberd *et al*.,1999). MBL

deficiency as a risk factor for meningococcal disease in early childhood is also reported in different studies (Eisen *et al*.,2003; Faber *et al*.,2007; Tully *et al*.,2006).

Previous studies have reported the binding of MBL with different serogroups of *N. meningitidis* (Jack *et al*., 2001; Van Emmerik., *et al* 1994; Kuipers *et al*., 2003). Clinical isolates of *N.meningitidis* belonging to serogroups B and C are able to show very high activation of MBL even at extremely low concentrations (Kuipers *et al*., 2003). However, the structure and sialylation of lipooligosaccharides (LOS) rather than the capsule structure are reported as the major determinants of MBL binding (Jack *et al*., 1998; Jack *et al*., 2001). MBL shows high binding to meningococcal strains with least sialylation and enhancement of sialylation of meningococci (by addition of sialic acid donor molecules i.e. CMP-NANA to growth media) results in decreased MBL binding (Jack *et al*., 2001). More recent work has shown that MBL binds to two major outer membrane proteins (i.e. PorB and Opa) of *N.meningitidis* (Estabrook *et al.*, 2004). Interestingly, the binding of MBL to Opa and PorB of *N.meningitidis* was not calcium dependent and was not inhibitable by mannose or GlcNAc, but it was sensitive to 0.5 M salt. While studying the complement activation in a whole blood model, Bjerre *et al.* (2002) found out that complement activation by *N. meningitidis* is dependent on the lectin and alternative pathways.

1.3 Thesis Aims

This work was conducted in order to achieve the following goals:

- To define and assess the role of lectin pathway of complement activation in systemic meningococcal infection by combining *in vitro* assays and experimental models of infection in a generated mouse strain with a total deficiency of lectin pathway functional activity.

- To investigate the therapeutic benefits of a transient inhibition of lectin pathway functional activity in murine model of meningococcal infection using inhibitory MASP-2–specific monoclonal antibodies.

- To determine the potential benefits of recombinant properdin administration to limit systemic meningococcal disease in an experimental murine model of *N.meningitidis* infection

- To investigate the combined therapeutic benefits of MASP-2 inhibition with properdin augmentation as a potential treatment strategy for systemic meningococcal infection.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, reagents and media

1kb plus DNA ladder	Invitrogen or New England Biolabs (NEB)
37% Formaldehyde solution	Sigma-Aldrich
Agar-Agar	Lab M
Agarose, electrophoresis grade	Melford
Barbital	Sigma-Aldrich
Blood agar base	Oxoid
Bovine serum albumin (BSA)	Sigma-Aldrich
Brain heart infusion (BHI) medium	Oxoid
Calcium chloride	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Defibrinated Horse blood	Oxoid
Deoxynucleotides, PCR grade (dNTPs)	Promega
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich
Disodium hydrogen phosphate	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich

Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich
Eosin	BDH laboratories
Ethanol	Fisher Scientific
Ethidium bromide	Sigma-Aldrich
Foetal bovine serum	Sigma-Aldrich
Foetal calf serum	Harlan
Formalin	Fisher Scientific
Haematoxylin	BDH laboratories
Hank's balanced salt solution (HBSS)	Gibco
Heparin	Sigma-Aldrich
High fidelity taq polymerase	New England Biolabs
Human serum albumin	Statens serum institute, Denemark
Iron Dextran	Sigma-Aldrich
Isopentane	Fisher Scientific
Low Molecular Weight DNA Ladder	New England Biolabs (NEB)
Magnesium chloride	Sigma-Aldrich
Mannan	Sigma-Aldrich
N-acetyl BSA	Promega
Oligo $(dT)_{23}$ anchored primers	Sigma-Aldrich
Oligonucleotides	Eurofin
Phenol/Chloroform for RNA extraction	Sigma-Aldrich

Phosphate Buffered Saline (PBS)	Oxoid
Propanol	Fisher Scientific
Proteinase K	Promega
QuantiTect SYBR Green Master Mix	Qiagen
RNase H	Promega
Rnase free DnaseI	NEB
RNaseOUT	Invitrogen
Sigma Fast p-Nitrophenyl Phosphate tablet	Sigma-Aldrich
Skimmed milk	Oxoid
Sodium chloride	Fisher Scientific
Sodium azide	Sigma-Aldrich
Sodium dihydrogen mono phosphate	Sigma-Aldrich
Superscript II reverse transcriptase	Invitrogen
Taq DNA polymerase	Thermo
TRIzol	Invitrogen
Tris-HCl	Sigma-Aldrich
Triton X-100	BDH laboratories
Trizma base	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Zymosan	Sigma-Aldrich

2.1.2.1 Buffers, solutions and media

Levinthal's supplement	200 ml of defibrinated horse blood was added to 400 ml of BHI medium and heated at 95°C for 40 minutes. After cooling, the mixture was centrifuged at 5350 xg for 25 minutes at 4°C. The supernatant was aliquoted into 40 ml lots and kept at -20°C until use.
DEPC-treated water	0.1% DEPC in nanopure water. Keep at RT overnight and then autoclave at 121°C for 20 minutes

2.1.2.2 Buffers used in ELISA

Coating buffer (pH 9.6)	15 mM Na_2CO_3 35 mM $NaHCO_3$
Tris buffered saline (TBS), (pH 7.4)	10 mM Tris-HCl 140 mM NaCl

MBL- binding buffer (pH 7.4)	20 mM Tris-HCl 10 mM $CaCl_2$ 1 M NaCl 0.05% (v/v) Triton X-100 0.1% (w/v) HSA
BSA-TBS blocking buffer (pH 7.4)	TBS with 1% (w/v) BSA
Barbital buffer saline (BBS), (pH 7.4)	4 mM barbital 145 mM NaCl 1 mM $MgCl_2$ 2 mM $CaCl_2$

2.1.3 Antibodies/proteins

Goat anti-human C1q polyclonal antibody	Atlantic Antibodies Scarborough (USA)
Chicken anti-human C4c-alkaline phosphatase	Immunsystem AB
Donkey anti-goat IgG (whole molecule) alkaline phosphatase antibody	Sigma-Aldrich

Goat anti-rabbit IgG (whole molecule) Alkaline phosphatase antibody	Sigma-Aldrich
Rat anti-mouse MBL-A monoclonal antibody	Hycult
Rat anti-mouse MBL-C monoclonal antibody	Hycult
Rabbit anti-human C3c polyclonal antibody	Dako
Anti-meningococcal immunotype L3,7,9 monoclonal antibody	NIBSC (UK)
Murine specific anti-MASP-2 antibody (AbD 04211)	Omeros Corporation Seatle (USA)
Recombinant murine properdin	Dr Youssif Mohammed Ali Department of infection, Immunity & inflammation, University of Leicester (UK)
Rat anti-mouse CL-11 antibody	Dr. Soren Hansen

	Department of Cancer and Inflammation Research, University of Southern Denmark (Denmark)
Mouse anti-human CL-11 antibody	Dr. Soren Hansen Department of Cancer and Inflammation Research, University of Southern Denmark (Denmark)

2.1.4 Kits

Oxidase test kit	Sigma-Aldrich
Restain quick diff. kit	Reagena
Wizard genomic DNA purification kit	Promega
Wizard plus SV minipreps DNA purification system	Promega

2.1.5 *Neisseria meningitidis* strains

42 clinical isolates of *N. meningitidis* belonging to different serogroups were used throughout the study. The details and characteristics of the isolates are presented in table 2.1. The concentration of meningococci was calculated by measuring the OD_{A260} of a lysate of bacterial suspension in 0.1 M NaOH/1% SDS. All the meningococcal strains and the

primers used to identify the bacterial virulence factors were kindly provided by Dr Chris Bayliss (Genetics Department, University of Leicester, UK).

2.1.6 LOS sialylation and distribution of various genes across different strains of *N.meningitidis*

PCR based gene profiling in the strains of *N.meningitidis* under investigation was performed. The encapsulated meningococcal strains contain a *ctrA* gene within the capsule-synthesis (*cps*) gene which is mainly responsible for capsular polysaccharide transport. In contrast, the unencapsulated meningococcal strains lack the genetic material necessary for capsule synthesis and bear a *cnl* (capsule null locus) sequence in place of the capsule biosynthesis operon (Claus *et al*., 2002).

The PCR based gene profiling showed expression of the *ctr*A (capsule transfer) gene in most of the clinical strains (see **Figure 2.2**). While expression of *cnl* yielded an amplicon only in the *N.meningtidis* carriage strain N56.1. This suggests that this gene is not expressed in *N.meningitidis* serogroup A strain Z2491 and serogroup B strain MC58. C*rgA* (contact-regulated gene A) encoding the CrgA protein of *N. meningitidis* was detected in meningococcal strains Z2491, MC58 and 6414 (see **Figure 2.2**).

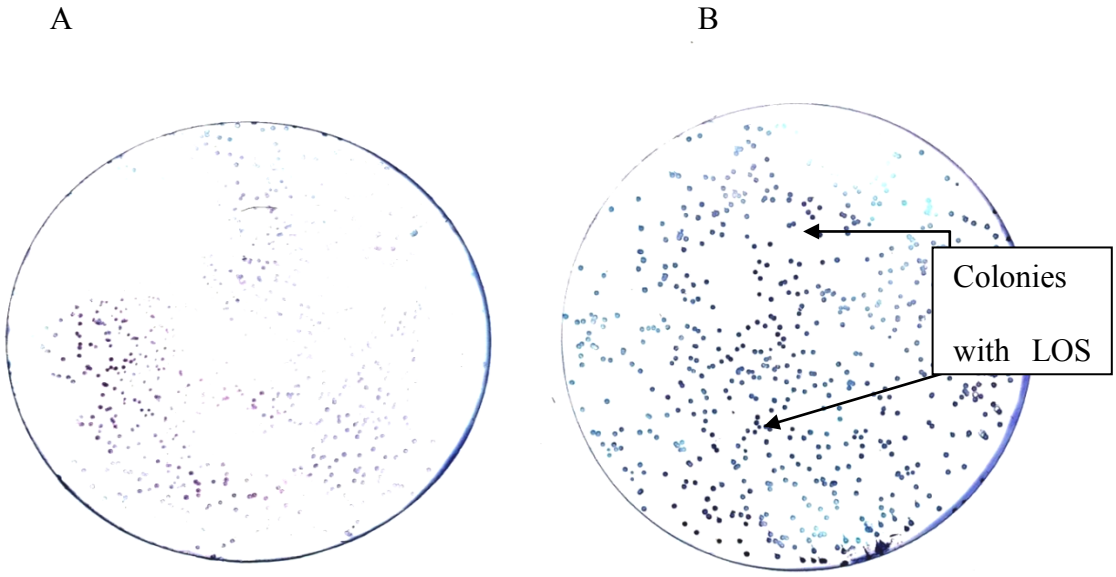

Figure 2.1: LOS sialylation of L3, 7 and 9 immunotypes of *N.meningitidis* serogroup B: Colonies of *N.meningitidis* serogroup B strain 8047 and MC58 were blotted on nitrocellulose membrane followed by blocking with 5% skimmed milk powder in PBST. The membranes were incubated with primary antibody directed against L3, 7 and 9 immunotypes having full sialylation. After washing with PBST, AP-conjugated secondary antibody (1; 5000) was then applied and finally membranes were developed with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). Faint staining of blotted colonies of *N.meningitidis* serogroup B strain 8047 on nitrocellulose membrane showing truncated LOS **(A)** while the same dilution of serogroup B strain MC58 shows dark colonies with intense LOS sialylation **(B).**

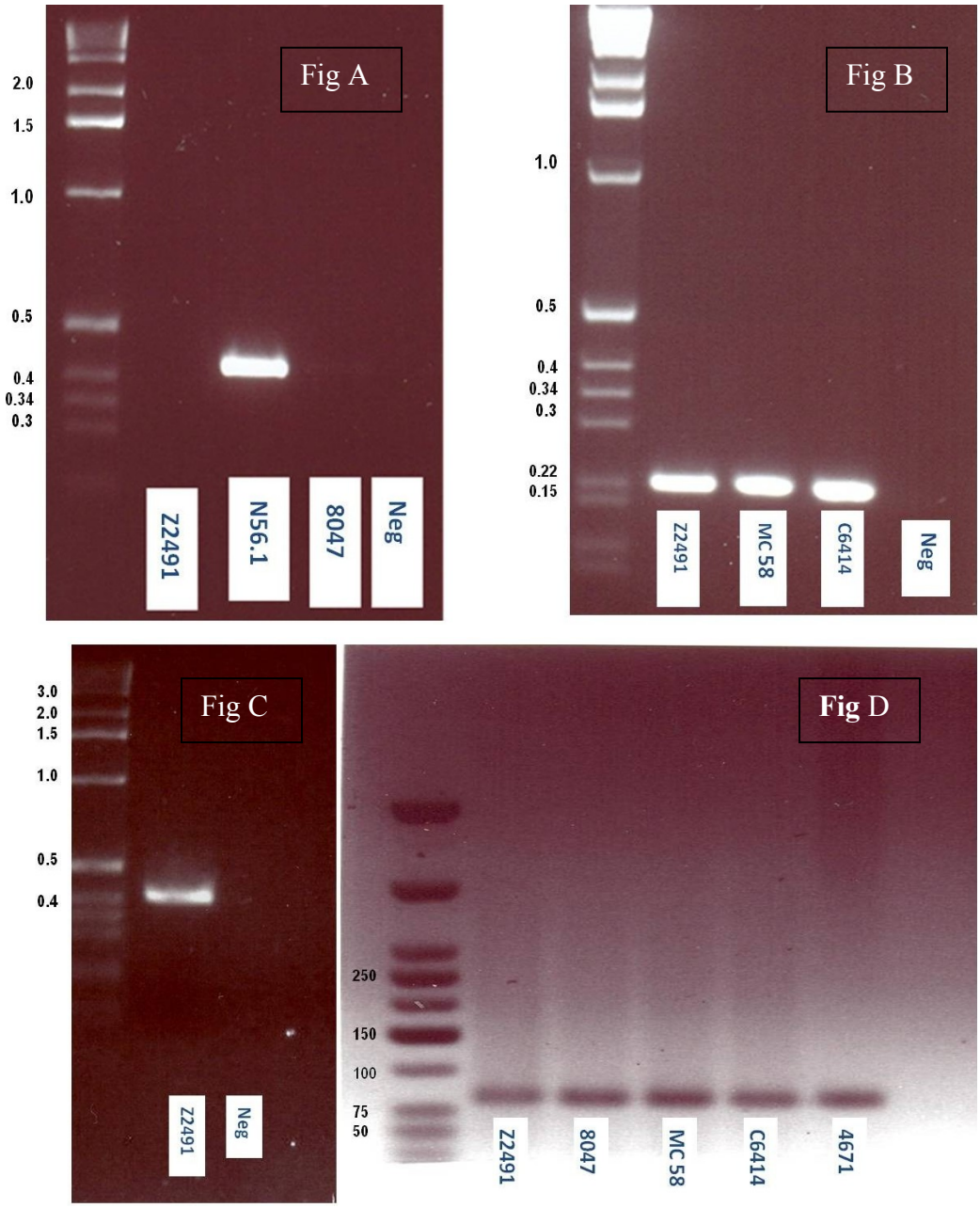

Figure 2.2: Characteristics of *N.meningtidis* strains used in this study: Fig A, *cnl* gene (capsule null locus) sequence is present in *N.meningtidis* carriage strain N56.1 only. **Fig B**, *crg* A (contact-regulated gene A) is possessed by *N.meningitidis* serogroup A strain Z2491, *N.meningitidis* serogroup B strain MC58 and *N.meningitidis* serogroup C strain 6414. **Fig C,** serogroup A Capsule synthesis gene (*cap*) gene is present only in *N.meningitidis* serogroup A Z2491. **Fig D,** *ctr* (capsule transfer gene) is present in all the invasive strains of different serogroups.

Table 2.1: Meningococcal Strains used in this study.

"number"	Date of isolation	Country of origin	Clinical description	Serogroup	Clonal complex
N137	1968	England	Invasive (unspecified/other)	29E	
Z5035	1979	China	Invasive (unspecified/other)	A	ST-1 complex/subgroup I/II
Z3515	1987	Saudi	Carrier	A	ST-5 complex/subgroup III
Z2491	1937	Africa	Invasive (unspecified/other)	A	ST-4 complex
Z1534	1941	England	Invasive (unspecified/other)	A	
Z1392	1968	Greece	Carrier	A	ST-1 complex
MC58	1985	UK	Invasive (unspecified/other)	B	ST-32 complex
N121		UK	Carrier		
H44/76	1976	Norway	Invasive (unspecified/other)	B	ST-32 complex
Z4664	1984	Netherlands	Invasive (unspecified/other)	B	ST-32 complex/ET-5 complex
Z4665	1977	Netherlands	Invasive (unspecified/other)	B	ST-1 complex
Z4667	1963	Netherlands	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z4671	1979	Netherlands	Invasive (unspecified/other)	B	ST-8 complex/Cluster A4
Z4672	1985	Netherlands	Invasive (unspecified/other)	B	ST-32 complex/ET-5 complex
Z4673	1986	Netherlands	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3

Z4674	1964	Netherlands	Invasive (unspecified/other)	B	ST-37 complex
Z4675	1940	Denmark	Invasive (unspecified/other)	B	
Z4676	1962	Denmark	Invasive (unspecified/other)	B	ST-37 complex
Z4677	1985	East Germany	Invasive (unspecified/other)	B	ST-18 complex
Z4678	1985	East Germany	Invasive (unspecified/other)	B	ST-18 complex
Z4682	1988	Norway	Invasive (unspecified/other)	B	
Z4683	1988	Norway	Invasive (unspecified/other)	B	
Z4684	1988	Norway	Invasive (unspecified/other)	B	ST-269 complex
Z4687	1988	Norway	Carrier	B	
Z4708	1977	China	Invasive (unspecified/other)	B	
Z6422	1994	Norway	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6423	1994	Chile	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6424	1993	Netherlands	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6425	1996	Norway	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6426	1991	New Zealand	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6427	1988	Scotland	Invasive (unspecified/other)	B	ST-41/44 complex/Lineage 3
Z6414	1994	New Zealand	Invasive (unspecified/other)	C	ST-8 complex/Cluster A4
Z4181	1989	Mali	Carrier	C	ST-11 complex
N86			capsule off	C	
N192	1968		Invasive (unspecified/other)	W-135	ST-2881

Z6432	1986	Norway	Carrier	W-135	ST-22 complex
Z6430	1986	Netherlands	Invasive (unspecified/other)	X	ST-750 complex
N51		UK	Carrier	Y	
N52		UK	Carrier	Y	
N54		UK	Carrier	Y	
N88		UK	Carrier	Y	
N343		UK	Carrier	Y	
N354		UK	Carrier	Y	
N369		UK	Carrier	Y	
Z6434	1986	Netherlands	Invasive (unspecified/other)	Y	ST-167 complex
Z6433	1994	Norway	Invasive (unspecified/other)	Y	ST-23 complex/Cluster A3
Z6433	1994	Norway	Invasive (unspecified/other)	Y	ST-23 complex/Cluster A3
Z6431	1989	Netherlands	Invasive (unspecified/other)	Z	ST-103 complex
N130		UK		--*	

*= Non groupable strains

2.2 Methods

2.2.1 *N. meningitidis* growth conditions

For growth in liquid media, *N. meningitidis* was grown overnight in 10 ml BHI (brain heart infusion medium) supplemented with 5% Levanthal's base at 37°C on shaking incubator. Next day, bacterial concentration was measured at A_{260} and $1x10^9$ cells were added to fresh BHI (brain heart infusion medium) supplemented with 5% Levanthal's base. The bacterial suspension was incubated at 37°C with shaking. Samples for bacterial OD_{260} and viable

count estimation were taken from the suspension after every 60 minutes until the OD_{260} reaches a plateau phase. For growth on solid media, *N. meningitidis* was streaked on brain heart infusion (BHI) agar with 5% Levanthal's supplement and incubated overnight at 37°C in the presence of 5% CO_2. Following day, a loopful of fresh colony growth was inoculated into 1ml of PBSB (phosphate-buffered saline [PBS] supplemented with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ pH 7.4) and OD_{260} of the sample was measured. Once the concentration of meningococci was determined, the stock was re-suspended and adjusted to desired concentration in PBSB or HBSS.

2.2.1.1 *N. meningitidis* fixation and preparation for *in-vitro* analysis

N. meningitidis was grown overnight at 37°C in BHI medium supplemented with 5% Levanthal's base in the presence of 5% CO_2. Next day, the broth medium containing bacteria was centrifuged at 4000 xg for 10 minutes. The cell pellet was washed three times with phosphate-buffered saline (PBS) before resuspending it in 10ml of 0.5% formalin (in PBS) and incubating for 60 minutes at room temperature. The cell pellet was washed twice with PBS. A loopful sample from the fixed bacteria was inoculated on chocolate agar plates, and the plates were incubated overnight at 37°C in the presence of 5% CO_2. Absence of growth on the plates after overnight incubation ensured the formalin-fixation. Finally the cell pellet was re-suspended in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% sodium azide, pH 9.6) for later *in-vitro* analysis (Lynch *et al*., 2004).

2.2.2 Preparation of mouse and human serum

The immuno-biochemical tests were run on sera, which was extracted from mouse and healthy human blood. For certain assays, serum obtained from mouse and human deficient in different complement components was used. Blood samples were kept on ice throughout the procedure in order to avoid the complement activation. Approximately 225 µl-700 µl of blood was collected from mouse (depending on its size) using a cardiac puncture bleed. Approximately 5 ml of blood was collected from healthy human individuals. The blood was collected into previously labelled 1.5 ml eppendorf tubes and put on ice immediately. Blood was then allowed to clot on ice for 4-6 hours before being centrifuged in a cooled microcentrifuge at 14,000 rpm for 7 minutes to separate serum. Serum was then pipetted as aliquots of 100 µl into previously labeled 0.5 ml eppendorf tube and was kept at -80°C for future analysis.

2.2.3 Complement pathway specific Enzyme Linked Immune Sorbent Assays (ELISAs)

2.2.3.1 MBL-A, MBL-C, Ficolin-A and CL-11 binding assays

Micro-titre ELISA (Maxisorb, Nunc) plates were coated with formalin fixed *N. meningitidis* strains (OD550=0.6) in the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% sodium azide, pH 9.6). 10 µg/ml Mannan (Sigma) in the coating buffer was coated as positive control in MBL-A and MBL-C binding assays while 10 µg/ml N-acetylated BSA (Promega) in the coating buffer was coated as positive control in ficoin-A

binding assay. 1 µg/ml Zymosan (Sigma) in coating buffer was coated as positive control in CL-11 and alternative pathway mediated C3 deposition assay. For some of the experiments, wells were coated with coating buffer which was used as negative control. The plates were incubated overnight at 4°C. On the following day, residual protein binding sites were blocked with 250 µl of TBS (10 mM Tris, 140 mM NaCl, pH 7.4) containing 1% w/v bovine serum albumin, incubated for 2 h at room temperature. Plates were washed three times with 250 µl of washing buffer (TBS with 0.05% tween-20 and 5mM $CaCl_2$). Two fold serial dilutions of serum samples starting with 1/80 dilution were made in the MBL binding buffer (20 mM Tris-HCl, 10 mM $CaCl_2$, 1 M NaCl, pH 7.4). Serum dilutions were added in duplicates to the corresponding wells in aliquots of 100 µl per well and incubated at 37°C for an hour. Wells receiving buffer instead of serum were used as negative controls (Celik *et al*., 2001; Takahashi *et al*., 2008; Schwaeble *et al*., 2011). Plates were washed three times followed by addition of 100 µl of rat anti-mouse MBL-A (Hycult; 1 mg/ml stock solution), rat anti-mouse MBL-C (Hycult; 1mg/ml stock solution), rabbit anti-mouse Ficolin A antibodies Prof. T. Fujita, Department of Immunology, Fukushima Medical University School of Medicine, Fukushima, Japan; 0.7 mg/ml stock solution) diluted 1/1000 and rat anti-mouse CL-11 (0.5 mg/ml stock solution) or mouse anti-human CL-11 (2.04 mg/ml stock solution) antibodies (kindly provided by Dr. Soren Hansen, Department of Cancer and Inflammation Research, University of Southern Denmark) in TBS with 0.05% tween-20 and 5mM $CaCl_2$. The plates were incubated for 90 min at room temperature and then washed with TBS with 0.05% tween-20 and 5mM $CaCl_2$. The secondary antibodies (goat α- mouse IgG-alkaline phosphatase conjugate, rat anti-mouse

MBL-A monoclonal antibody, rat anti-mouse MBL-C monoclonal antibody and goat α-rabbit IgG-alkaline phosphatase conjugate) were then added in 1/10000 dilutions (100 µl/well) into the corresponding wells. The plates were incubation for 90 min at room temperature. After washing, alkaline phosphatase (AP) was detected by addition of 100 µl of substrate solution (Fast pNPP tablet sets, Sigma). Hydrolysis of substrate was monitored by measuring absorption at 405nm, using a BioRad micro-titre ELISA plate reader.

2.2.3.2 C3 deposition assays

Microtiter wells were coated and blocked as previously described. After washing the wells with TBS containing 0.05% tween-20 and 5 mM $CaCl_2$, two-fold serial dilutions of serum were prepared in barbital buffered saline (BBS; 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and added to the wells. Wells receiving buffer instead of serum were used as negative controls (Celik *et al*., 2001; Takahashi *et al*., 2008; Schwaeble *et al*., 2011). After incubation for 1 hour at 37°C, the plates were washed 3 times with the washing buffer. Then 100 µl of 1/5000 dilution of rabbit anti-human C3c (Dako code No.0062) in washing buffer was added to the wells, and plates were incubated for another 90 min at 37°C. After washing the plates, 1/10000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) in washing buffer was added (100 µl/well) followed by incubation for 90 min at room temperature. After washing the plates, a hundred microlitre per well of pNPP solution was added and incubated at room

temperature. The optical density was measured at 405 nm, using a micro-titre ELISA plate reader.

2.2.3.3 Alternative pathway mediated C3 deposition assays

The same procedure was followed as in C3c deposition assay except for starting serum dilution (1/2) which was prepared in BBS buffer without $CaCl_2$ in the presence of 10 mM EGTA. A washing buffer without calcium chloride was also used in all washing steps before the serum dilutions were removed.

2.2.3.4 C1q deposition assay

Micro-titre plate wells (Maxisorb, Nunc) were coated with formalin fixed *N. meningitidis* strains (OD550=0.6) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% sodium azide, pH 9.6) while a positive control of BSA/anti BSA immune-complex was prepared *in situ*. Wells for positive control were coated with 1 µg of BSA in 100 µl of coating buffer per well. For some of the experiments, wells were coated with coating buffer which was used as negative control. The plates were incubated overnight at 4°C. After blocking and washing, 100 µl of rabbit anti-BSA (2 µg/ml) was added to each well and incubated for 90 minutes at room temperature. After another wash, two fold serial dilutions of mouse serum were prepared in BBS buffer starting with 1/80 dilution and added to the corresponding wells in duplicates. Wells receiving buffer instead of serum were used as negative controls

(Celik *et al*., 2001; Takahashi *et al*., 2008; Schwaeble *et al*., 2011). The plates were then incubated for 1 hour at 37°C. Following washing step, goat anti-human C1q (2 µg/ml) was added to the plates (100 µl/well) and incubated for a further 90 min at 37°C. After another washing step, anti-goat IgG-alkaline phosphatase conjugate was added in 1/10000 dilutions (100 µl/well) into the corresponding wells, incubated for further 90 min at room temperature and washed. Finally, pNPP substrate solution was added and absorbance at 405 nm was measured in BioRad ELISA micro-titre reader.

2.2.3.5 LP- mediated C4 deposition assay

Microtiter wells were coated and blocked as previously described. After washing the wells with washing buffer (TBS containing 0.05% tween-20 and 5 mM $CaCl_2$), mouse serum diluted in MBL binding buffer (20 mM Tris-HCl, 10 mM $CaCl_2$ 1M NaCl, pH 7.4) beginning with 1/80 dilution was added to the wells and incubated overnight at 4°C. For the negative control, serum was replaced by buffer only (Celik *et al*., 2001; Takahashi *et al*., 2008; Schwaeble *et al*., 2011). Following day, 100 µl of 1µg/ml human C4 diluted in BBS was added to each well, incubated for 90 minutes at 37°C. After another wash, the deposition of C4c was detected by addition of 100 µl of alkaline phosphatase-conjugated chicken anti-human C4c antibody (Immunsystem AB) diluted 1; 10000 in washing buffer. Following incubation for 90 min at room temperature, the plates were washed and the bound chicken anti-human C4c-alkaline phosphatase antibody was detected by using pNPP

substrate. Hydrolysis of substrate was monitored by measuring absorption at 405nm, using a BioRad micro-titre ELISA plate reader (Petersen *et al.,* 2001).

2.2.4. Serum Bactericidal Assay (SBA)

A modification of the method of Estabrook *et al* (1997) was used to assess the sensitivities of meningococcal strains to SBA of different serum samples. Stocks of *N. meningitidis* were re-suspended in HBSS to obtain required bacterial concentration. The viable bacterial count was confirmed by diluting the samples in PBS and plating on to BHI agar with 5% Levanthal's supplement in 5% CO_2 at 37°C for overnight. Suspension of known concentration of *N. meningitidis* prepared in HBSS was then mixed with desired concentration of serum sample in 1.5 ml eppendorf tube. The reaction mixture was incubated for 120 minutes at 37^{o}C with shaking at 120 rpm. Samples from each SBA reaction were taken at 0, 15, 30, 60, 90 and 120 minutes, which were serially diluted in HBSS and plated onto chocolate agar or BHI agar with 5% Levanthal's supplement plates followed by incubation at 37^{o}C in 5% CO_2 for overnight.

2.2.5 *In vivo* studies

2.2.5.1 Mice

Mice ranging in age from 8 to 12 weeks old were used in all of *N. meningitidis* infection studies. Two mouse genotypes were used; MASP-2 deficient mice and MASP-2 sufficient

C57BL/6 wild-type mice. MASP-2 deficient mice, established in our lab are presently the only available mice with a total deficient of the lectin pathway functional activity. All the mice used in the study were housed in the University of Leicester Biomedical Services facility under specific pyrogen and pathogen-free conditions and were fed standard laboratory food.

2.2.5.2 *N. meningitidis* infection study

Strains from three different serogroups of *N. meningitidis* were used for the infection study which included; *N. meningitidis* serogroup A-Z2491, *N. meningitidis* serogroup B-MC58 and *N. meningitidis* serogroup C-6414.

2.2.5.3 Preparation of *N. meningitidis* for infection study

N. meningitidis growth depends on the availability of iron, and it is now well established that virulence of Meningococci can be enhanced in experimental infections by the injection of iron compounds into the animal host (Perkins-Balding *et al*., 2004).

Two C57BL/6 wild-type mice were injected with iron dextran (400 mg/kg body weight) 12 hours before the infective dose. *N. meningitidis* was grown overnight in 10 ml of brain heart infusion (BHI) broth with 5% Levanthal's supplement at 37°C with shaking. Next day, bacteria were pelleted and re-suspended in 5 ml phosphate buffered saline (PBS, pH

7.4). 100 µl of the bacterial suspension (10^9 CFU/ml) co-administered with iron dextran (400 mg/kg body weight) was injected into the peritoneal cavity of C57BL/6 mice. Once mice developed signs of terminal illness (12-16 hours post infection), they were culled by cervical dislocation and blood samples were immediately collected by cardiac puncture. Blood samples were streaked on brain heart infusion (BHI) agar with 5% Levanthal's supplement in 5% CO_2 at 37°C overnight. Following day, single colonies were picked from the culture and were characterised by gram stain and oxidase test. A loopful of colonies was inoculated into 1 ml of PBSB (phosphate-buffered saline [PBS] supplemented with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ pH 7.4) and OD_{260} of the sample was measured, which corresponded to $\geq 10^9$ CFU/ml. 500 µl aliquot was then inoculated from the stock into new tube containing 10 ml BHI broth with 5% Levanthal's supplement. The bacterial suspension was incubated at 37°C in the presence of 5% CO_2 with shaking at 200 rpm for 300 minutes (mid-log phase). After this time, a sample was taken out and optical density was measured at 260 nm to check the count. 20% v/v filtered fetal calf serum (Harlan) was added to the bacterial culture which was then distributed in 500 µl aliquots in sterile cryotubes. The bacterial stocks were kept at -80^{o}C until further use. Viable count of *N. meningitidis* stock was determined by surface drop method in random aliquots before freezing and after freezing at different time points to study the effect of freezing on viability of meningococci. For each infection experiment, aliquots of *N. meningitidis* stock were thawed at room temperature, spun down and re-suspended in PBS to obtain the required viable count. The viable bacterial count of the infective dose was confirmed by

diluting the samples in PBS and plating on to brain heart infusion (BHI) agar with 5% Levanthal's supplement in 5% CO_2 at 37°C for overnight.

2.2.5.4 Virulence testing of *N. meningitidis*

To determine the appropriate infective dose, groups of age-matched mice (all on the pure C57BL/6 background) were given serial 10-fold dilutions of passaged *N.meningitidis* co-administered with iron dextran (400 mg/kg body weight) via intraperitoneal (i.p.) route. For *N.meningitidis* serogroup A-Z2491, each group received an estimated dose of between 1 x 10^6 – 1 x 10^9 CFU/mouse bacteria diluted in 100 µl sterile PBS where as *N.meningitidis* serogroup C-6414 received an estimated dose of between 1 x 10^5 – 1 x 10^8 CFU/mouse. For *N.meningitidis* serogroup B-MC58, the injected dose amongst different groups of mice was between 1 x 10^5 – 1 x 10^7 CFU/mouse diluted in 100 µl sterile PBS. Following infection, mice were observed regularly for at least three times daily for a maximum period of 72 hours. The signs of illness were monitored and were scored based on the scheme of Fransen *et al* (2010) with slight modifications (see **Table 2.2**). Infective dose with significant morbidity range (60-80 % mice affected) was used in subsequent experiments.

Table 2.2: Disease severity scores associated with clinical signs in infected mice

Signs	Score
Normal	0
slightly ruffled fur	1
ruffled fur, slow and sticky eyes	2
ruffled fur, lethargic and eyes shut	3
very sick and no movement after stimulation	4
Dead	5

2.2.5.5 Infection of mice with *N.meningitidis*

Female MASP2$^{-/-}$ mice and MASP-2$^{+/+}$ wild-type mice ranging in age from 8 to 12 weeks were used in *N.meningitidis* infection experiments. Both groups of mice were subjected to two main experiments, the survival experiment and time course experiment. Mice to be infected with *N.meningitidis* were injected with iron dextran (400 mg/kg body weight) via intraperitoneal route of infection 12 hours before the infective dose. At the time of infection, groups of MASP-2 deficient and MASP-2 sufficient mice were injected with 100 µl of the calculated *N.meningitidis* dose co-administered with iron dextran (400 mg/kg body weight) by intraperitoneal route. After injection of mice, they were placed in negative pressure infectious isolators dedicated to infection experiments. Mice were regularly monitored for at least three times for illness symptoms. Mice that progressed to terminal disease signs (very sick and no movement after stimulation; score 4) were immediately culled according to Home Office Regulations.

2.2.5.6 Determination of blood and organs bacterial burden

At pre-chosen time points following infection, a number of randomly selected mice from each group were culled by cervical dislocation and blood was collected immediately afterwards by cardiac puncture. Mice were then dissected under aseptic conditions and organs (livers, spleens, kidneys, lungs and brains) intended to be used for bacterial load determination were removed separately into 5 ml of sterile PBS, weighed, and then homogenised in the Ultra-Turrax T10 basic IKA- Werke homogenizer. Viable counts in blood and organs' homogenates were determined by serial dilution in sterile PBS and plating on to brain heart infusion (BHI) agar with 5% Levanthal's supplement and incubated overnight at 37°C in the presence of 5% CO_2. Organs intended to be used for total RNA preparation were snap-frozen in pre-cooled isopentane (Fisher Scientific) on dry ice and stored at -80°C until used.

2.2.6 Molecular Biology techniques

2.2.6.1 RNA methods

2.2.6.1.1 Extraction of total RNA

Organs for total RNA extraction were aseptically removed from mice, rapidly flash frozed and then stored at -80°C. For total RNA preparation, 50-100 mg of organ was cut and then homogenised in 1 ml of TRIzol reagent (Invitrogen), using Ultra-Turrax T10 basic IKA-

Werke homogenizer (Germany). In order to ensure complete dissociation of nucleoprotein complexes, organ homogenate was left at room temperature for 5 minutes. Tissue homogenate was then centrifuged at 12000 xg for 10 minutes at 4°C to get rid of any insoluble protein residues, and supernatant was transferred to a sterile RNase-free microcentrifuge tube. 200 µl of chloroform was added to the samples, mixed vigorously for 15 seconds and then centrifuged at 12000 xg for 15 minutes at 4°C. Upper aqueous phase containing RNA was carefully transferred into a new sterile RNase-free microcentrifuge tube which was then precipitated by mixing with 500 µl of iso-propanol. RNA samples were incubated at room temperature for 10 minutes and spun down at 12000 xg for 10 minutes at 4°C. The supernatant was subsequently removed, and the RNA pellet was washed with 1ml of 75% ethanol. RNA pellet was air dried before dissolving it in 100 µl of DEPC-treated water. To ensure complete dissolution, the RNA samples were stored overnight at -20°C. RNA concentration and purity were measured at wavelengths 260 and 280 nm on the NanoDrop 3300 spectrophotometer where an absorbance ratio A_{260}/A_{280} above 1.8 indicated a pure preparation.

2.2.6.1.2 Purification of RNA

Contaminating DNA in the isolated RNA samples was degraded by RNase-free DNaseI (Promega). 10 µg of RNA was digested with 2 µl RNase-free DNase I (Promega), 20 µl 10x DNase buffer in a final volume of 200 µl with DEPC water (RNase-free water) for 30 minutes at 37°C. After digestion, RNA solution was extracted with 200 µl of

phenol/chloroform/iso-amyl alcohol mixture (Sigma). RNA samples were vigorously shaken for 15 seconds and spun down at 10000 xg for 10 minutes. The upper aqueous phase containing RNA was carefully removed and transferred into a new sterile RNase-free microcentrifuge tube. The RNA was precipitated with 500 µl of absolute ethanol and 20 µl of 3 M sodium acetate before centrifuging it at 10000 xg. The purified RNA was dried at room temperature for 15 minutes and subsequently resuspended in DEPC water. RNA concentration and purity was measured again at wavelengths- 260 and 280 nm on the NanoDrop 3300 spectrophotometer where the absorbance ratio A_{260}/A_{280} of 1.7-2 represents good RNA preparation.

2.2.6.1.3 Synthesis of cDNA by Reverse transcriptase PCR (RT- PCR)

For cDNA synthesis, 1 µl of Oligo $(dT)_{23}$ anchored primers (Sigma) were added to 10 µl of RNA solution equivalent to 1 µg of RNA. The reaction mixture was incubated for 10 minutes at 70°C. After that, the temperature was decreased to 45°C. A master mix of 1 µl of SuperScript II Reverse Transcriptase (Invitrogen), 2 µl of 10x reverse transcriptase buffer (Invitrogen), 2 µl of 25 mM $MgCl_2$ (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), 1 µl of 10 mM deoxyribonucleoside triphosphate mixture (dNTPs) (Promega) and 0.5 µl of RNaseOUT (40 U/µl, Invitrogen) (Promega) was prepared, added to each RNA sample and incubated for another 60 minutes at 45°C. The temperature of reaction mixture was then raised to 70°C for 10 minutes in order to inactivate the reverse transcriptase enzyme. Finally, the temperature was dropped to 4°C. After cDNA preparation, the prepared cDNA

was treated with 1 µl RNase H (Promega) before incubating the reaction tubes at 37°C for 30 minutes. Volume of cDNA was made up to 50 µl with DEPC treated water and stored at -20°C.

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

Quantitative real time PCR is a powerful and quick technique used for studying gene expression levels through precise quantification of mRNA transcripts via the prepared cDNA using a set of gene-specific primers. The principal of qRT-PCR is based on using a fluorescent DNA-binging dye; SYBR green (Qiagen), which binds to the double strand DNA after primer annealing and emits up to 20- to 100-fold enhanced fluorescence. In solution, the SYBR green that does not bind cDNA emits a minor fluorescence. Amplification of cDNA can be monitored by measuring the increase in the fluorescence throughout the amplification cycles.

2.2.6.1.5 Analysis of gene expression by quantitative qRT-PCR

Quantitative real-time PCR was conducted to determine the gene expression levels of different complement proteins and cytokines using Roche Applied Science LightCycler Real Time PCR instrument (Mannheim, Germany) according to the instruction manual. The mRNA expression levels of different complement proteins, including C3, C4, C1q, MBL-A, MBL-C and properdin were analyzed. The mRNA expression profiles of different

proinflammatory and anti-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IL-1β, IL-6, IL-10, IL-17 and macrophage inflammatory protein-2 (MIP-2) in different organs were analyzed. The expression levels were computed by normalising each cytokine concentration to the concentration of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample.

Five microlitre aliquots from the prepared cDNA (from WT and MASP-2$^{-/-}$ infected mice) were pooled together before being used for the construction of a standard curve for each cytokine (see **Figure 2.3**), which was then used to determine the relative cytokine expression in different samples. Each 15 µl of PCR reaction contained 1 µl of pooled cDNA dilutions or sample, 1.5 µl of each primer (5pmol/µl), 3.5 µl of sterile nano-pure water and 7.5 µl of QuantiTect SYBR Green Master Mix (Qiagen). The reaction mixture in each LightCycler tube was spun down at 3000rpm for a minute before being subjected to 45 amplification cycles in qRT-PCR machine (see **Table 2.3 & Table 2.4**). Fluorescence signal was automatically measured by the machine at the end of each cycle. Melting curve analysis was used to confirm the specificity and purity of the amplicon in each PCR (see **Figure 2.4**). Results were analyzed using the Fit Points option in the LDCA software supplied with the machine which also allows the baseline to be set above the noise bands to minimize the error in calculation of the standard curve (see **Figure 2.5**). The corresponding standard curve is automatically generated, which is used to calculate the relative expression levels of each cytokine after normalizing to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the sample.

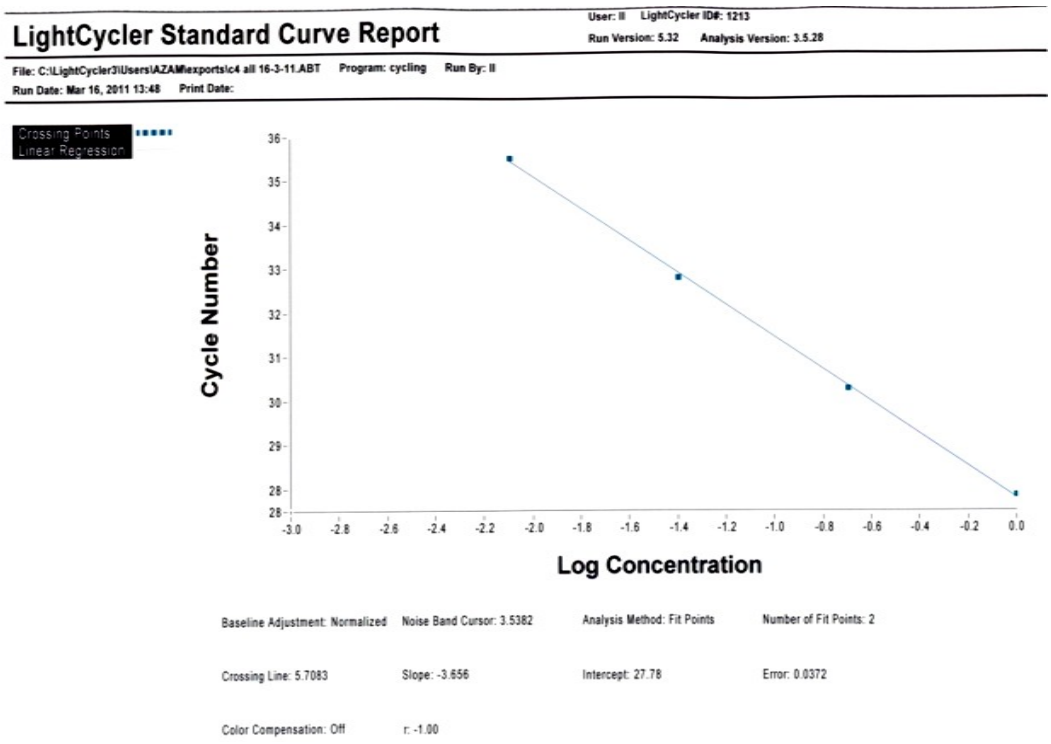

Figure 2.3: Standard curve of C4 expression levels generated by the Light Cycler and used for calculation of relative expression levels of other samples.

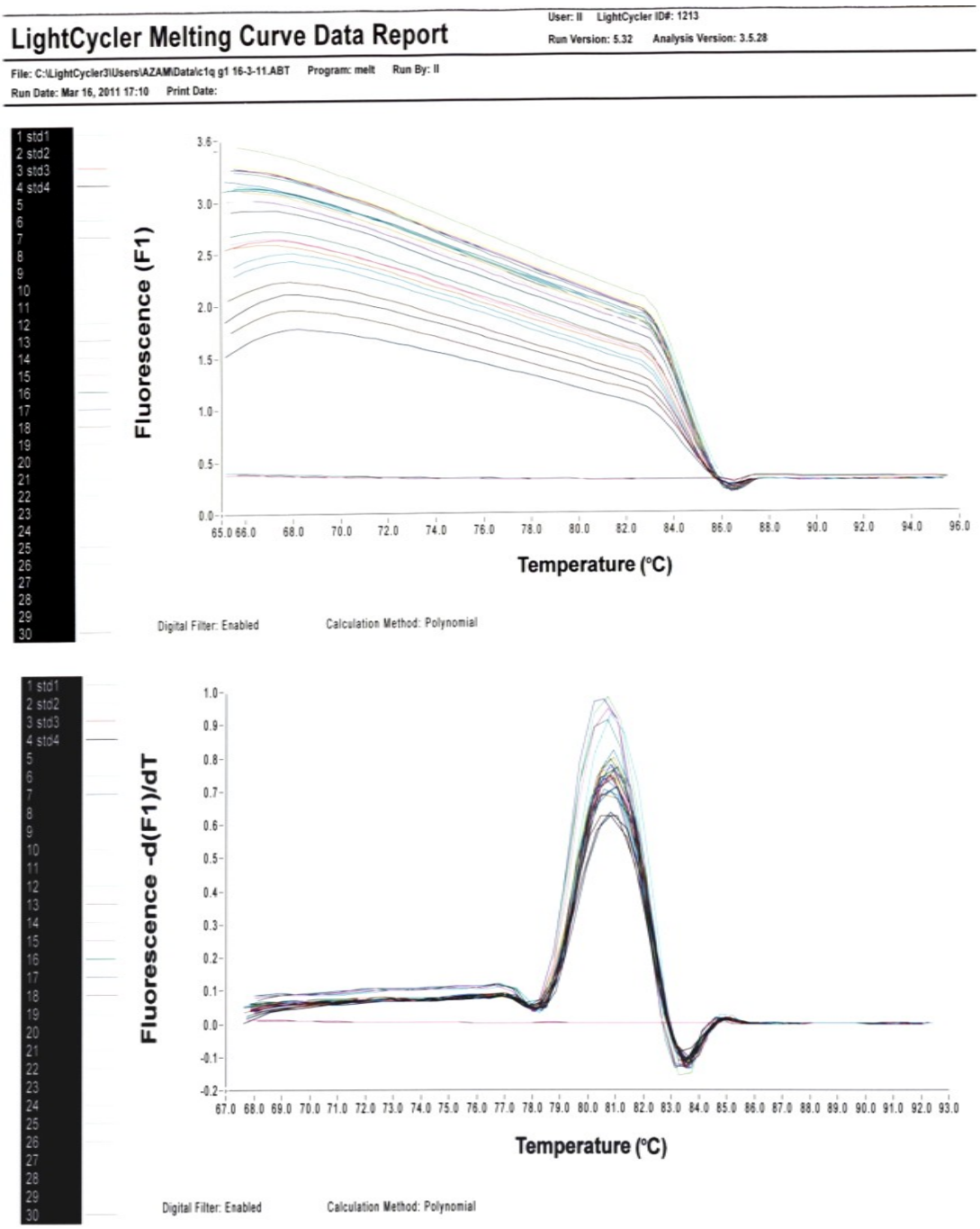

Figure 2.4: Melting curve analysis of C4 cDNA copies with a linear negative control line (red) showing absence of non-specific amplication.

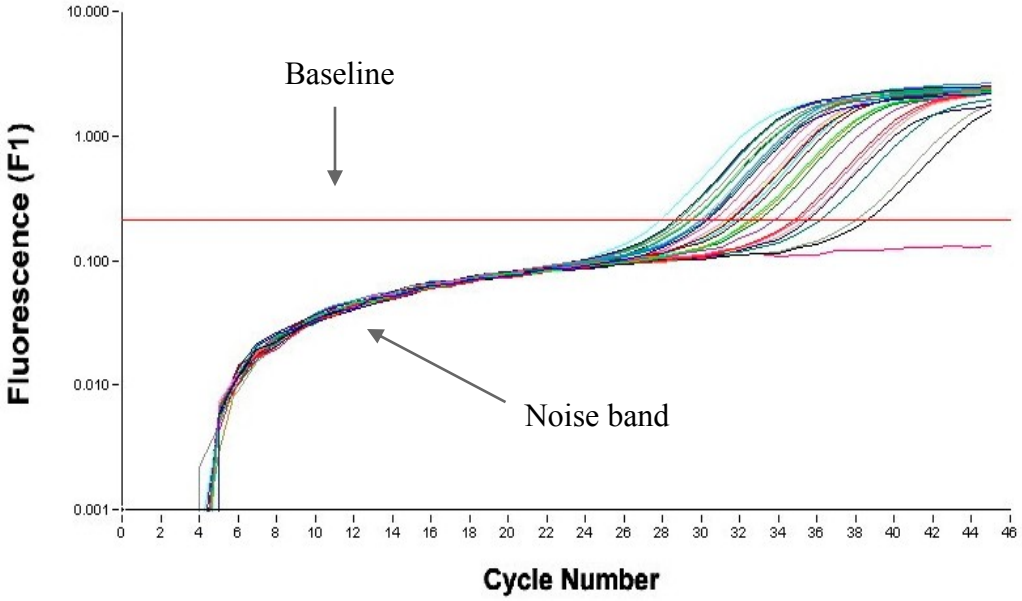

Figure 2.5: C4 amplification curves using the Light Cycler. Each colored line represents a sample. Curves were analysed by Fit Points method where the baseline was adjusted to remove the noise band background.

Table 2.3: Sequence of primers used for cytokine expression levels in qRT-PCR.

Primer	Sequence (5' to 3')	Product size
rmC1qB_F rmC1qB_R	CTTCGAAAAGGTGATCACCA CTGTGGCCTGCAGGTGA	266 bp
IL-1β_F IL-1β_R	CACTCATTGTGGCTGTGGAGA AGGTGGAGAGCTTTCAGCTCA	247 bp
TNFα_F1 TNFα_R2	CCTCACACTCAGATCATCTTCTCA GTGGGTGAGGAGCACATAG	237 bp
GAPDH_F2 GAPDH_R1	GTGCTGCCAAGGCTGTG 3 AGACAACCTGGTCCTCAGTGTA	211bp

C3_F C3_R	AGTGCTGACCAGTGAGAAGACA GAAGTTTGCCACCACTGTCA	136 bp
C4_F C4_R	ATGCCCTTCAGGTGTTCCAG ACTGGCCCAACTTCTCACTGAC	156 bp
IL6_F IL6_R	CAAAGCCAGAGTCCTTCAGA CACTCCTTCTGTGACTCCA	95 bp
IL10_F IL10_R	CTTGCACTACCAAAGCCACA TAAGAGCAGGCAGCATAGCA	86 bp
IL-12_F IL12_R	CTT GCA CTA CCA AAG CCA CA AGT CCC TTT GGT CCA GTG TG	---
INFγ_F INFγ_R	CCTGCGGCCTAGCTCTGA CAGCCAGAAACAGCCATGAG	81bp
MIP2_F MIP2_R	ATCCAGAGCTTGAGTGTGAC AAGGCAAACTTTTTGACCGCC	90bp
MBL-A_F MBL-A_R	CAGGGTCACAAACCTGTGAG TGCAACTTGTTGGTTAGCT	295 bp
MBL-C_F MBL-C_R	GACCTTAACCGAAGGTGTTCA CAGTTTCTCAGGGCTCTCAG	305 bp
CFP_F CFP_R	CAA CGC TGC CTA TGC CTT CCA G TGC TGA CCA TTG TGG AGA CCT GC	---

Table 2.4: qRT-PCR touchdown program used for amplification of cDNA of different genes using Light Cycler.

Program	Denaturation			Type	None	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC/Sec)	2 Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	95	900	20	0	0	0	None
Program	Cycling			Type	None	Cycles	45
Segment Number	Temperature Target (C)	Hold Time (Sec)	Slope (C^O/Sec)	2 Target Temp (C)	Step Size (C)	Step Delay (Cycles)	Acquisition Mode
1	95	15	20	0	0	0	None
2	55	20	20	58	0.8	0	None
3	72	15	15	0	0	0	Single
Program	Melt			Type	Melting curves	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC /Sec)	2 Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	95	2	20	0	0	0	None
2	65	10	1	0	0	0	None
3	95	0	0.1	0	0	0	Continuous
Program	Cool			Type	None	Cycles	1
Segment Number	Temperature Target (^oC)	Hold Time (Sec)	Slope (^oC /Sec)	2^o Target Temp (^oC)	Step Size (^oC)	Step Delay (Cycles)	Acquisition Mode
1	40	30	20	0	0	0	None

2.2.6.2 Bacterial DNA isolation and identification

The bacterial DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. Overnight grown cultures of *N. meningitidis* were centrifuged at 4000 xg for 10 minutes. The cell pellet was washed three times with PBS and finally re-suspended in 5 ml of PBS. 20 µl sample from bacterial suspension was added to 180 µl of 1% SDS-0.1% NaOH and then OD_{260} of the suspension was performed using the NanoDrop 3300 spectrophotometer.

500 µl of bacterial suspension having a count of $\geq 10^9$ cells/ml was harvested by centrifuging for 5 min at 3000 xg and the pellet was resuspended in 180 µl of Buffer ATL. 20 µl of Proteinase K was added to the suspension and after mixing thoroughly by vortexing, the tubes were incubated at 56°C for 30 min with shaking in a thermomixer. Following a brief vortex, 200 µl Buffer ATL was added and the tubes were thoroughly vortexed. Then, 200 µl ethanol (96–100%) was added to the suspension and mixed thoroughly. The mixture was then pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube. Following a brief centrifugation at 6000 xg for 1 min, flow-through alongwith collection tube was discarded, and DNeasy Mini spin column was placed in a new 2 ml collection tube. 500 µl Buffer AW1 was added to the column and the tube was centrifuged for 1 min at 6000 x g (8000 rpm). The flow-through along with the collection tube was discarded, and DNeasy Mini spin column was placed in a new 2 ml collection tube. 500 µl of Buffer AW2 was added to the tube and centrifugtion for 3 min at 20,000 x g was provided in order to dry the DNeasy membrane. The collection tube along with flow-

through was discarded, and column was placed in a new 2 ml collection tube. 200 µl Buffer AE was pipette directly onto the DNeasy membrane and the column was incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000 xg (8000 rpm) to elute the DNA.

Table 2.5: Primers used for bacterial virulence factor genes

Primer	Sequence	Amplicon size
CtrA_F CtrA_R	5'-GCT GCG GTA GGT GGT TCA A-3' 5'-TTG TCG CGG ATT TGC AAC TA-3'	110 bp
Cnl_F Cnl_R	5'-CGC GCC ATT TCT TCC GCC-3' 5'-GGT CGT CTG AAA GCT TGC CTT GCT C-3'	400 bp
CrgA_F CrgA_R	5'-GCT GGC GCC GCT GGC AAC AAA ATT-3' 5'-CTT CTG CAG ATT GCG GCG TGC CGT-3'	229 bp
CapA_F CapA_R	5'-CGC AAT AGG TGT ATA TAT TCT TCC-3' 5'-CTG AAT AGT TTC GTA TGC CTT CTT-3'	395 bp

2.2.6.3 Genotyping of MASP-2 deficient mice

2.2.6.3.1 Isolation of genomic DNA from mouse ear snips

Genomic DNA extraction from mouse ear snips was done by using the Wizard gDNA Purification Kit (Promega) according to manufacturer's instructions.

Approximately, 0.3 cm mice ear snips were incubated overnight at 55°C in 300 µl of lysis buffer (50 µl 0.5M EDTA solution + 250 µl of Nuclei Lysis Solution) along with 10 µl of 20 mg/ml Proteinase K (Qiagen) with gentle shaking. On the following day, 1.5 µl of RNase solution (4 mg/ml) was mixed to the nuclear lysate by inverting the tube (2.5 times) and then the tubes were incubated for 30 minutes at 37°C. To the room temperature cooled sample, 200 µl of protein precipitation solution was added and tubes were subjected to high-speed vortex for 20 seconds followed by centrifugation in a chilled centrifuge for 4 minutes at 13,000 rpm. Avoiding the white protein pellet, supernatant containing the DNA was removed carefully and transferred to a previously labelled clean 1.5 ml microcentrifuge tube. 300 µl of room temperature isopropanol was added to the tubes. Inverting the tube several times precipitated the genomic DNA which was then collected by centrifugation at 13000 rpm for 5min. After carefully removing the clear supernatant, the DNA pellet was washed by 300 µl of 70% ethanol and then DNA was re-precipitated by centrifugation at 13000rpm for 5 minutes. The DNA pellet was air dried for 15-30 minutes and 100 µl of DNA re-hydration solution was added. The prepared genomic DNA was then stored at 4°C in a fridge.

2.2.6.3.2 Polymerase Chain Reaction

The polymerase chain reaction is a powerful and quick tool to amplify a segment of DNA that lies between two regions of a known sequence. Two synthetic oligonucleotide primers are required for the purpose. The forward primer is complementary to a specific sequence

on the anti-sense strand at the 5' end, and the reverse primer is complementary to the sense strand at the 3' end of the region to be amplified. In PCR, template DNA is mixed with a thermostable DNA polymerase, molar excess of each of the primers and the four dNTP's in the presence of optimised molar concentration of $MgCl_2$.

2.2.6.3.3 Multiplex PCR for genotyping of MASP-$2^{-/-}$ mice

MASP-2 deficient mice and MASP-2 sufficient wild-type mice were identified using a standard PCR reaction.

Table 2.6: Primers used for genotyping of MASP-$2^{-/-}$ mouse colony

Primer name	Primer sequence
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'

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

The reaction mixture is first heated to 95°C to denature the template and then cooled to a temperature that permits the primers to anneal to their target sequences on the DNA strand. The reaction mixture is then incubated at an optimal temperature at which new DNA strands are synthesized by thermophilic DNA polymerase. A final elongation step ensures the complete synthesis of DNA strands. The denaturation, annealing and DNA synthesis steps are repeated 35 times using an automated thermal cycler in order to allow exponential amplification of sequence of interest since the products of each cycle serve as templates for the synthesis of new strands.

2.3 Statistical analysis

Statistical significance of differences in bacterial loads, SBA of different sera, relative cytokine expression and average illness scores at different time points were determined using Student's unpaired t-test. Survival data was analysed by Kaplan-Meier plots and compared by log rank test/Gehan-Wilcoxon method or one way ANOVA (where indicated). All statistical analyses were done using Graphpad Prism, Version 5.0 (Graphpad Software). Differences were considered significant at P values of <0.05.

Chapter 3. Results: *In vitro* study

3.1 Role of lectin pathway of the complement system in *Neisseria meningitidis* infection

The interactions of *N.meningitidis* with the mouse complement system were studied *in vitro* as well as in experimental mouse models of meningococcal infection.

3.1.1.1 Complement pathway specific Enzyme Linked Immune Sorbent Assays (ELISAs)

3.1.1.1.1 Binding of carbohydrate recognition molecules of the lectin pathway to *N.meningitidis*

As the lectin pathway-mediated complement activation requires the binding of the lectin pathway specific carbohydrate recognition subcomponents such as MBL, ficolins or CL-11 to signal through MASPs, therefore, the binding of the murine lectin pathway recognition molecules i.e. MBL-A, MBL-C, CL-11 and ficolin-A to the surface of different strains of *N.meningitidis* was studied. The results from current study revealed that both MBL-A and MBL-C were able to bind to different strains of *N.meningitidis* (see **Figure 3.1-7**), whereas ficolin-A showed no binding to any of the *N.meningitidis* strains tested (see **Figure 3.8** & **Figure 3.9**). A limited binding of CL-11 has been observed to different strains of *N.meningitidis* (see **Figure 3.10**).

3.1.1.1.1.1 Binding of MBL-A/ MBL-C to different strains of *N.meningitidis*

The ability of different strains of *N.meningitidis* to bind to murine MBL-A and MBL-C was determined using ELISA. It was previously reported that MBL recognizes and binds to two of the outer membrane proteins (i.e. to Opa and PorB) of *N.meningitidis* (Estabrook *et al.*, 2004). However, the structure and sialylation of lipooligosaccharide (LOS) are also identified as the major determinants of MBL binding (Jack *et al*., 1998; Jack *et al*., 2001). Following ELISAs based binding assays utilised in current study, different species belonging to different serogroups of *N.meningitidis* exhibited varying degrees of MBL binding. Following incubation with normal mouse serum, *N.meningitidis* serogroups A strain Z2491 showed high but equal levels of binding to MBL-A and MBL-C (see **Figure 3.1**). The capsular polysaccharide in members of meningococci serogroup A is composed of a homopolymer of *O*-acetylated, α1→6-linked N-acetylmannosamine (ManNAc) 1-phosphate. The surface exposed ManNAc in serogroups A capsule is known to be a good ligand for MBL (Drogari-Apiranthitou *et al.,* 1997; Van Emmerik *et al.,* 1994).

On the other hand, the capsules of *N.meningitidis* belonging to serogroups B and C are composed of homopolymers of α(2,8)- and α(2,9)-linked sialic acid respectively (Edwards *et al.,* 1994). Interestingly, *N.meningitidis* serogroups B strain MC58 showed a significant binding to mouse MBL-A and MBL-C (see **Figure 3.2**). Likewise, *N.meningitidis* serogroups C strain 6414 bearing a highly immunogenic capsule, showed high levels of binding to mouse MBL-A and MBL-C (see **Figure 3.3**).

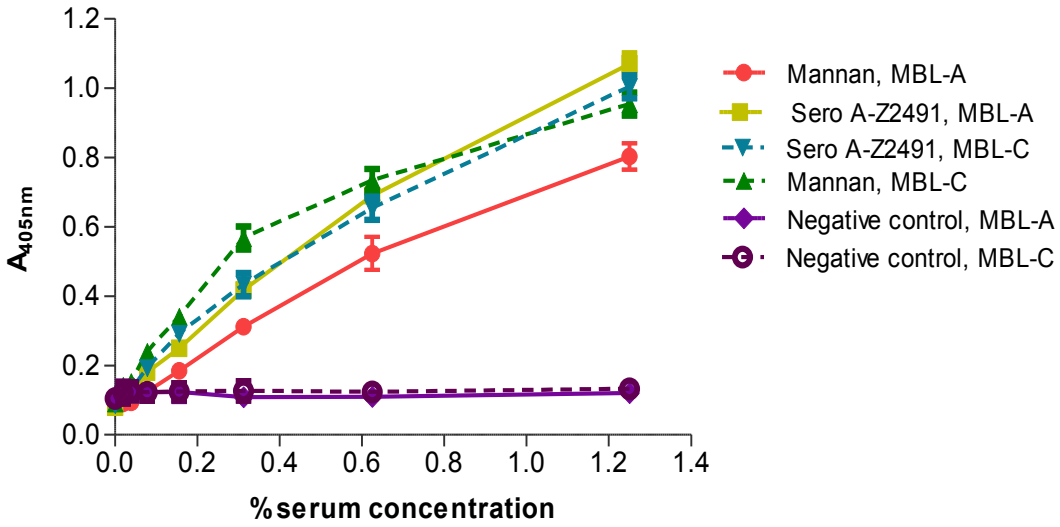

Figure 3.1: MBL-A and MBL-C deposition on the surface of *N.meningitidis* serogroup A strain Z2491 using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

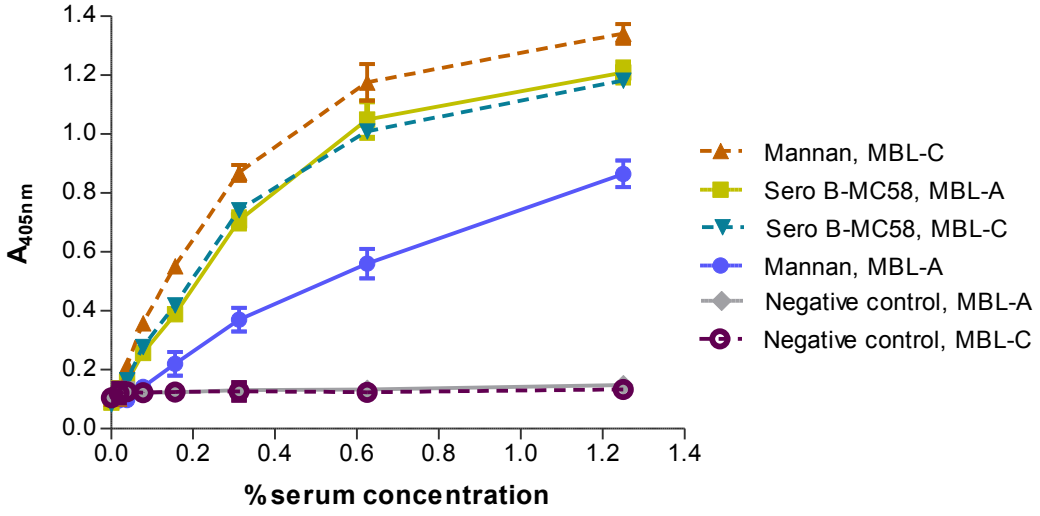

Figure 3.2: MBL-A and MBL-C deposition on the surface of *N.meningitidis* serogroup B strain MC58 using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

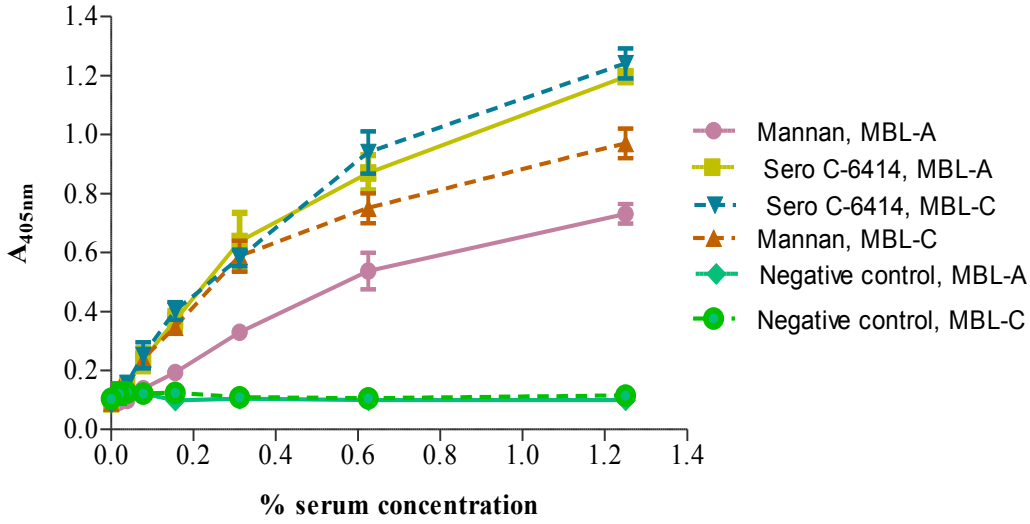

Figure 3.3: MBL-A and MBL-C deposition on the surface of *N.meningitidis* serogroup C strain 6414 using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

The asymptomatic carrier rate of *N.meningitidis* has been reported to be in the range of approximately 10-15% of healthy population (Anderson *et al*., 1998). To investigate the effect of the carriage status of latent meningococcal strains in activating the lectin pathway of complement system, the binding of MBL with carrier strains belonging to different serogroups of meningococci was also tested. *N.meningitidis* strain 4181 is a carrier strain which belongs to serogroup C. Interestingly, strain 4181 showed high levels of binding to both MBL-A and MBL-C (see **Figure 3.4**). The *N.meningitidis* serogroup B strain 8047 bearing a truncated LPS glycoform, showed less binding to both MBL-A and MBL-C when

compared to strain 4181 (see **Figure 3.4**). The carrier strain belonging to serogroups B (strain 4687) showed high levels of binding with both MBL-A and MBL-C when compared to the carrier strain belonging to serogroups A meningococci (strain 3515) (see **Figure 3.5**).

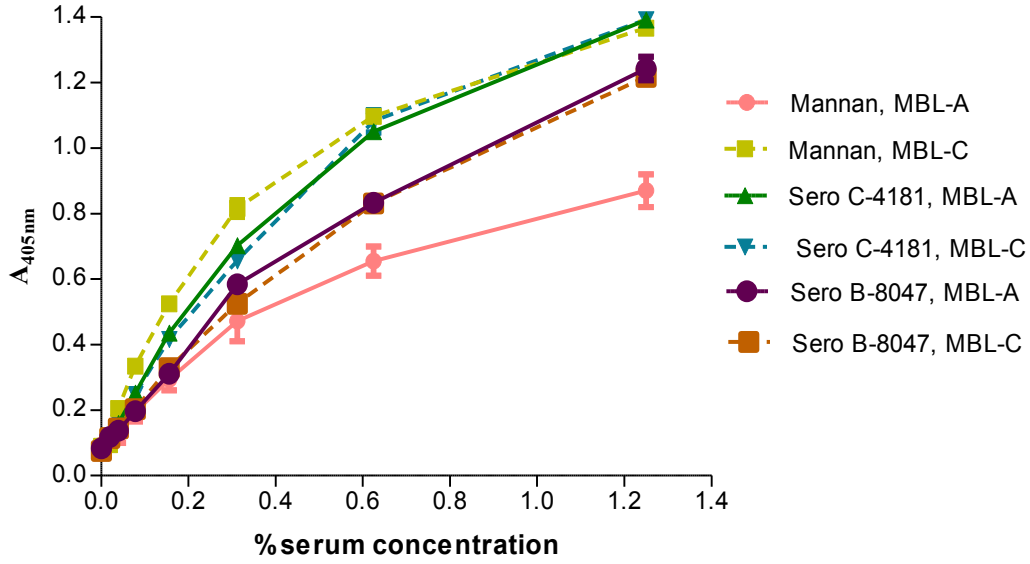

Figure 3.4: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

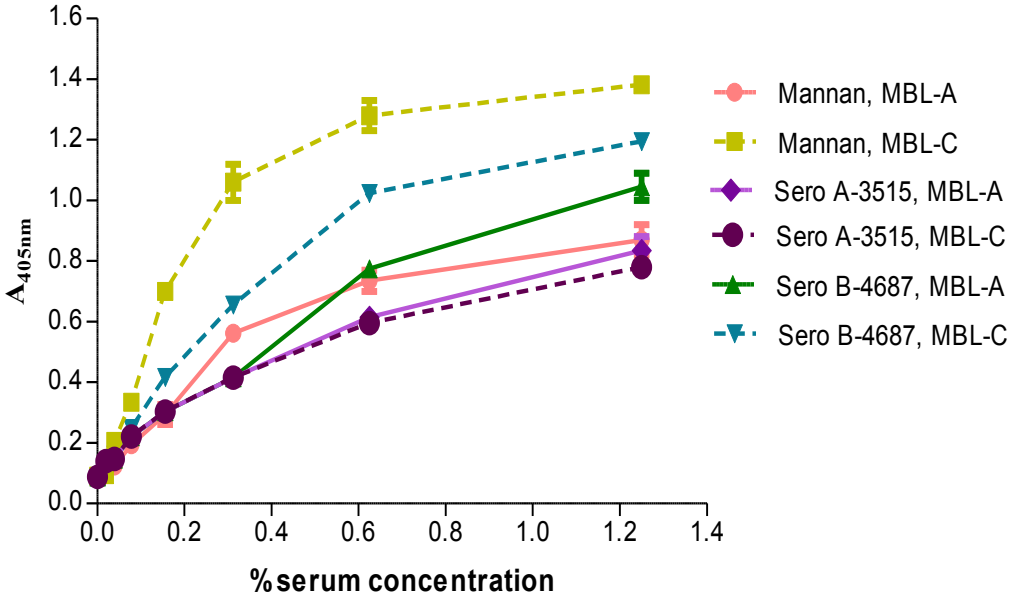

Figure 3.5: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

N.meningitidis serogroups B strain H44/76 is an isolate of a patient with invasive meningococcal disease. Interestingly, strain H44/76 showed higher binding for murine MBL-A than MBL-C (for which a very limited binding was observed). Meningococcal strain 6433 is an invasive strain of serogroup Y and showed high binding levels to MBL-A compared to MBL-C (see **Figure 3.6**).

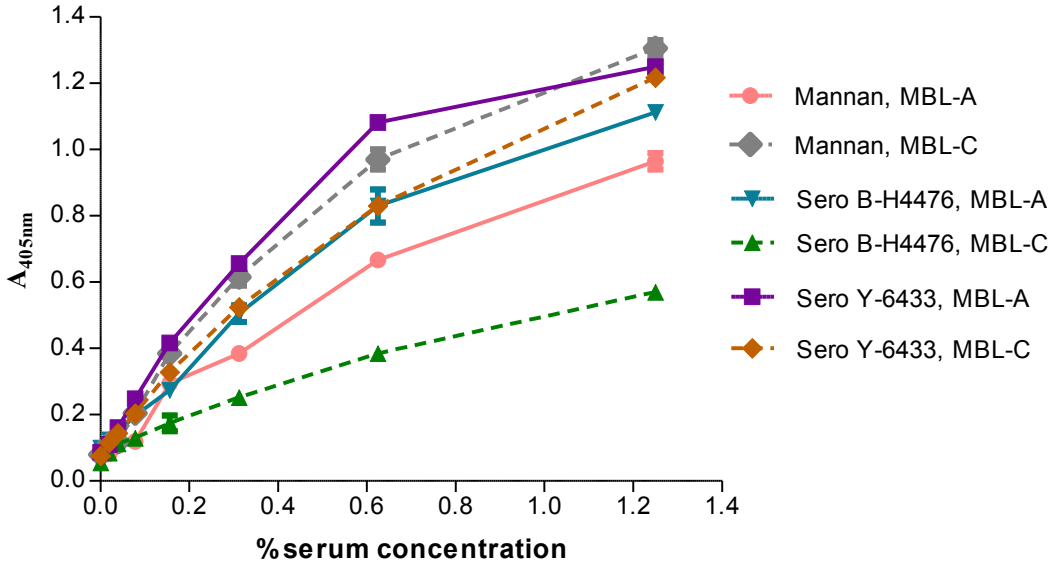

Figure 3.6: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

The carrier strains of the *N.meningitidis* serogroup Y (strain 88 & strain 343) show very limited binding to murine MBL-A and MBL-C with the exception of strain 52 (which shows high binding to MBL-A) (see **Figure 3.7**).

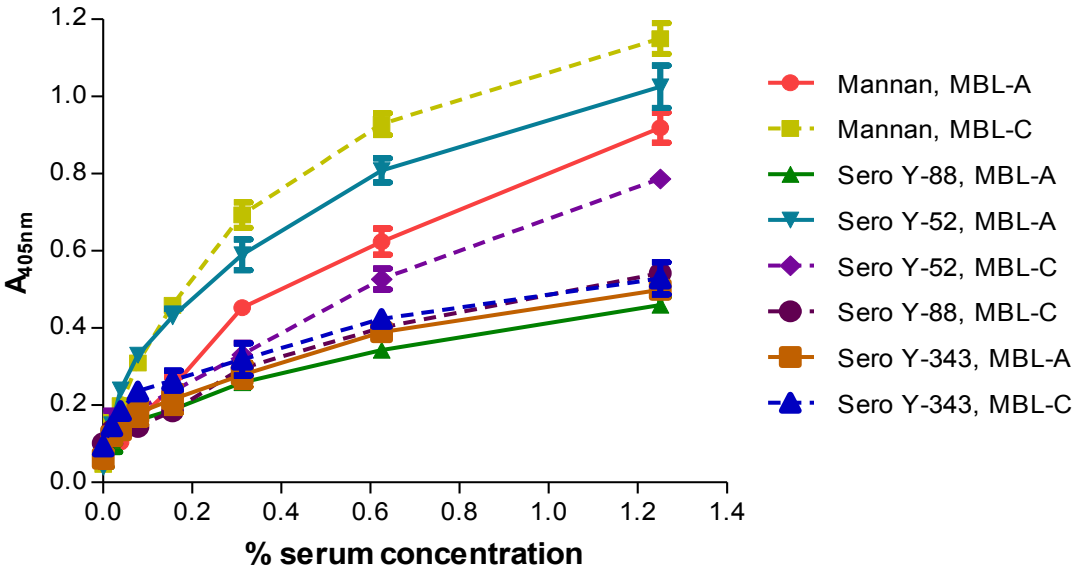

Figure 3.7: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.1.2 Binding of ficolin-A to different strains of *N.meningitidis*

Ficolins are the carbohydrate recognition molecules of the lectin pathway which selectively recognize and bind to *N*-acetylglucosamine (GlcNAc) residues of oligosaccharides on pathogen surface. Two types of ficolins have been identified in mice, designated as ficolin A and ficolin B. Ficolin A is expressed mainly in the liver and is present in the circulation, whereas Ficolin B is expressed in cells of myeloid cell lineage in bone marrow. Only ficolin A can associate with mannan-binding lectin-associated serine proteases (MASPs) and activate the lectin pathway of complement on pathogen surface.

Following ELISA based binding assays, it was found that none of tested strains of *N.meningitidis* showed any binding to murine ficolin A (see **Figure 3.8 & Figure 3.9**).

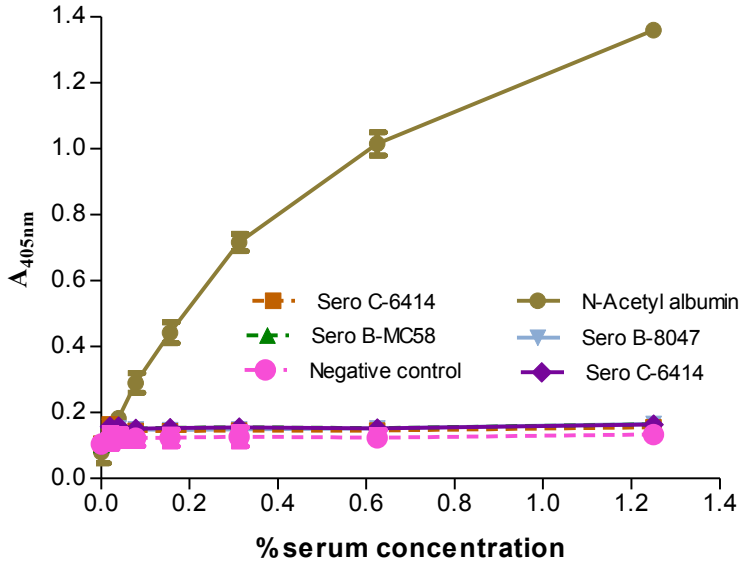

Figure 3.8: Ficolin-A deposition on the surface of different strains of *N.meningitidis* using dilutions of wild-type mouse serum. N-Acetyl albumin coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

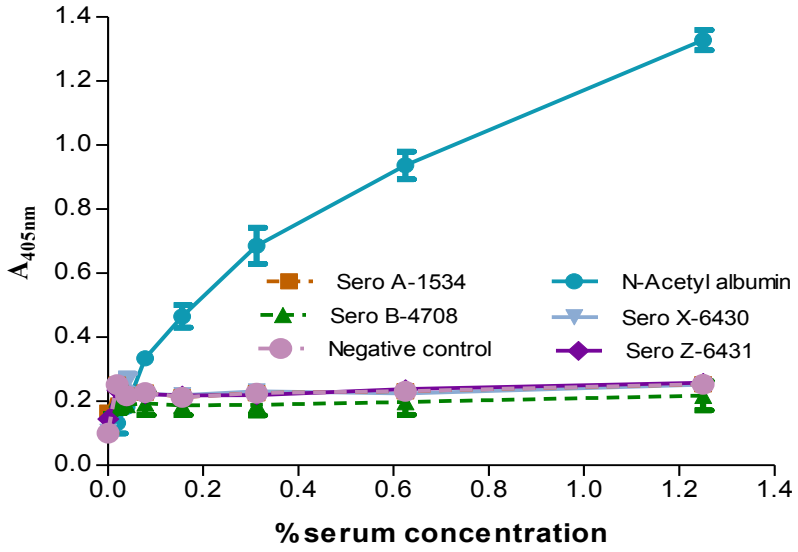

Figure 3.9: Ficolin-A deposition on the surface of different strains of *N.meningitidis* using dilutions of wild-type mouse serum. N-Acetyl albumin coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.1.3 Binding of CL-11 to different strains of *N.meningitidis*

Collectin 11 (CL-11) or collectin kidney (CL-K1) is, like MBL, a member of the collectin family of proteins and was only recently identified as one of the carbohydrate recognition molecules of the lectin pathway. CL-11 selectively binds to l-fucose and d-mannose sugars on the surface of a broad range of microorganisms (including bacteria, fungi and viruses) and activate the lectin pathway of complement activation.

The ELISA-based assay used in current study has revealed a very limited level of murine CL-11 binding to *N.meningitidis* serogroup A strain Z2491, *N.meningitidis* serogroup B strain MC58 and *N.meningitidis* serogroup C strain 6414 (see **Figure 3.10**).

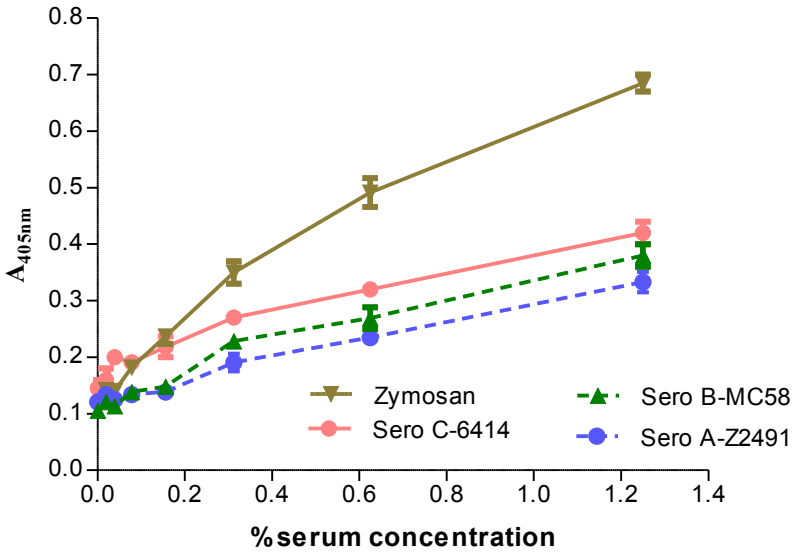

Figure 3.10: CL-11 deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Zymosan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.2 C3 deposition assays

The ability of meningococci to activate the complement system was assessed by measuring the deposition of C3 on the surface of different strains of *N.meningitidis*. In order to achieve this, a relative quantification of C3 cleavage was achieved by detecting the C3 cleavage products (C3c as well as C3c part of C3b and iC3b) binding to the surface of the ELISA plate utilizing an antibody directed against C3c. Under the chosen assay conditions, C3 cleavage on the surface of bacteria is either achieved by the classical or the lectin activation pathway. The alternative pathway mediated activation of C3 is not detected in the assay described due to high plasma/serum dilutions which render the alternative

pathway dysfunctional. All the tested strains of *N.meningitidis* triggered activation of the complement system in diluted mouse serum and substantial C3 deposition was observed on all of the tested strains. Amongst serogroup A-Z2491, serogroup B-MC58 and serogroup C-6414, *N.meningitidis* serogroup B strain MC58 showed the highest C3 deposition on its surface followed by *N.meningitidis* serogroup A strain Z2491 while a comparatively low levels of C3 deposition was observed on the surface of *N.meningitidis* serogroup C strain 6414 (see **Figure 3.11**).

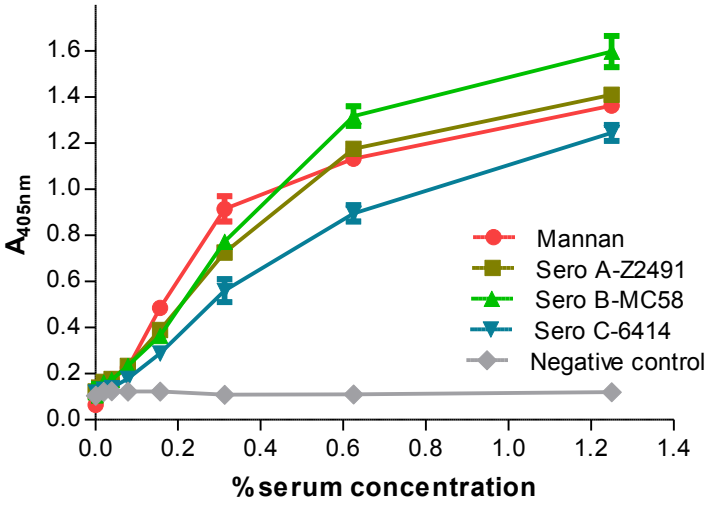

Figure 3.11: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions that allowing both the classical pathway and lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

A varying level of C3 deposition was observed on the surface of carrier strains belonging to serogroups A, B and C meningococci. *N.meningitidis* serogroup A strain 3515 and

serogroup B strain 4687 showed only limited C3 deposition on their surface whereasa, high levels of C3 deposition was observed on the surface of strain 4181 (see **Figure 3.12**).

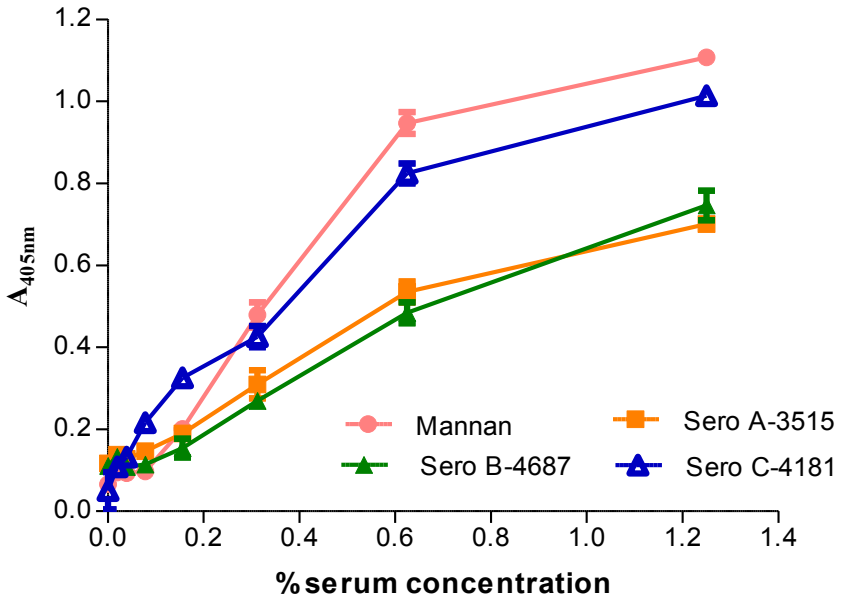

Figure 3.12: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

Different levels of C3 deposition was observed on the surface of different invasive strains belonging to serogroup B (4667), 29E (N137), W135 (N192) and Y (6433) meningococci. *N.meningitidis* strain N137 showed the highest levels of C3 deposition followed by strain N192, while strain 4667 and strain 6433 showed limited amount of C3 deposition on their surface (see **Figure 3.13**).

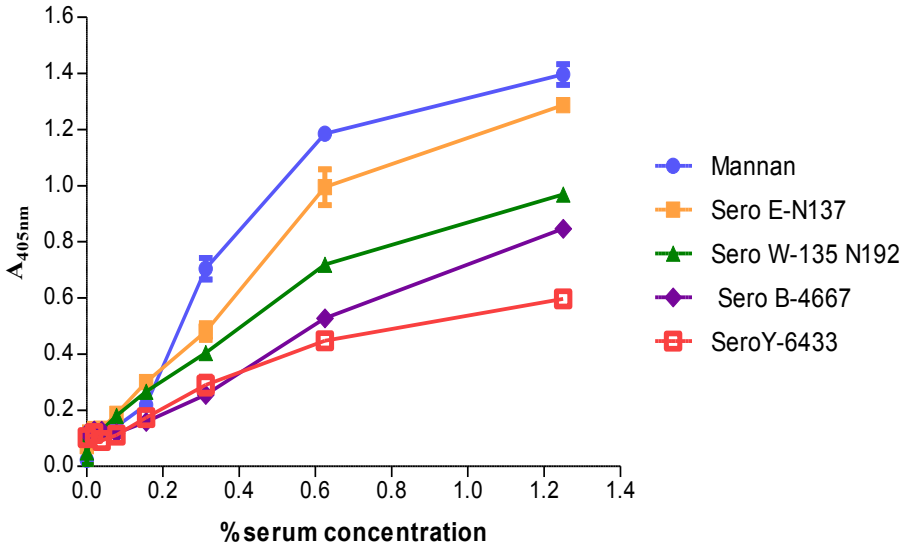

Figure 3.13: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

In order to define the role of the lectin pathway in the deposition of C3b on *N.meningitidis*, this ELISA based C3 cleavage assay was performed using sera from wild-type MASP-2 sufficient as well as MASP-2 deficient mouse. Interestingly, no C3 deposition on the surface of *N.meningitidis* was observed in diluted sera of MASP2$^{-/-}$ mice at low serum concentration i.e. ≤0.4% (**Figure 3.14-16**). However, a limited C3 deposition on the surface of *N.meningitidis* strains Z2491, MC58, 6414, 354, 4678 and 4708 was observed with MASP2$^{-/-}$ serum at high serum concentration (i.e. at 1.2%) (see **Figure 3.14 & 3.16**) which can be mediated via classical or alternative pathway of complement system.

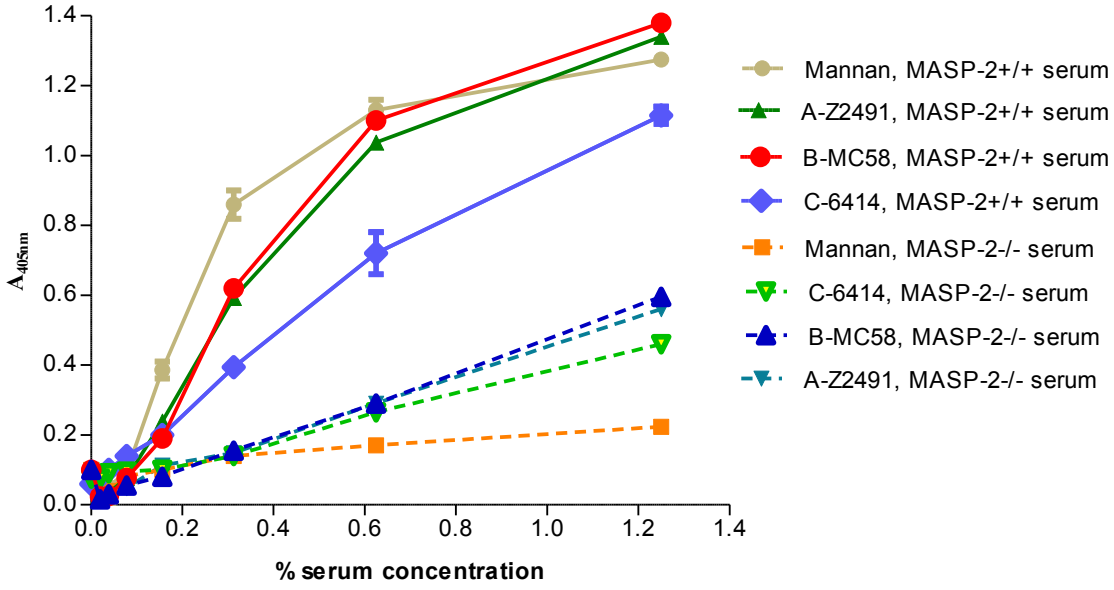

Figure 3.14: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

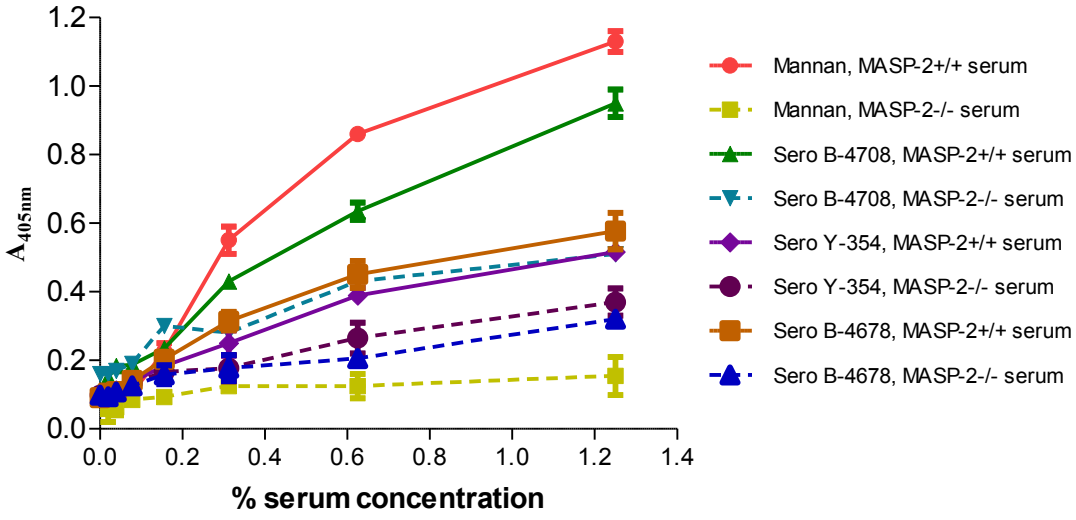

Figure 3.15: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

In contrast, some of the strains belonging to serogroup B meningococci (4664, 4675 and 4677) did not show any C3 deposition even in high concentration of MASP2$^{-/-}$ serum (see **Figure 3.16**).

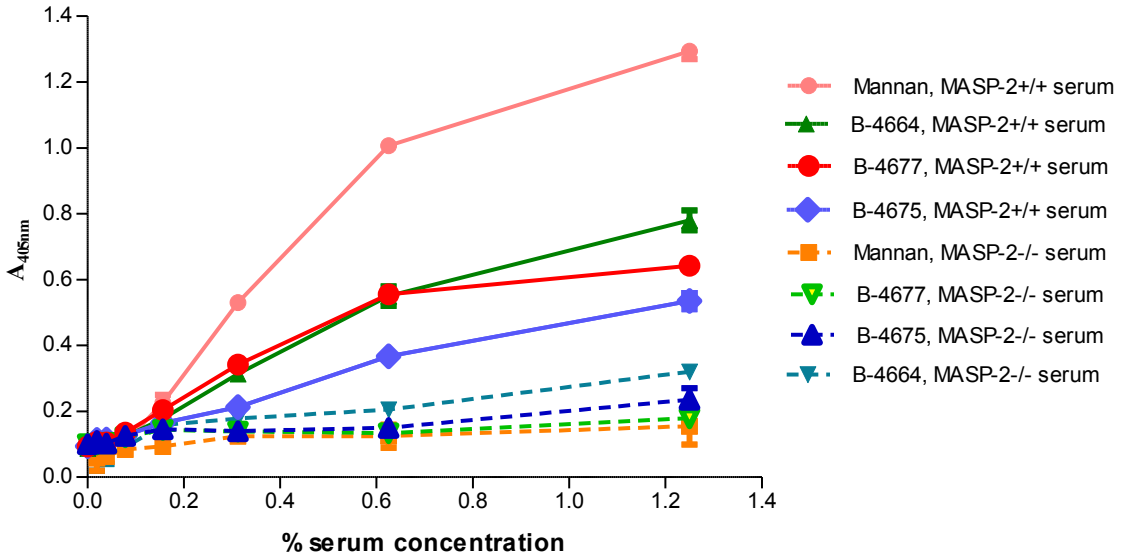

Figure 3.16: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

To further investigate the role of different complement pathways in C3 deposition and opsonisation of *N.meningitidis* serogroup A strain Z2491, C3 cleavage assay was carried out using sera/plasma from C1q$^{-/-}$, MASP1/3$^{-/-}$ and MASP2$^{-/-}$ mice (see **Figure 3.17**) under experiment conditions allowing C3 cleavage via either classical pathway or the lectin

pathway. Significant amount of C3 deposition was observed in sera of C1q$^{-/-}$ which can be mediated by lectin pathway. A very limited amount of C3 deposition on the surface of *N.meningitidis* serogroup A strain Z2491 using MASP1/3$^{-/-}$ mice was observed which suggests an important role of alternative pathway against meningococci as MASP-1/3$^{-/-}$ lack a functional alternative pathway activity (Takahashi *et al*., 2010). In contrast, very low C3 deposition was observed in sera from MASP-2$^{-/-}$ mice at high serum dilution suggesting an important role of lectin pathway in activating complement on the surface of *N.meningitidis.*

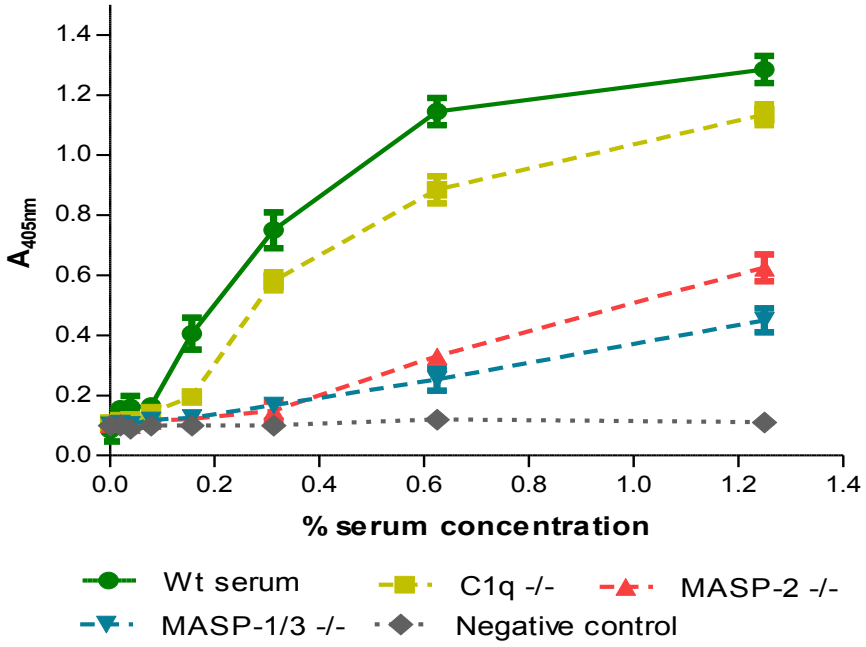

Figure 3.17: C3c deposition Assay on the surface of *N.meningitidis* serogroup A strain Z2491 using dilutions of wild-type, C1q deficient, MASP1/3 deficient, Ficolin-A deficient and MASP-2 deficient mice serum diluted in BBS. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

In order to assess C3 deposition on the surface of *N.meningitidis* also at high serum concentration where the alternative pathway is active, an alternative specific assay to measure C3 deposition on the surface of different strains of *N.meningitidis* in mouse (**Figure 3.18, Figure 3.19 & Figure 3.20**) and human sera (see **Figure 7.18** & **Figure 7.19 in appendices**) was carried out. Under permissive conditions for alternative pathway to occur, a high level of C3 deposition was observed on the surface of *N.meningitidis* serogroup B strain MC58. This suggests an important role of alternative pathway in driving complement on the surface of serogroup B meningococci. A moderate deposition of C3 was observed on the surface of *N.meningitidis* serogroup A strain Z2491 and *N.meningitidis* serogroup C strain 6414 (see **Figure 3.18)**.

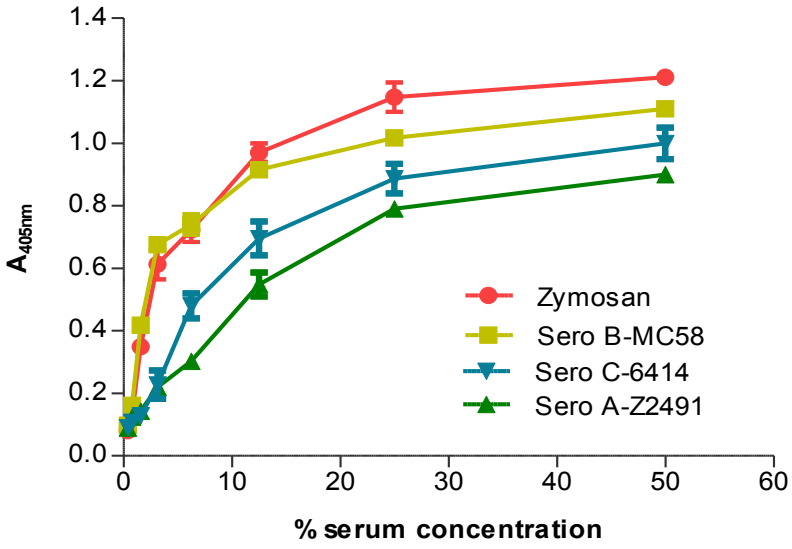

Figure 3.18: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of mouse serum under conditions that selectively allow activation via the alternative pathway. Zymosan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

Very high levels of alternative pathway-mediated C3 deposition was observed on the surface of *N.meningitidis* seorgroup B strain 4667 as illustrated in **Figure (3.19**). In contrast, a very limited amount of alternative pathway-mediated C3 deposition was observed on the surface of *N.meningitidis* serogroup B 4708 which suggests a degree of inter-species variability with respect to C3 deposition within the serogroups. *N.meningitidis* Serogroup A strain 3515 showed significant levels of C3 depostion on its surface (see **Figure 3.19**).

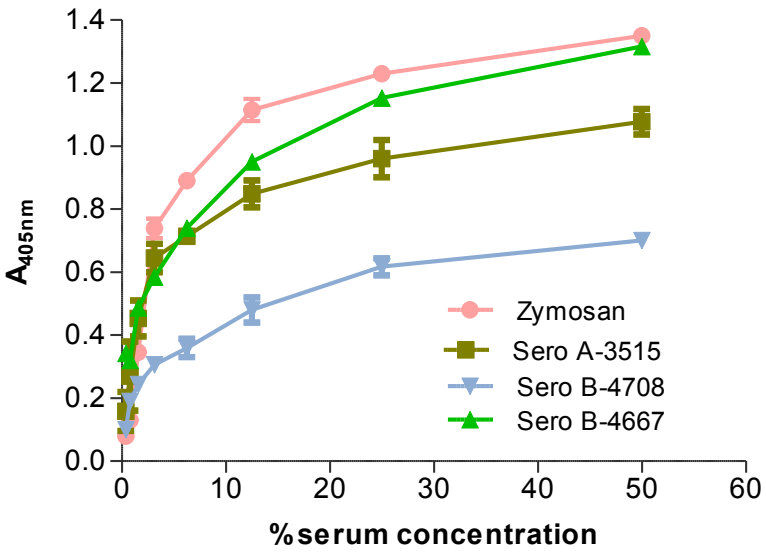

Figure 3.19: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of mouse serum under conditions that selectively allow activation via the alternative pathway. Zymosan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

Similarly, varying levels of alternative pathway-driven C3 deposition were observed on the surfaces of different strains of meningococci with *N.meningitidis* serogroup A strain 1534, *N.meningitidis* serogroup B strain H44/76, *N.meningitidis* serogroup C strain N86 and *N.meningitidis* serogroup Y strain 6433 showing high levels of C3 deposition, whereas, a moderate level of C3 deposition was observed on the surface of *N.meningitidis* serogroup A strain 1392 (see **Figure 3.20**).

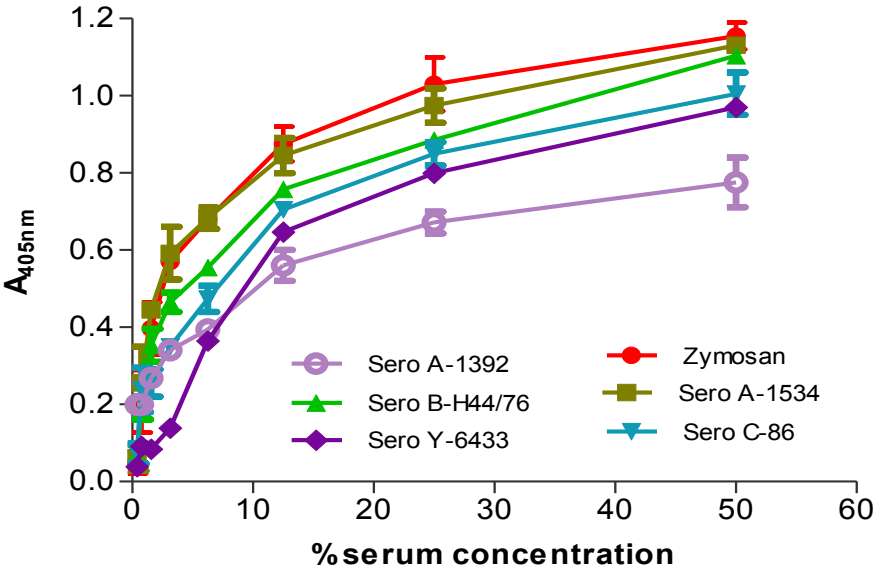

Figure 3.20: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of mouse serum under conditions that selectively allow activation via the alternative pathway. Zymosan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

Furthermore, a high level of C3 deposition was observed on the surface of different strains of *N.meningitidis* when using high concentrations of wild-type MASP-2 sufficient and

MASP-2 deficient mouse sera (see **Figure 3.21)**. Following the high levels of binding of the lectin pathway carbohydrate recognition molecules (MBL-A/MBL-C) to *N.meningitidis* (see **Figure 3.1 & 3.2**), a substantial amount of C3 deposition was observed when using MASP-2 deficient mouse serum at high serum concentrations. This observation is in support of the recently published hypothesis that the remaining lectin pathway enzymes MASP-1 and MASP-3 might play role in mediating C3 deposition on *N.meningitidis* via alternative pathway of complement system in MASP-2 deficient mouse serum (Iwaki *et al.,* 2011).

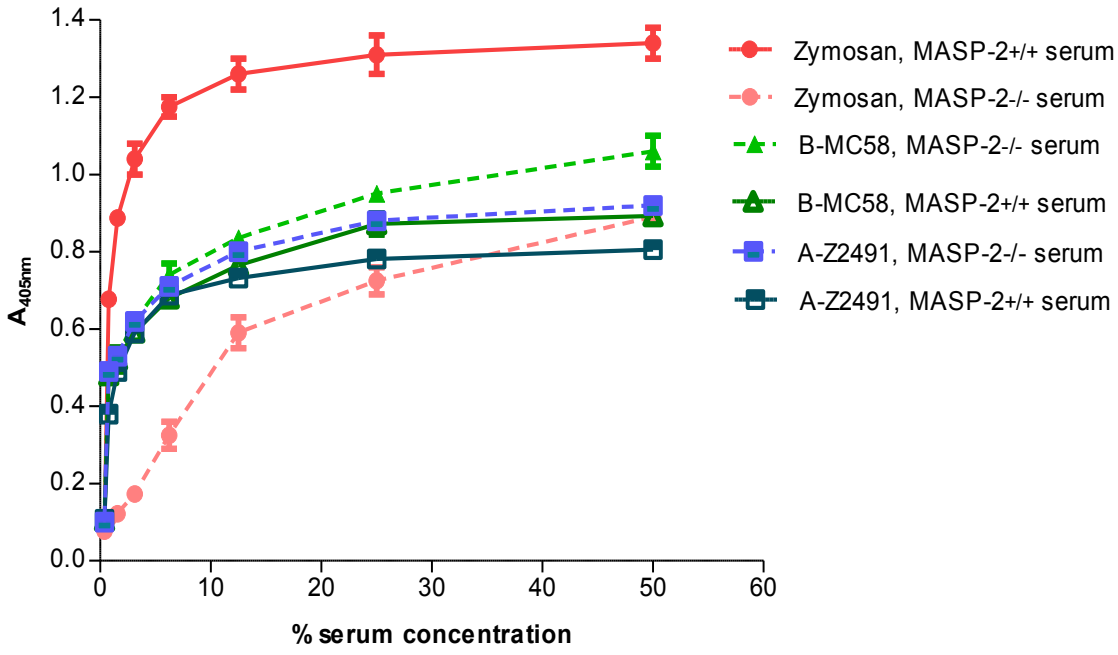

Figure 3.21: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of mouse serum under buffer conditions allowing complement activation through all three pathways. Zymosan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.3 C1q binding assay

In order to investigate a possible role of the classical activation pathway of complement in fighting meningococcal infections, the amount of C1q binding on the surface of different strains of *N.meningitidis* was measured. This further helped to investigate the mechanism responsible for the deposition of C3c on *N.meningitidis* in the absence of alternative pathway functional activity. A minor contribution of classical pathway towards the deposition of C3c on meningococci was observed in the assays described (see **Figure 3.22**).

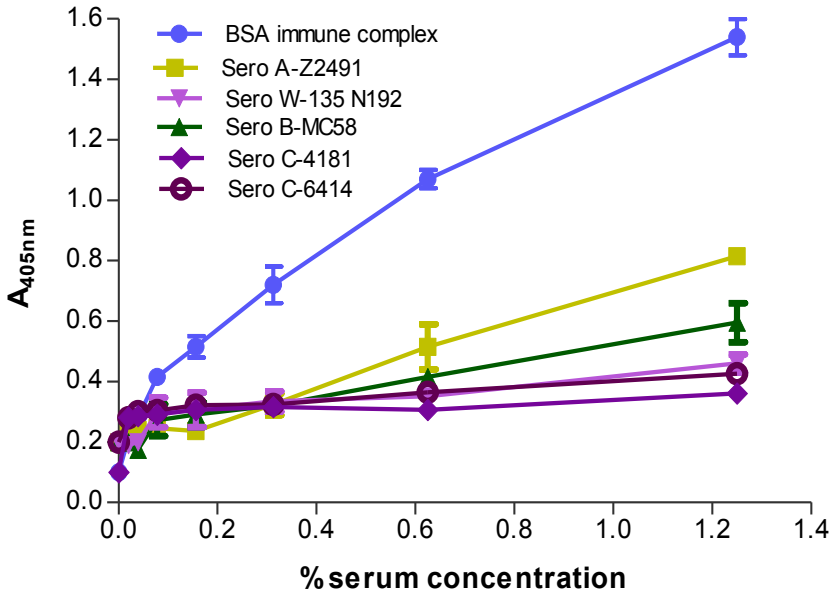

Figure 3.22: C1q deposition on the surface of different strains of *N.meningitidis* using dilutions of mouse serum. BSA anti-BSA coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.4 LP-dependant C4 deposition assays

The low amount of detectable C1q binding to the surface of *N.meningitidis* suggested that in the absence of specific antibodies binding to this pathogen, the classical pathway may contribute little towards C3 activation on the surface of *N.meningitidis*. I, then assessed to what degree MASP-2 dependent-lectin pathway activation complexes are mainly responsible for complement activation on the surface of *N.meningitidis* in the absence of antibodies. The amount of C4c depositions on the surface *N.meningitidis* via lectin pathway activation was quantified by incubating *N.meningitidis* in serum dilutions with high salt concentration (1M NaCl) (MBL binding buffer). The high salt concentration dissociates the C1 complex and also prevents activation of endogenous C4. These conditions render the classical activation pathway dysfunctional and allow complement activation only to occur via the lectin pathway. The results show that strains of *N.meningitidis* serogroup A (Z2491) showed higher lectin pathway mediated C4 deposition on their surface when compared to strains belonging to serogroup B (MC58) and serogroup C (6414) meningococci (see **Figure 3.23**).

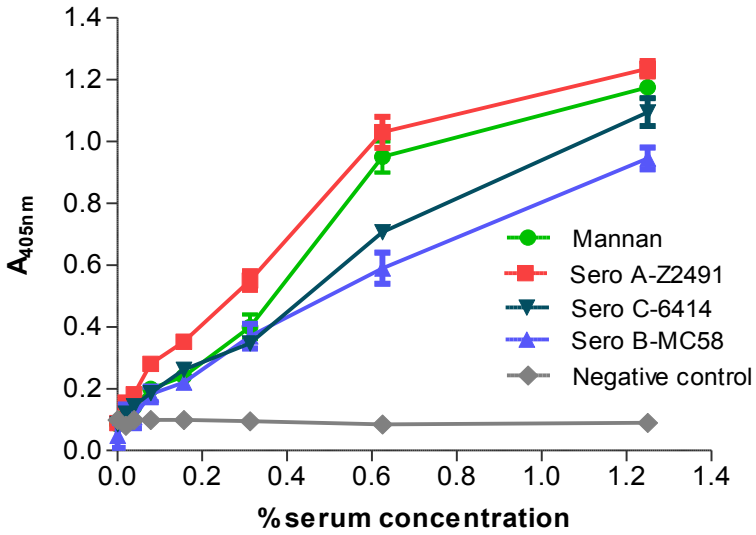

Figure 3.23: C4c deposition on the surface of different strains of *N.meningitidis* exposed to mouse serum diluted in high salt buffer i.e. conditions allowing only the lectin pathway specific activation. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

Meningococcal carrier strain belonging to serogroups A (1392) showed high levels of C4 binding when compared to meningococci serogroup C carrier strain (4181). Likewise, *N.meningitidis* serogroups B strain 8047 also showed a significant amount of C4 binding (see **Figure 3.24**).

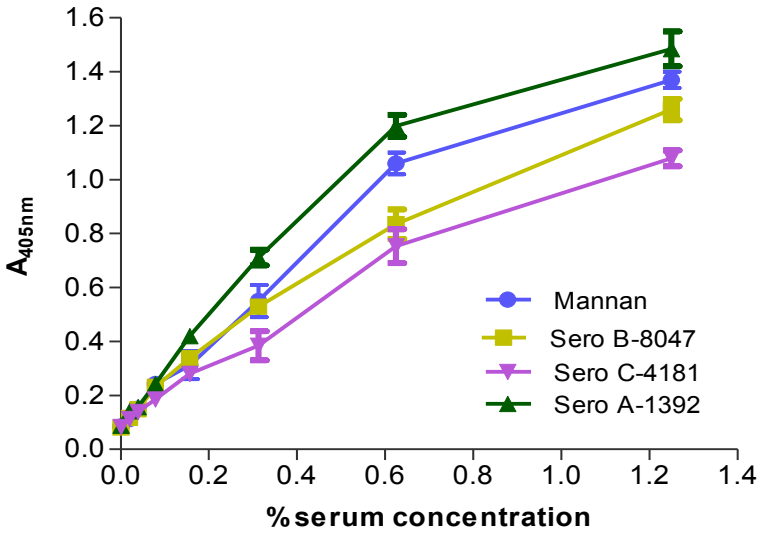

Figure 3.24: C4c deposition on the surface of different strains of *N.meningitidis* exposed to mouse serum diluted in high salt buffer i.e. conditions allowing only the lectin pathway specific activation. Mannan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiments for each indicated serum concentration and the relative OD values are expressed as means±SEM.

3.1.1.1.5 Characterisation of different strains of *N.meningtidis* used in this study

3.1.1.1.5.1 Growth characteristics of *N.meningitidis* serogroups A strain Z2491, serogroup B strain MC58 and serogroup C strain 6414

The growth of *N.meningitidis* serogroup A strain Z2491, serogroup B strain MC58 and serogroup C strain 6414 was compared *in vitro* in BHI serum broth supplemented with 5% Levanthal's base over a period of 8 h and the OD260 was measured throughout (see Materials and methods). All strains exhibited almost similar growth patterns (see **Figure 3.25**).

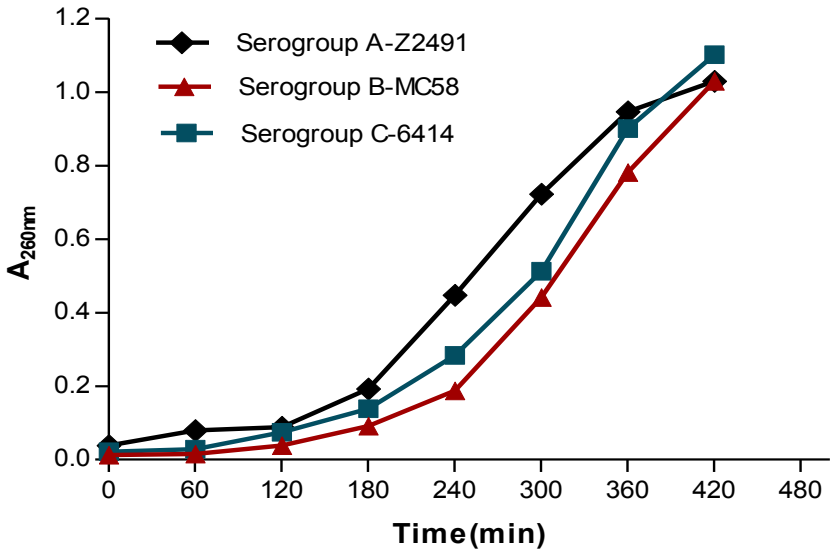

Figure 3.25: *N.meningitidis* serogroup A strain Z2491, serogroup B strain MC58 and serogroup C strain 6414 were grown in BHI serum broth supplemented with 5% Levanthal's base at 37°C and orbital shaking at 200 rpm . Growth was followed on an 8-h period and the OD260 was measured throughout.

3.1.1.1.6 Serum bactericidal assay

Sera from MASP-2 sufficient (WT) type and MASP-2 deficient mice were used to assess the serum bacteriolytic activity against *N.meningitidis* serogroup A strain Z2491, serogroup B strain MC58 and serogroup C strain 6414. *N.meningtidis* serogroup A strain Z2491 was extremely sensitive to serum lysis in MASP-2 deficient mice. The bactericidal activity of MASP-2 deficient serum was significantly higher than that of MASP-2 sufficient WT serum: while only half of the original CFUs of *N.meningtidis* serogroup A strain Z2491 were recovered in 30% (v/v) WT serum after 90 minutes incubation, the same serum concentration of MASP-2 deficient sera was able to kill all bacteria within 60 minutes (**Figure 3.28**).

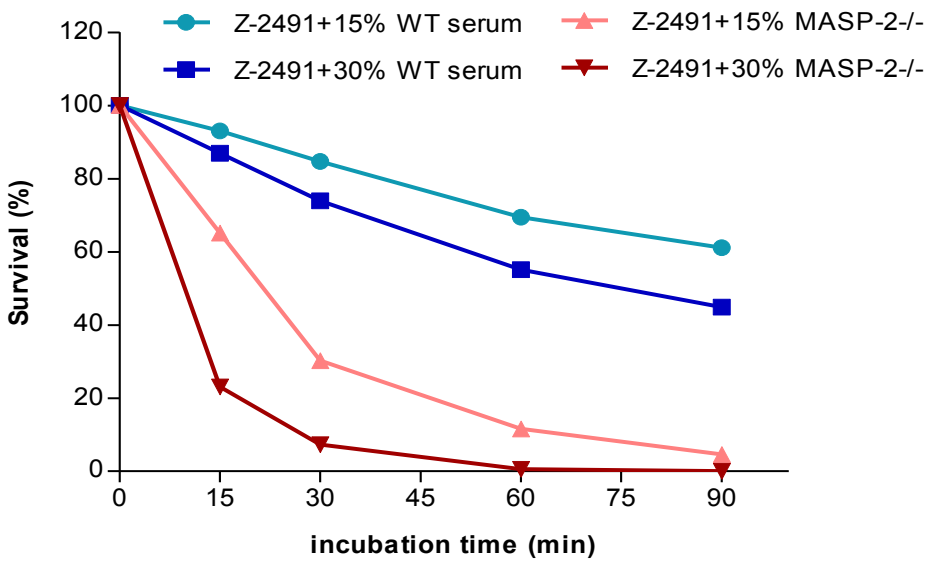

Figure 3.28: Determination of viable counts of *N.meningitidis* serogroup A strain Z2491 following incubation with 15% and 30% serum of either MASP-$2^{-/-}$ or MASP-$2^{+/+}$ mice. Survival was calculated as a percentage relative to survival in HIS at the corresponding time points.

Also at serum concentration of 15% (v/v), *N.meningitidis* serogroup B strain MC58 showed better survival in MASP-2 sufficient WT serum as compare to MASP-2 deficient serum (**Figure 3.29**). Again, sera from MASP-2 sufficient WT mice showed a significantly lower serum bactericidal activity (SBA) compared to MASP-2 deficient sera when incubated with *N.meningitidis* serogroup C strain 6414 (**Figure 3.30**).

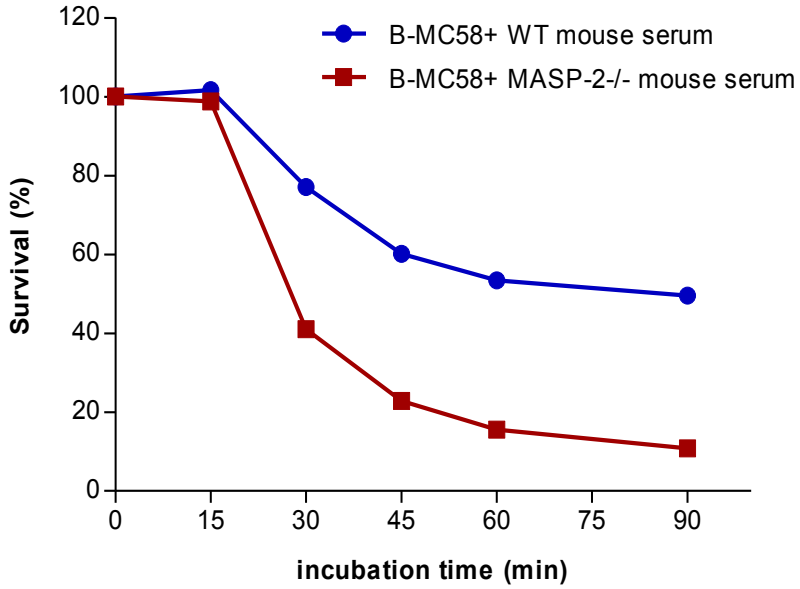

Figure 3.29: Determination of viable counts of *N.meningitidis* serogroup B strain MC58 following incubation with 15% serum of either MASP-$2^{-/-}$ or MASP-$2^{+/+}$ mice. Survival was calculated as a percentage relative to survival in HIS at the corresponding time points.

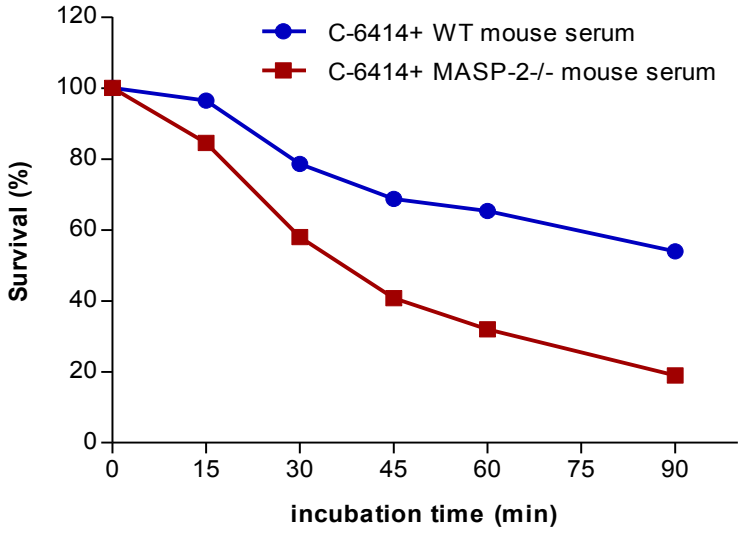

Figure 3.30: Determination of viable counts of *N.meningitidis* serogroup C strain 6414 following incubation with 15% serum of either MASP-$2^{-/-}$ or MASP-$2^{+/+}$ mice. Survival was calculated as a percentage relative to survival in HIS at the corresponding time points.

Following MBL-A/C binding (see **Figures 3.1-3**), a very high level of C3 deposition was observed on different meningococcal strains (see **Figures 3.11**) which was dramatically reduced when MASP-2 deficient serum was used in high dilutions allowing only the classical pathway and the lectin pathway route to work (see **Figures 3.14**). In contrast, a very high level of alternative pathway-mediated C3 deposition was observed in MASP-2 deficient serum when 10-50% serum concentration was used (see **Figure 3.18**). This is in line with the high serum bactericidal activity seen in these sera (see **Figures 3.28, 3.29 & 3.30**) suggesting that the alternative pathway of complement activation is compensating for the absence of the lectin pathway functional activity in MASP-2 deficient mice. To analyse this observation further, serum bactericidal activity was also tested against *N.meningitidis* serogroup B strain MC58 using various complement deficient mice sera. MASP-1/3 deficient serum showed no killing of meningococci whatsoever when compared to WT serum (see **Figure 3.31**) which further confirms the important role of the alternative complement pathway against *N.meningitidis* as MASP-1/3 deficient mice were previously shown to lack a fully functional alternative pathway activity (Takahashi *et al*., 2010). Similar impairment of bactericidal activity was observed in MBL-A/C deficient serum (see **Figure 3.31**). Recent work by Teizo Fujita's team has suggested that MASP-3 complexed with lectin pathway activation complexes is able to induce alternative activation pathway of complement system by cleaving C3b bound factor B directly on the pathogen surface and thus forming an alternative pathway C3 convertase (Iwaki *et al.,* 2011). The results from current study strongly support this hypothesis and suggest that MASP-1/ MASP-3 along with MBL are important components in driving alternative pathway activation on the surface of meningococci.

The essential contribution of the alternative activation pathway is underlined by the observation shown in Figure (**31**) demonstrating that enhanced killing was achieved when adding recombinant properdin, an essential positive regulator of alternative pathway activation, to WT serum. Statistical analysis revealed a highly significant difference ($P<0.01$) between SBA of WT serum added with different concentrations of recombinant properdin (i.e. 5 µg/ml and 10 µg/ml) when compared to SBA of WT serum or MASP-2$^{-/-}$ serum at time points 60 minutes and 90 minutes post incubation (see **Figure 3.31**).

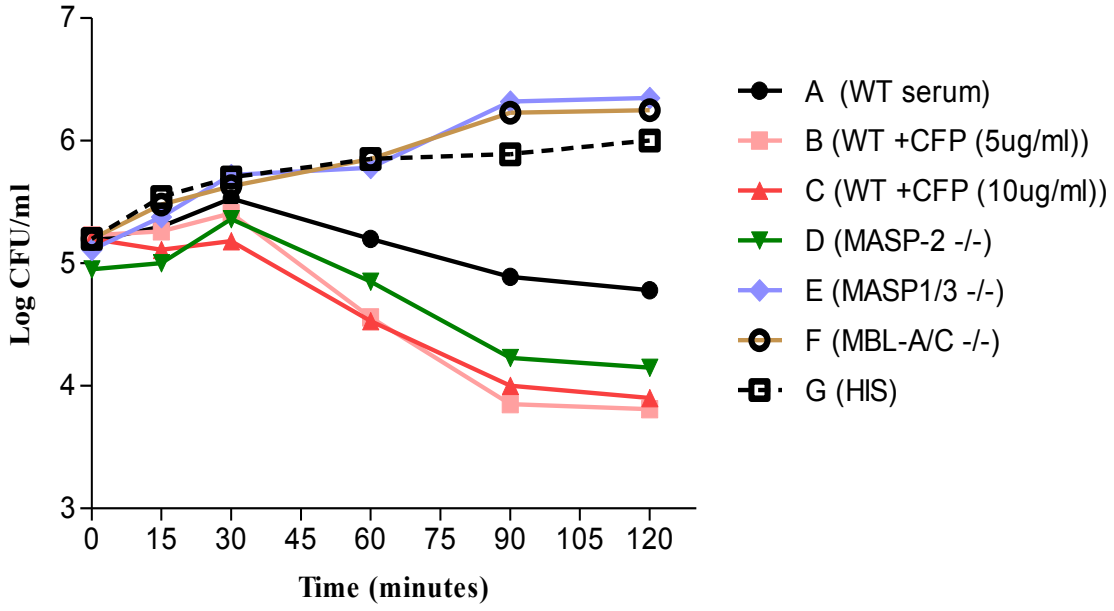

Figure 3.31: Determination of viable count of *N.meningitidis* serogroup B strain MC58 following incubation with different mouse sera (20% concentration) at 37°C with shaking. Samples were taken 0, 15, 30, 45, 60, 90 and 120 mins interval, plated out and viable counts were calculated. Heat inactivated wild-type serum was used as a negative control.

Table 3.1: Statistical significance between SBA of different sera following incubation with *N.meningitidis* serogroup B strain MC58 using the Student's t-test. ***=p<0.0001

Student's t-test (time point 60min)			
	Mean Diff.	Significant? P < 0.05?	P value Summary
A vs B	0.6477	Yes	***
A vs C	0.6725	Yes	***
A vs D	0.3596	Yes	***
B vs C	0.02483	Yes	** (0.0081)

Table 3.2: Statistical significance assessed between SBA of different sera following incubation with *N.meningitidis* serogroup B strain MC58 using the Student's t-test.

Student's t-test (time point 90min)			
	Mean Diff.	Significant? P < 0.05?	P value Summary
A vs B	1.15	Yes	** (0.0076)
A vs C	0.9331	Yes	** (0.0026)
A vs D	0.6741	Yes	*** (0.001)
B vs C	-0.2166	No	ns (0.0466)

Serum bactericidal activities of different complement deficient human sera were also tested against *N.meningitidis* serogroup B strain MC58. Consistent with the findings for mouse sera, human MASP-3 and MBL deficient sera also showed no serum killing of meningococci whatsoever using serum concentration of 20% when compared to normal human serum (NHS) (see **Figure 3.32**). These findings strongly suggest an important role of the lectin pathway recognition molecule MBL and of lectin pathway serine protease MASP-3 in driving the alternative pathway on the surface of *N.meningitidis*. These novel roles of lectin pathway components MBL and MASP-3 are independent of MASP-2 mediated lectin pathway activation. In addition, a significantly higher SBA was observed following incubation of *N.meningitidis* serogroup B strain MC58 with normal human serum added with recombinant properdin when compared to normal human serum alone at the time point 60 minutes post incubation (see **Figure 3.32**).

As MASP-3 is not an autoactivating enzyme like MASP-1 or MASP-2, it is likely that MASP-3 requires to be cleaved by MASP-1 to be converted into its enzymatically active form (Iwaki *et al*., 2011). It is hypothesised that a MASP-1 bearing lectin pathway activation complex has to bind to the pathogen surface in close proximity to a MASP-3

bearing lectin pathway recognition complex to allow MASP-1 to activate MASP-3. If this is the molecular sequence of activation events of this novel route of the lectin pathway-mediated initiation of alternative pathway-mediated complement activation on the pathogen surface, then the likelihood of MASP-1 bearing complexes to bind in close proximity to MASP-3 bearing complexes would significantly be higher in MASP-2 deficient sera and explain why these sera show higher bactericidal activity than wild-type serum.

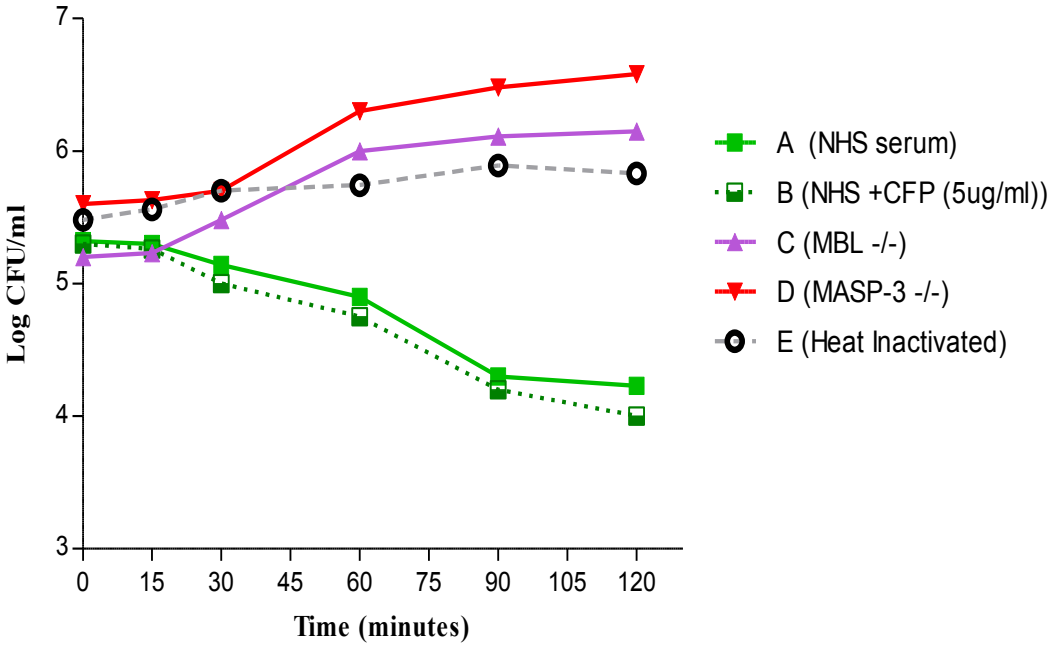

Figure 3.32: Determination of viable count of *N.meningitidis* serogroup B strain MC58 following incubation with different complement deficient human sera (20% concentration) at 37°C with shaking. Samples were taken at different time points, plated out and viable counts were calculated. Heat inactivated human serum was used as a negative control.

Table 3.3: Statistical significance assessed between SBA of different sera following incubation with *N.meningitidis* serogroup B strain MC58 using the Student's t-test. ***=p<0.0001

Student's t-test (time point 60 min)			
	Mean Diff.	Significant? P < 0.05?	P value Summary
A vs B	0.1552	Yes	*** (0.0005)
A vs C	-1.1489	Yes	***
A vs D	-1.323	Yes	*** (0.0005)
B vs C	-1.303	Yes	***

Table 3.4: Statistical significance between SBA of different sera following incubation with *N.meningitidis* serogroup B strain MC58 using the *Student's t-test.* ***=p<0.0001

Student's t-test (time point 90 min)			
	Mean Diff.	Significant? P < 0.05?	P value Summary
A vs B	0.1095	ns	0.407
A vs C	-1.822	Yes	***
A vs D	-2.185	Yes	***
B vs C	-1.932	Yes	***

Chapter 4. Results: *In vivo* infection study

4.1 Animal models of meningococcal infection

A complete understanding of meningococcal disease requires an animal model that mimics the natural progression of disease in the human host. Animal models of disease are helpful in studying the impact of host–pathogen relationship on whole tissues during the course of disease. Besides, the interactions between both humoral and cellular immune responses following infection are better studied in an animal model. The virulence factors, iron acquisition and colonization mechanisms of *N.meningitidis* are highly adapted to humans and have been difficult to study in animal models. Humans are the only natural hosts for *N. meningitidis* (Gorringe *et al*., 2005). Meningococcal surface structures also show exquisite specificity for interaction with human receptors (Crosa, 1984). These include: the interaction of *N. meningitidis* Opa and Opc with human CEACAM1 receptors: pili with human CD46 receptors, and transferin-binding proteins (i.e. TbpA and TbpB) of *N.meningitidis* which help in iron acquisition only through human transferrin (Holbein, 1980; Mickelsen and Sparling**,** 1981; Zhu *et al.*, 2005).

4.1.1 Animals models of meningococcal infection

Various experimental animal models using monkeys, mice, rats, or rabbits have been evaluated in different studies. There are certain limitations associated with these models such as the use of either infant animals or supplementation with exogenous iron since

(Holbein, 1980). Presently, two different routes of infection have been tested in these models which include the intranasal (i.n.) and intraperitoneal (i.p.) infection route (Holbein, 1980; Mackinnon *et al*., 1993).

These models closely mimic the natural progression of disease, i.e. from colonization of nasopharynx, entrance and multiplication into blood, and finally movement to the subarachnoid space of brain. Intranasal infection of mouse in the presence of iron dextran leads to consistent colonization of nasal tissue followed by blood infection (Gorringe *et al*., 2005). However, animals infected via i.n. route develop lung infection before sepsis which is, in contrast to infection in human where meningococcal disease is not presented with pneumonia. Besides, the i.n. models require the use of infant rodents with premature immune system which also limit the understanding of disease by this model (Mackinnon *et al*., 1993; Zhu *et al*., 2005). It is widely accepted that the intraperitoneal (i.p.) challenge of mouse model does not represent the natural pathogenesis of the disease (*N. meningitidis* is transmitted in human via aerosolic route), however, it does model the overwhelming septicaemia that is characteristic of invasive meningococcal disease (Gorringe *et al*., 2005). Intraperitoneal model can be used to recover a large numbers of bacteria from the blood and different organs of infected animals (Hong *et al.*, 2008).

One of the very first aims of current study was to develop a murine model of meningococcal infection in 8-12 weeks old C57BL/6 mice following intraperitoneal route. Preliminary experiments using different doses of *N. meningitidis* serogroup A-Z2491 were unsuccessful in recovering viable bacteria from the blood, which can be explained by two

possibilities. First, the usage of higher infective dose to induce systemic meningococcal infection was associated with challenging the mice with higher doses of bacterial OMVs (which include LOS and outer membrane proteins) which induce a strong inflammatory response leading to death of mice at much earlier time points of infection experiments (6-12 hours post infection), therefore, shortening the infection window period. A low infective dose, on the other hand, was cleared by the innate immune system of the mature mice (8-12 weeks old). The adult murine model of meningococcal infection was subsequently tested with different i.p. doses of iron dextran. It was observed that iron supplementation in the form of iron dextran enhanced the bacterimia in adult mice when mice were pretreated with i.p. injection of iron dextran at a dose of 400 mg/kg body weight followed by a second dose (400 mg/kg body weight) co-administered with the infection dose. The model provided valuable insight into understanding the different events of meningococcal disease.

4.1.2 Genotyping of MASP-2$^{-/-}$ mice

MASP-2 deficient mice were generated and housed in the University of Leicester Biomedical Services facility in a specific pyrogen free environment.

A multiplex PCR protocol was used to determine the genotype of the breeding pairs and their off springs. Three oligonucleotide primers specific for the murine *MASP-2* gene were used for this purpose. The targeted allele of the murine *MASP-2* gene was disrupted by insertion of a neomycin cassette in the targeting construct. This neomycin cassette is used

to detect the disrupted allele with the reverse primer to amplify the targeted allele being chosen to hybridise to this neomycin cassette (Schwaeble *et al*., 2011). The second reverse primer (to detect the wild-type allele) is designed to hybridize to the genomic sequences of wild-type gene in the region replaced by the neomycin cassette in the disrupted allele. A band of 750 bp is amplified by the forward and reverse primer in the absence of a neomycin cassette, identifying the wild-type phenotype. A single band of 750 bp is, therefore, an indication of a mouse homozygous for the wild-type allele ($MASP2^{+/+}$). The targeted genome carrying a neomycin cassette is amplified by the forward and neomycin specific reverse primer. A band of approximately 500bp shows a mouse genome homozygous for the disrupted allele ($MASP\text{-}2^{-/-}$). DNA from heterozygous mice is identified through two bands of 750 and 500bp (see **Figure 4.1**).

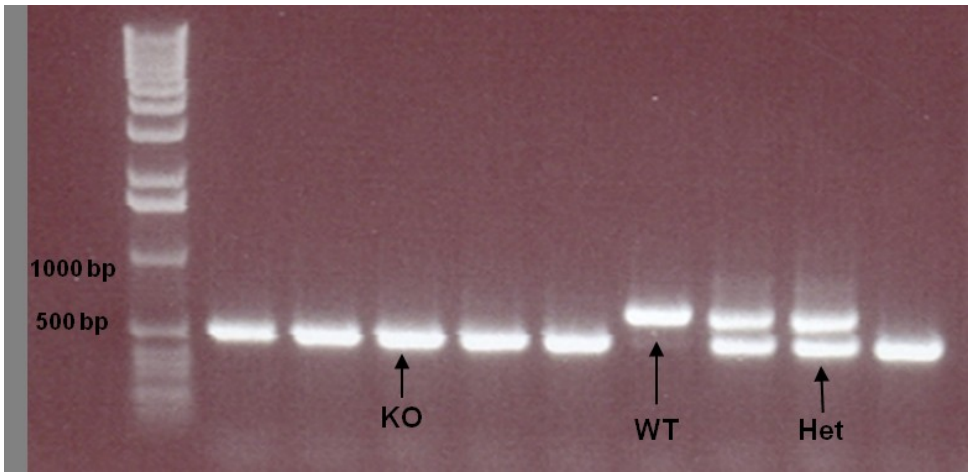

Figure 4.1: Genotyping results for the MASP-2 targeted mouse line identifying the wild-type and the targeted allele. $MASP\text{-}2^{+/+}$ wild-type and $MASP\text{-}2^{-/-}$ mice show PCR amplified products of approximately 750 bp and 500 bp respectively, while heterozygous mice give two bands at 750 bp and 500 bp. 1 Kbp DNA ladder was run along with the samples as a molecular weight marker in a 1% agarose TAE-gel.

4.1.1.3 Experimental murine model of *N.meningitidis*

Strains belonging to three different serogroups of *N. meningitidis* were tested in a mouse model of meningococcal infection which included; *N. meningitidis* serogroup A-Z2491, *N. meningitidis serogroup* B-MC58 and *N. meningitidis* serogroup C-6414.

4.1.2 Experimental murine model of *N.meningitidis* serogroup A strain Z2491 infection

The optimal infective dose of *N.meningitidis* serogroup A strain Z2491 for infection experiments was calculated by challenging group of mice ranging between 8 to 10 weeks of age (all on pure C57BL/6 background) following the intra-peritoneal route of infection. The infective dose was adding iron to a final concentration of 400 mg/kg body weight (**Figure 4.2**).

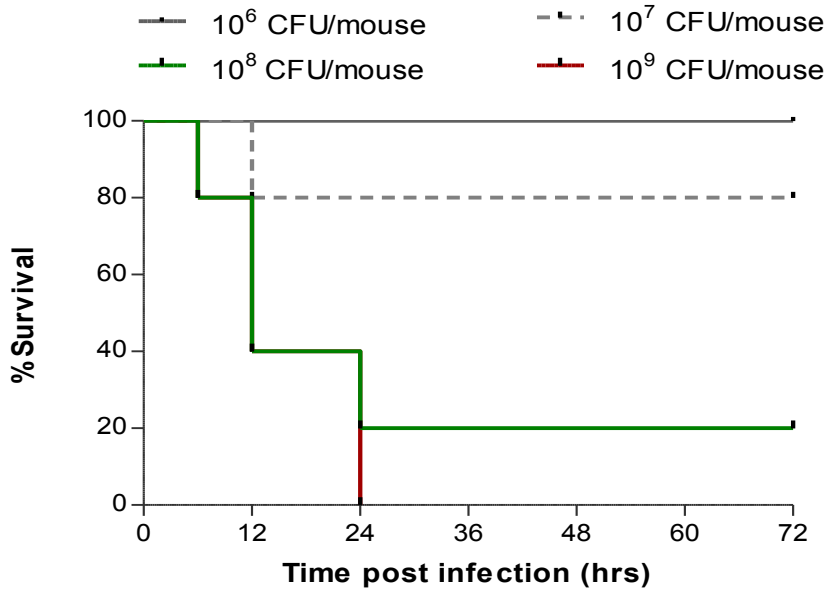

Figure 4.2: Comparison of C57BL/6 wild-type mice survival with different doses of *N.meningitidis* serogroup A strain Z2491. The infective dose was adding iron to a final concentration of 400 mg/kg body weight. For evaluation of the optimal lethal dose, bacteria were injected in four different doses. Experiments included at least five mice per group.

4.1.2.1 Survival of MASP-2$^{+/+}$ wild-type and MASP-2$^{-/-}$ mice (on C57BL/6 backgound) following infection with *N.meningitidis* serogroup A strain Z2491

To assess the role of lectin pathway activation towards the host immune response to meningococcal infection, groups of MASP-2 deficient and MASP-2 sufficient mice were challenged by intraperitoneal infection with a high dose of *N.meningitidis* serogroup A strain Z2491 ($5x10^7$ CFU/mouse). The time-course of infection was monitored and then the survival of MASP-2 deficient mice was compared with that of MASP-2 sufficient wild-type mice. In survival experiment, at 12 hrs post infection, 10% of MASP-2 sufficient mice showed severe signs of disease and had to be euthanised, while none of the MASP-2

deficient mice showed severe disease signs (see **Figure 4.4**). At 24 hours post-infection, 40% of MASP-2 sufficient mice progressed to terminal disease signs and had to be culled adding up to a total mortality rate of 50% for the group of wild-type mice (see **Figure 4.3**). None of MASP-2 deficient mice had progressed to a lethargic end stage despite showing moderate signs of infection. The remaining 50% of MASP-2 sufficient mice and all of the MASP-2 deficient mice recovered and survived the total observation period of 72 hours of the experiment.

Using the Log-rank (Mantel-Cox) statistical analysis test revealed a significant difference in the survival between the MASP-2 deficient group and MASP-2 sufficient group, with MASP-2 deficient mice being more resistant to meningococcal infection as illustrated in Figure (**4.3**). The scoring results of disease severity signs (**Figure 4.4**) also show that MASP-2 deficient group showed significantly less severe scores of disease severity through out the course of infection when compared to the disease severity scores of MASP-2 sufficient group.

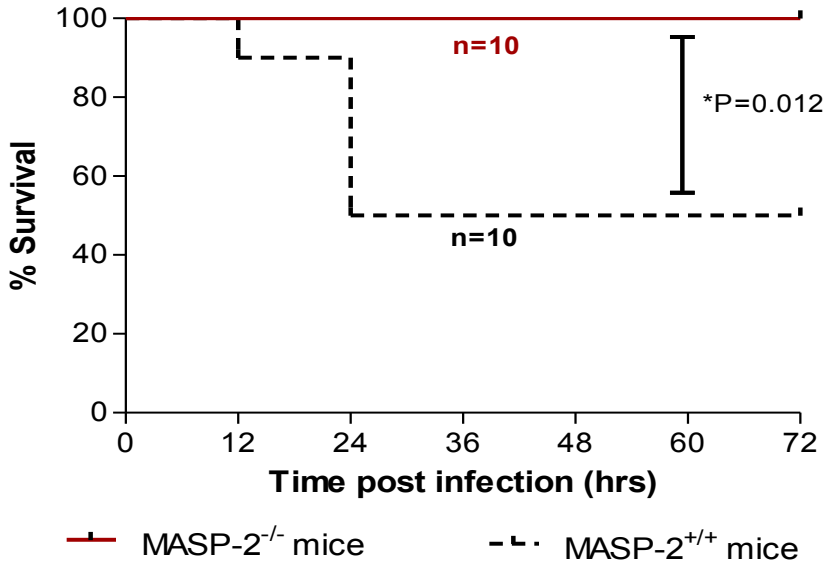

Figure 4.3: Survival of MASP-$2^{+/+}$ wild-type and MASP-$2^{-/-}$ mice (on C57BL/6 backgound) following i.p. injection with a high dose ($5x10^7$ CFU/mouse) of *N.meningitidis* serogroup A strain Z2491. The infective dose was adding iron to a final concentration of 400 mg/kg body weight.

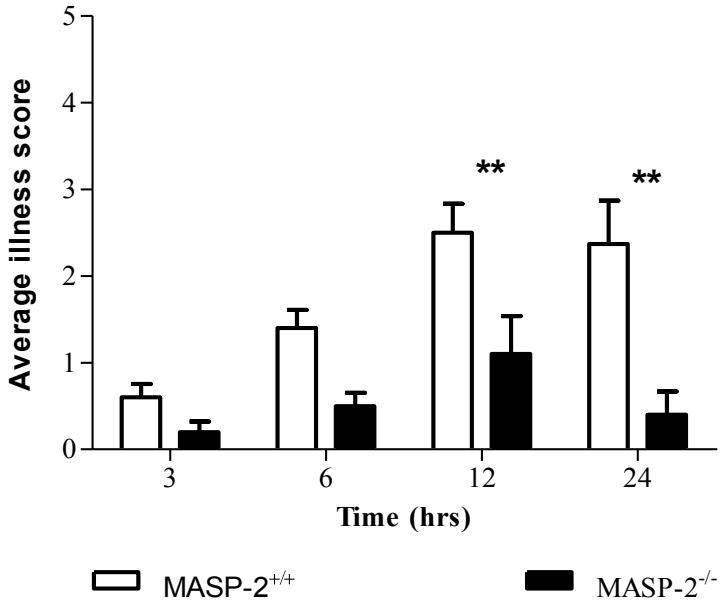

Figure 4.4: Average illness score of MASP-$2^{+/+}$ wild-type and MASP-$2^{-/-}$ mice following i.p. injection with a high dose ($5x10^7$ CFU/mouse) of *N.meningitidis* serogroup A strain Z2491. The results are expressed as means±SEM. **p=0.0074, **p=0.0022.

4.1.2.2 Viable bacterial load of *N.meningitidis* serogroup A strain Z2491 in blood and different organs of infected mice

Two groups of age-matched female MASP-2 deficient and MASP-2 sufficient mice were challenged with a sublethal dose of $4x10^7$ CFU/mouse of *N.meningitidis* serogroup A strain Z2491 via the intraperitoneal infection route. Mice were culled at time points 0, 3, 6, 12, 24 36 and 48 hours post-infection to assess the viable bacterial load in blood and different organs. MASP-2 sufficient mice showed a significantly higher bacterial load in blood compared to MASP-2 deficient mice (**Figure 4.5**). Likewise, MASP-2 sufficient mice developed the symptoms of severe infection at 12 hours post-infection while the MASP-2 deficient mice recovered from the clinical symptoms of infection at this time. At 24 hrs post-infection, the MASP-2 deficient mice started to clear the infection from blood while the majority of MASP-2 sufficient mice showed signs of terminal disease due to high bacteremia and therefore, had to be euthanized in line with the Home Office regulations.

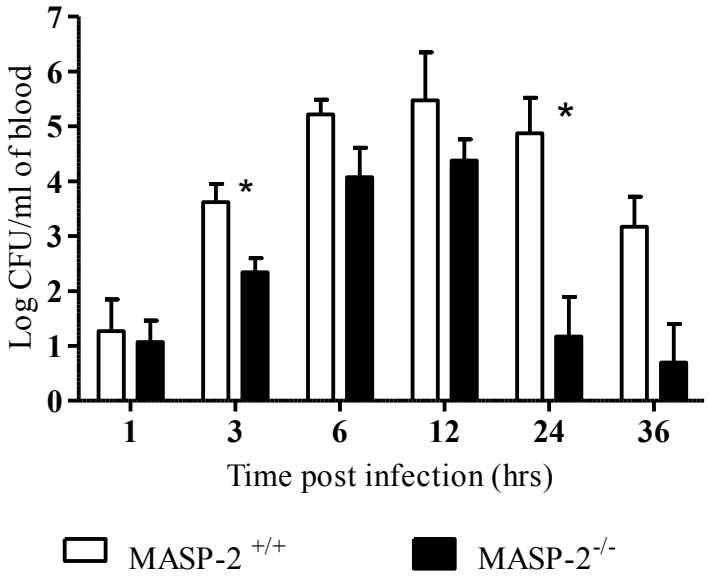

Figure 4.5: Time course showing the bacterial load of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in blood of MASP-2 deficient and MASP-2 sufficient mice at time points 1, 3, 6, 12, 24 and 36 hours post infection. The results are expressed as means±SEM. n=3 at time 1, 3, 6, 12, 24 and 36 hours for both mice groups.

* p=<0.05

Monitoring the viable bacterial burdens in spleen tissues of mice post-infection revealed significantly higher viable bacterial loads in spleens of MASP-2 sufficient mice when compared to MASP-2 deficient mice. It was noticed the MASP-2 sufficient mice showed a decrease in the bacterial counts in spleen tissues 12 hours post-infection, followed by a rapid increase at 24 hours post-infection (see **Figure 4.6**). In contrast, bacterial counts in spleens of the MASP-2 deficient mice decreased continuously until the end of the observation period.

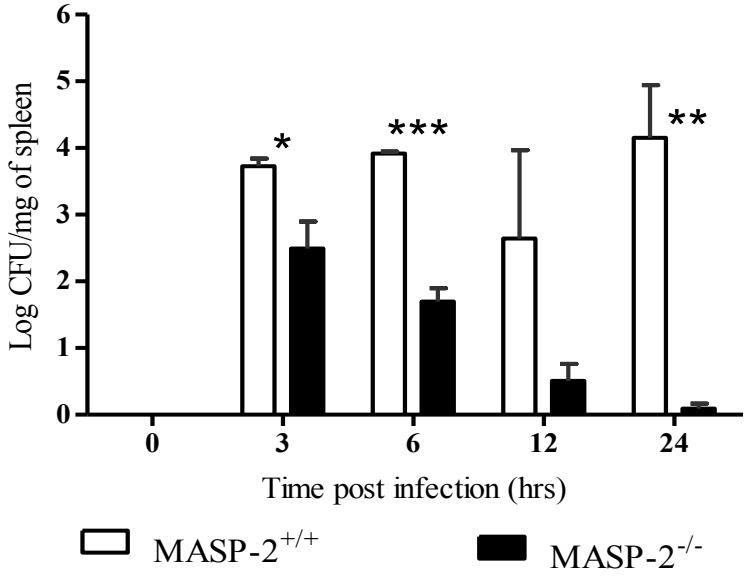

Figure 4.6: Time course showing the bacterial load of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in spleen tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 3, 6, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, 3, 6, 12 and 24 hours for both mice groups.

$p<0.05$, ** $p=0.0069$, *** $p<0.0001$

The viable bacterial burdens in livers of MASP-2 sufficient mice were significantly increased with a 2.4 and 2.34 log difference following 12 and 24 hours post-infection respectively in comparison with MASP-2 deficient mice livers as illustrated in **Figure (4.7)**.

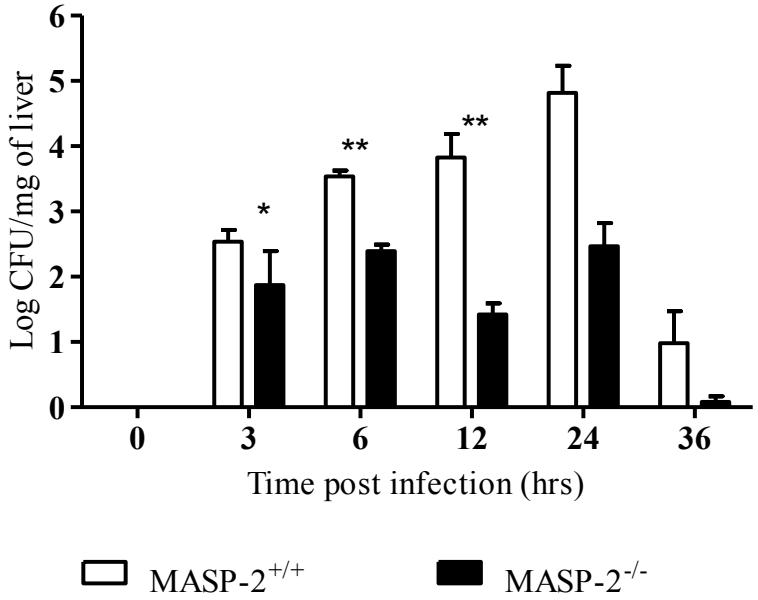

Figure 4.7: Time course showing the bacterial load of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in liver tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 3, 6, 12, 24 and 36 hours post-infection. The results are expressed as means±SEM. n=3 at time 0, 3, 6, 12, 24 and 36 hours for both mice groups. *$p<0.05$ and **$p<0.003$

In kidneys of infected MASP-2 deficient type mice, the bacterial counts reached to a peak level at 6 hours post-infection after which there was a significant drop indicating bacterial clearance (see **Figure 4.8**). MASP-2 sufficient wild-type mice, on the other hand, were compromised in clearing *N.meningitidis* from kidney tissues. The viable meningococcal burdens of wild-type mice were significantly higher (2.1 and 2.6 log difference after 12 and 24 hours post-infection respectively) when compared to MASP-2 deficient mice.

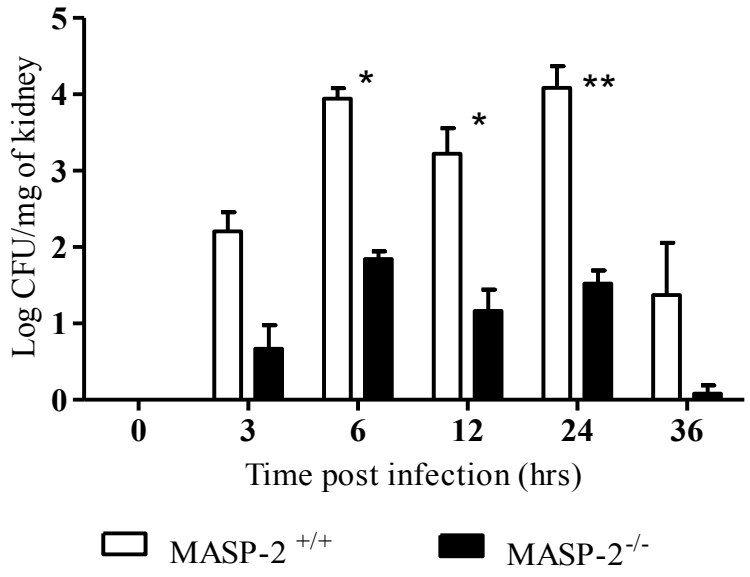

Figure 4.8: Time course showing the bacterial load of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in kidney tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 3, 6, 12, 24 and 36 hours post infection. The results are expressed as means±SEM. n=3 at time 0, 3, 6, 12, 24 and 36 hours for both mice groups. * $p<0.05$ and **$p=0.006$

The viable bacterial burden in the brain tissues of mice post-infection revealed a slow increase in viable bacterial load in both MASP-2 sufficient and the MASP-2 deficient group starting from 6 to 24 hours post-infection. MASP-2 sufficient wild-type mice showed a significantly higher bacterial burden in brain tissues when compared to MASP-2 deficient at time points 12 and 24 hours post-infection (see **Figure 4.9**).

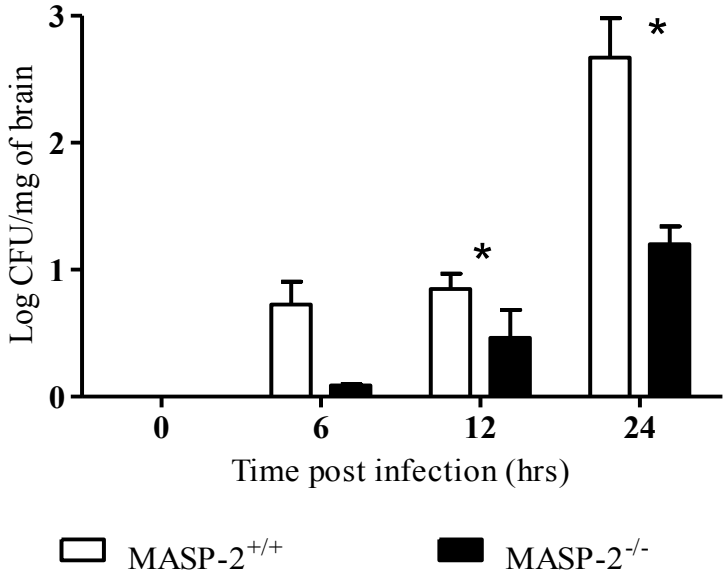

Figure 4.9: Time course showing the bacterial load of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in brain tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 6, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, 6, 12 and 24 hours for both mice groups. * $p<0.05$

Viable bacterial counts in lung tissue of the infected mice started to appear very late during the course of meningococcal infection experiments. *N.meningitidis* started to appear 12 hours post-infection in both mouse groups and the after reaching to a peak value at 24 hours post-infection, the viable meningococcal count started to decline slowly at 36 and 48 hours post-infection. MASP-2 sufficient wild-type mice showed a significantly higher bacterial load in lung tissue compared to MASP-2 deficient mice wild-type mice as illustrated in Figure (**4.10**).

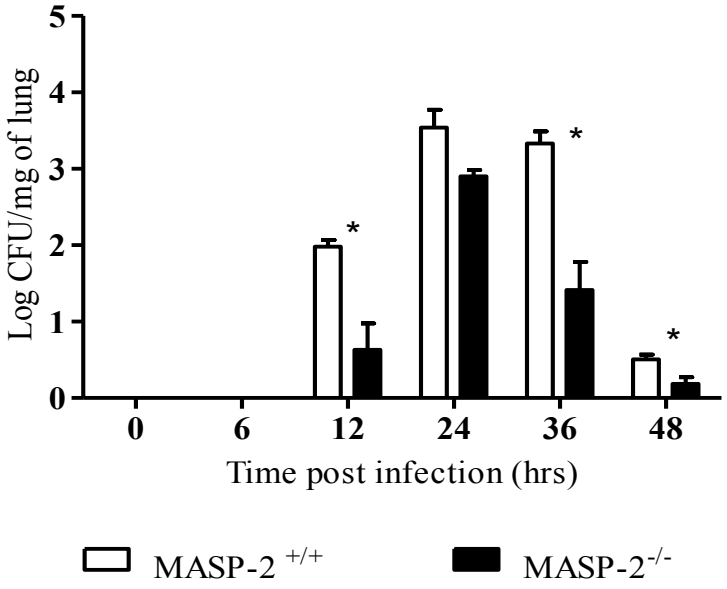

Figure 4.10: Time course showing the bacterial burden of *N.meningitidis* serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse in lung tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 6, 12, 24, 36 and 48 hours post infection. The results are expressed as means±SEM. n=3 at time 0, 6, 12, 24, 36 and 48 hours for both mice groups. * $p<0.05$

4.1.2.3 mRNA expression profiles in mouse tissues post infection with *N.meningitidis* serogroup A strain Z2491

4.1.2.3.1 mRNA expression for complement components

The mRNA expression profiles of different complement genes in different organs including spleens, kidneys, livers and brains were determined in $MASP2^{+/+}$ and $MASP2^{-/-}$ mice at time points 0, 12 and 24 hours after intraperitoneal infection with *N.meningitidis* serogroup A strain Z2491 using qRT-PCR.

In spleen tissues (see **Figure 4.11**), mRNA expression levels of C3, C1q and properdin were significantly up-regulated in MASP-2 deficient mice when compared to the MASP-2 sufficient control group at 24 hours post infection. In contrast, the C4 mRNA expression levels were significantly down-regulated in MASP-2 deficient mice compared to the MASP-2 sufficient control group at 24 hours post infection. The mRNA expression profiles for MBL-A and MBL-C were nearly identically up-regulated in both MASP-2 sufficient and MASP-2 deficient at time points 12 and 24 hours post infection. The mRNA expression profiles of complement genes in liver and kidney tissues (see **Figure 4.12 and Figure 4.13**) were more or less similar to those of spleens, where C3 and properdin mRNA expression were significantly up-regulated in MASP-2 deficient mice compared to MASP-2 sufficient mice at 24 hours post infection. The C4 mRNA expression levels were down-regulated in MASP-2 deficient mice whereas no significant differences in MBL-A and MBL-C mRNA expression were observed between MASP-2 deficient mice compared to MASP-2 sufficient mice at 24 hours post infection.

In brains tissues (see **Figure 4.14)**, the mRNA expression of C1q, properdin and C3 were significantly higher in MASP-2 deficient mice compared to MASP-2 sufficient control group at 24 hours post infection. The C4 and MBL-C expression profiles were almost similar in both MASP-2 sufficient and MASP-2 deficient mice, while MBL-A mRNA expression levels were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 12 hours post infection.

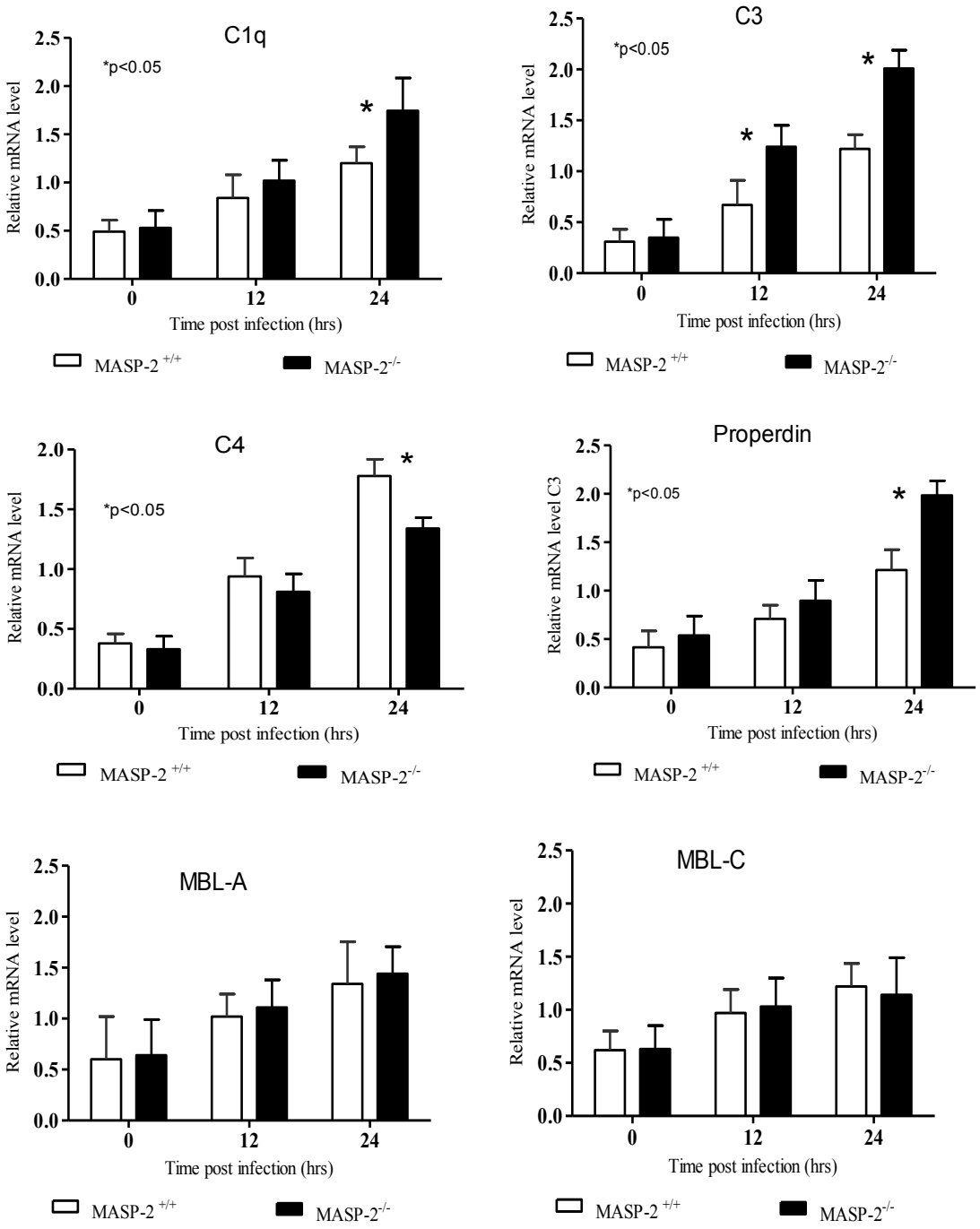

Figure 4.11: Relative mRNA expression profile of complement genes in spleens of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups.

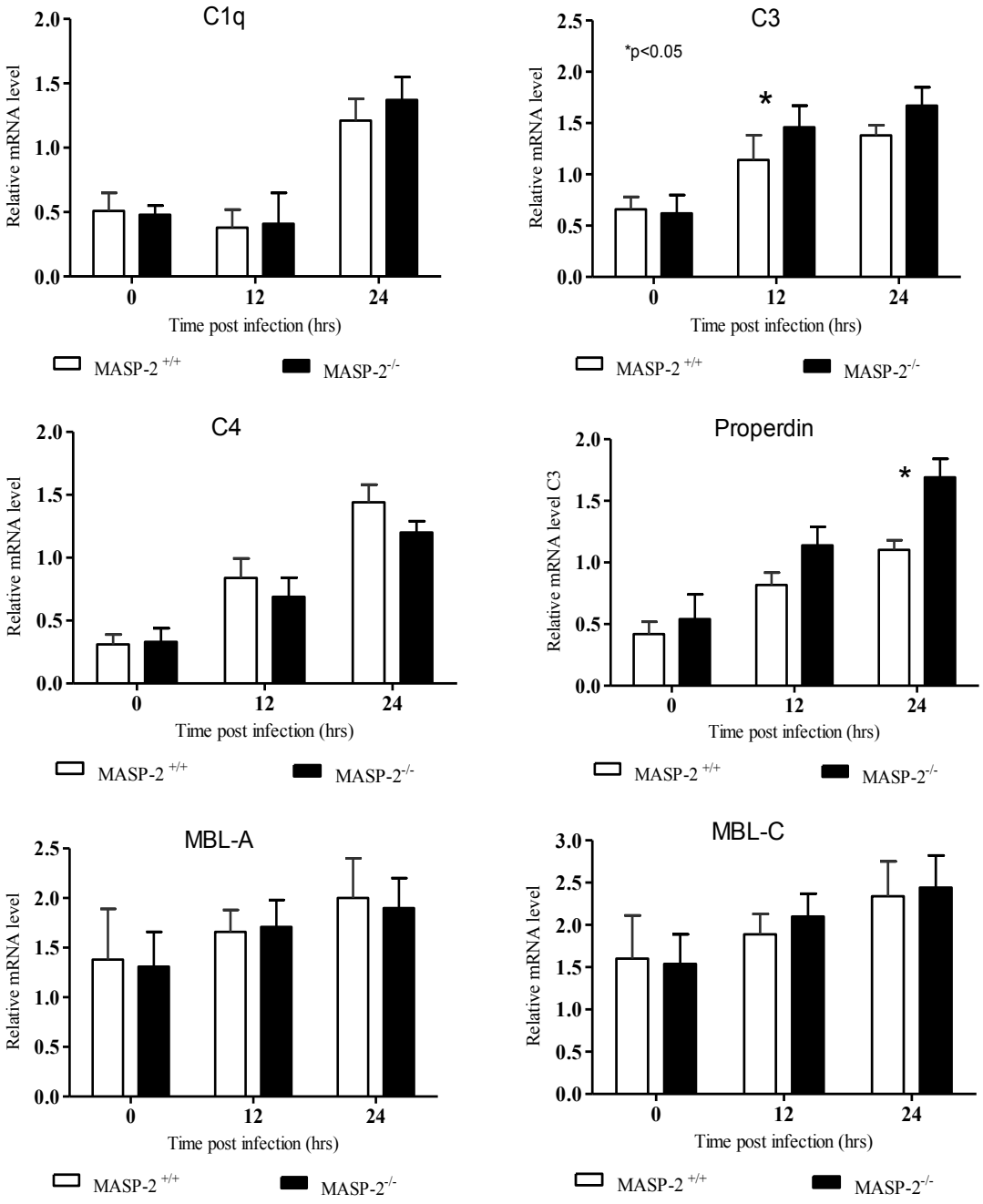

Figure 4.12: Relative mRNA expression profile of complement genes in livers of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups.

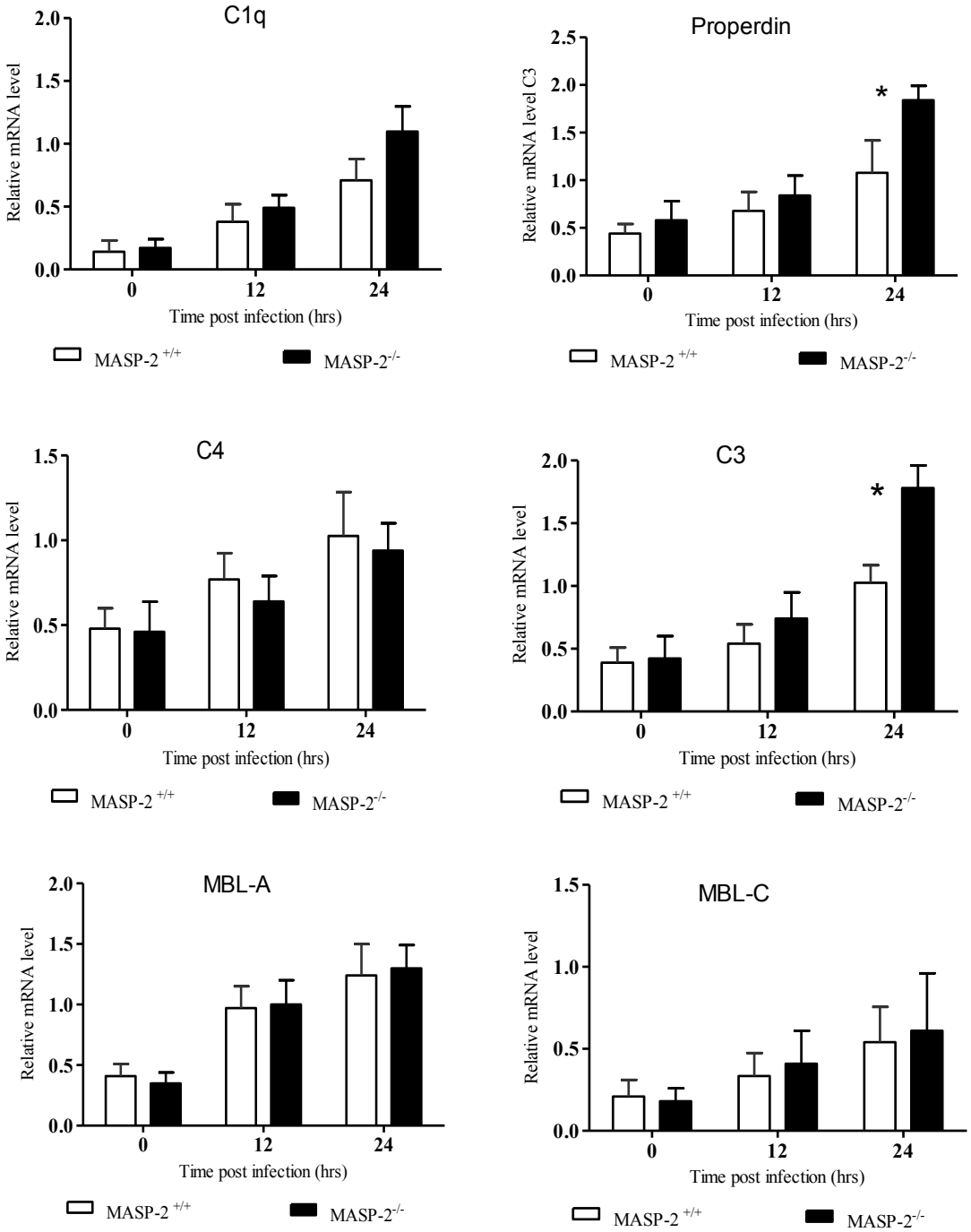

Figure 4.13: Relative mRNA expression profile of complement genes in kidneys of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups.

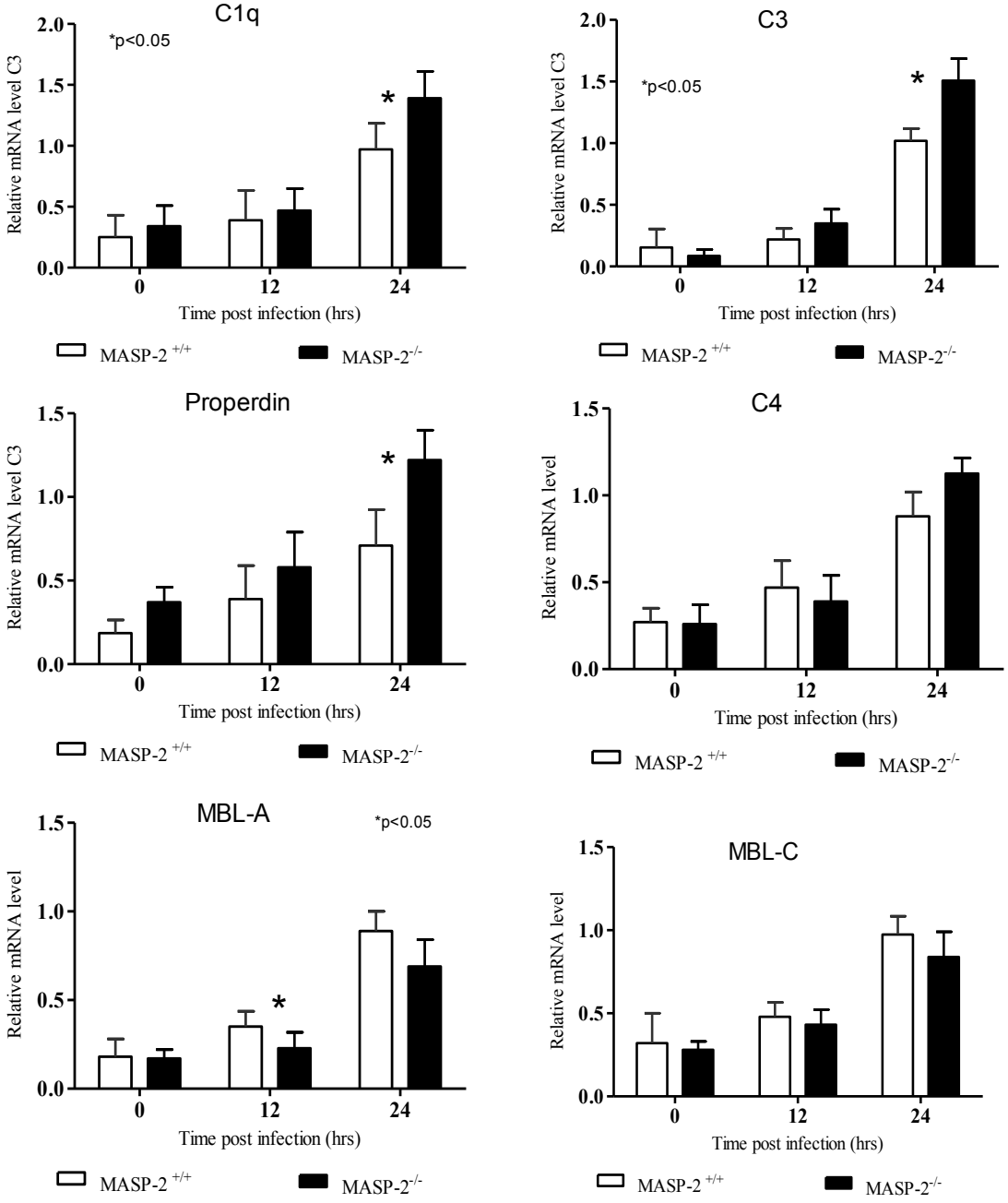

Figure 4.14: Relative mRNA expression profile of complement genes in brains of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups.

4.1.2.3.2 mRNA expression for different cytokines

The mRNA expression profiles of different proinflammatory and anti-inflammatory cytokines in different organs, including spleens, kidneys, livers and brains were determined in MASP-$2^{+/+}$ and MASP-$2^{-/-}$ mice at time points 0, 12 and 24 hours after intraperitoneal infection with *N.meningitidis* Z2491 (serogroup A) using Light Cycler based qRT-PCR technique.

The mRNA expression levels of MIP-2 and IFN-γ in spleen tissues (see **Figure 4.15**) were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 12 hours post infection. In contrast, mRNA expression levels of TNF-α and IL-1β were nearly identical in both MASP-2 deficient mice and MASP-2 sufficient control group at 12 and 24 hours post infection. The mRNA expression profiles of IL-6 and IL-10 tissues were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 24 hours post infection.

In liver tissues (see **Figure 4.16**), the mRNA expression levels of MIP-2 and IL-10 were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 12 and 24 hours post infection. While IL-1β and TNF-α mRNA expression levels were up-regulated in both MASP-2 deficient and MASP-2 sufficient mice at 12 hours post infection, still no significant difference was observed between MASP-2 deficient and MASP-2 sufficient mice. The IL-6 mRNA expression was significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 24 hours post infection.

In kidneys (see **Figure 4.17**), the mRNA expression profiles for IFN-γ, IL-1β, IL-6 and TNF-α were nearly identical in both MASP-2 sufficient and MASP-2 deficient mice at time points 12 and 24 hours post infection. The abundance of MIP-2 and IL-10 mRNA were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 12 hours post infection.

Compared to cytokine mRNA expression profiles in kidneys, the cytokine expression in the brains of mice post *N.meningitidis* infection were slightly different (see **Figure 4.18**). The mRNA expression profiles of TNF-α, IL-10 and MIP-2 were significantly lower in MASP-2 deficient mice compared to MASP-2 sufficient control group at 24 hours post infection. While IL-6 mRNA expression levels remained similar in both MASP-2 sufficient and MASP-2 deficient mice, the mRNA expression profiles for IFN-γ was significantly higher in MASP-2 deficient mice compared to MASP-2 sufficient control group at 24 hours post infection. Moreover, the abundance of IL-17 mRNA was significantly higher in MASP-2 deficient mice compared to the MASP-2 sufficient control group at 24 hours post infection.

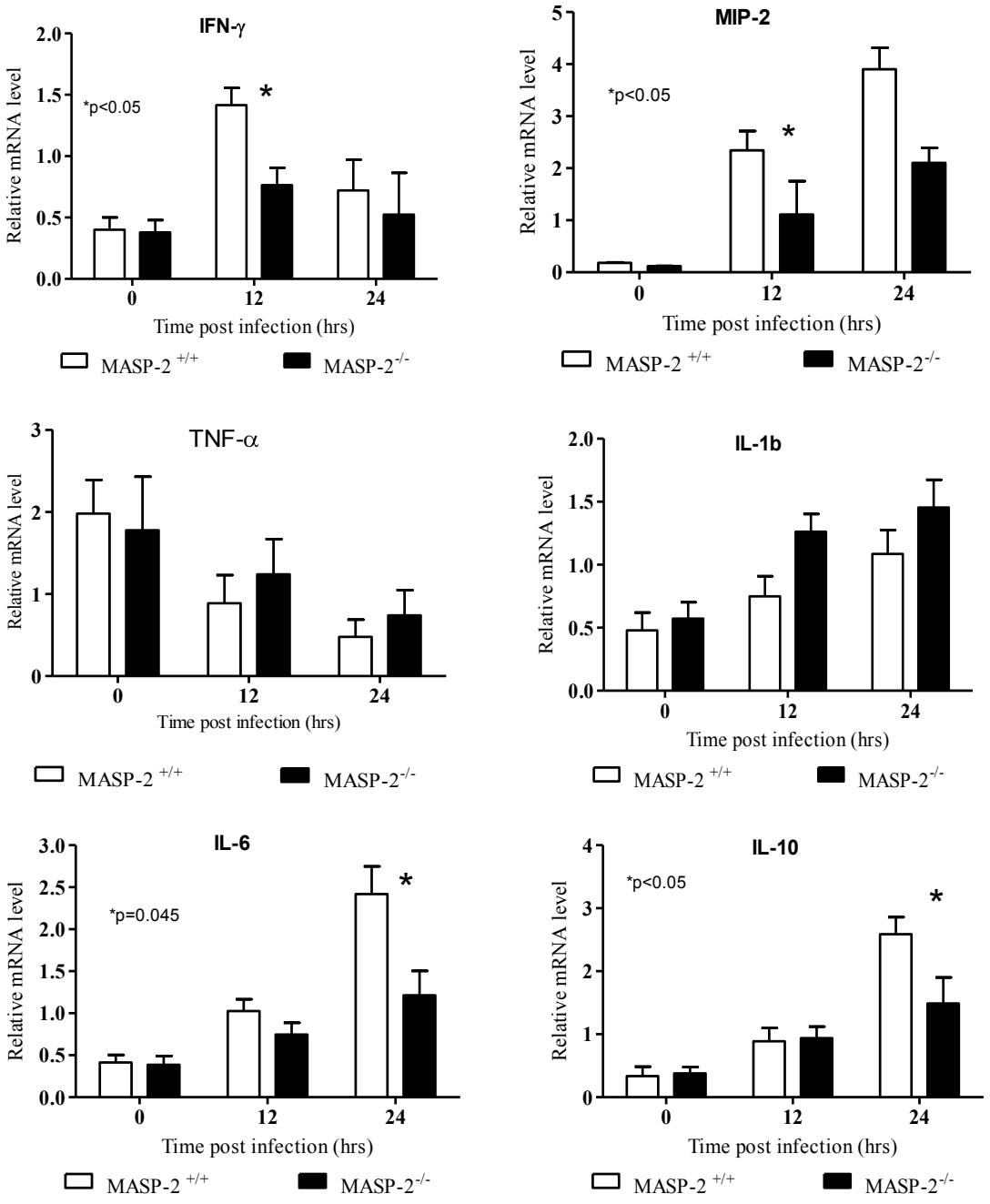

Figure 4.15: Relative cytokine mRNA expression levels in spleens of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups

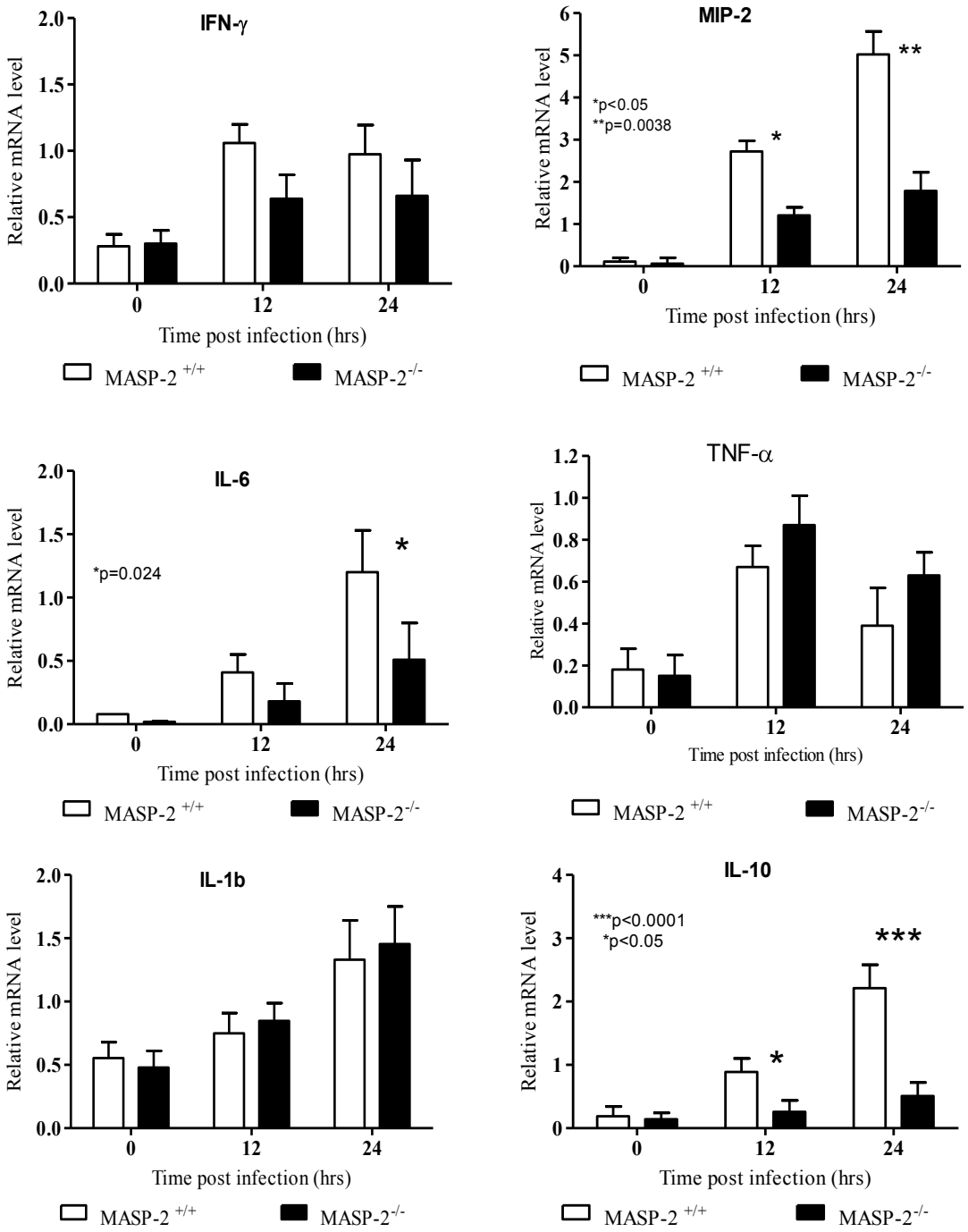

Figure 4.16: Relative cytokine mRNA expression levels in livers of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. (n=3) for each time point for both groups. The results are expressed as means±SEM.

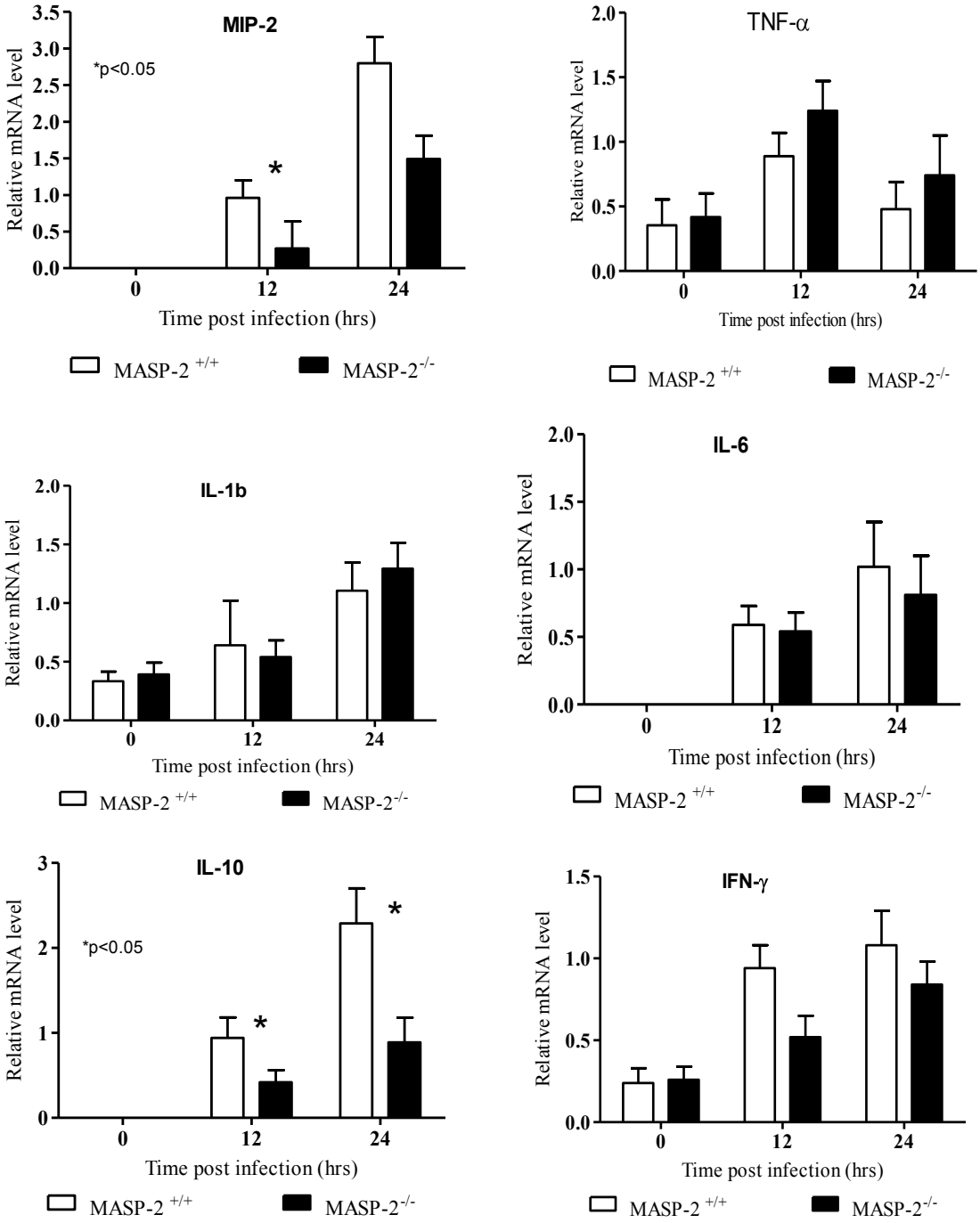

Figure 4.17: Relative cytokine mRNA expression levels in kidneys of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups

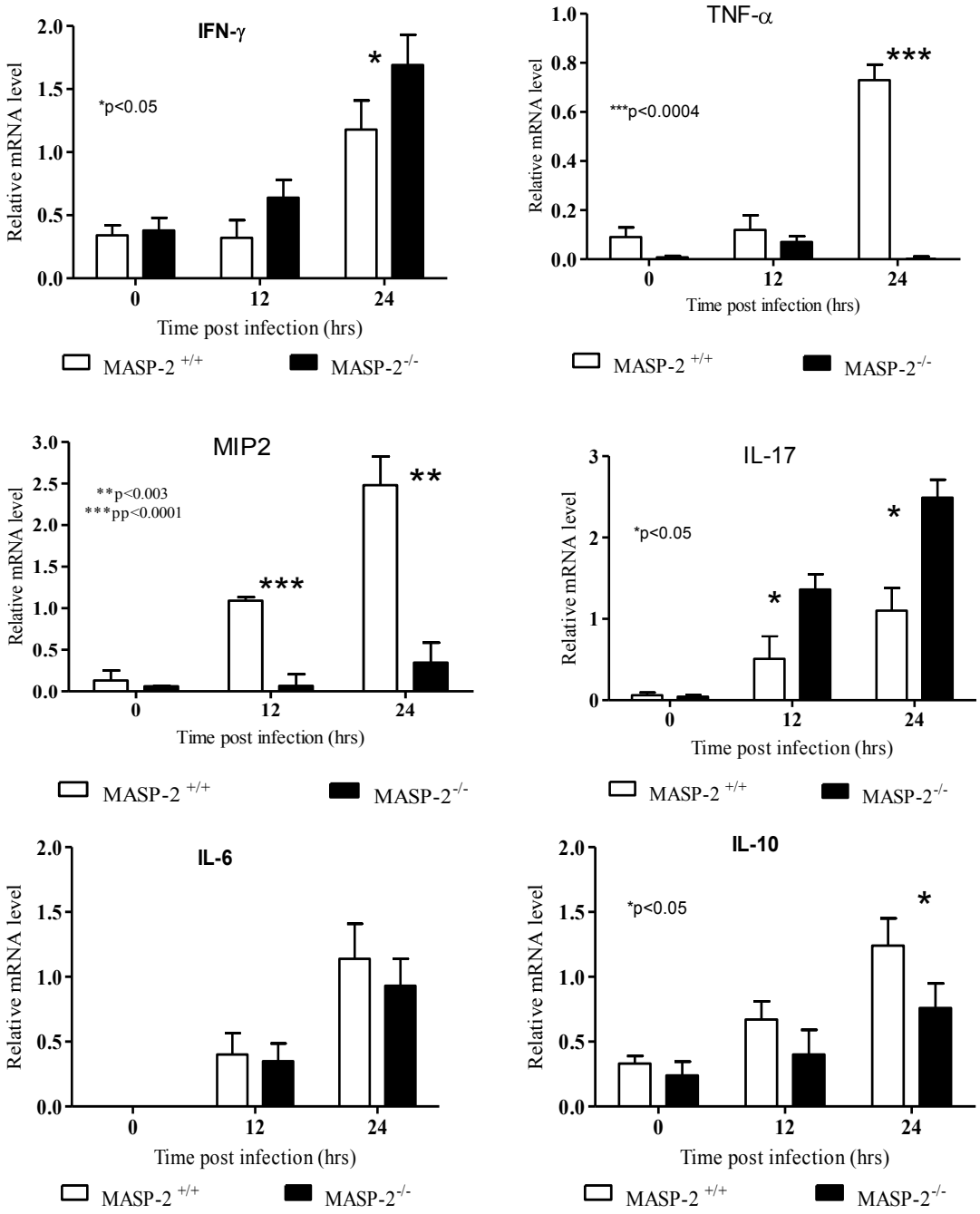

Figure 4.18: Relative cytokine mRNA expression levels in brains of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup A strain Z2491 given i.p. at a dose of $4x10^7$ CFU/mouse) over different time points. The results are expressed as means±SEM. n=3 for each time point for both groups

4.1.3 Experimental murine model of *N.meningitidis* serogroup C strain 6414 infection

Four groups of age-matched (8 to 10 weeks) C57BL/6 mice were challenged with *N.meningitidis* serogroup C strain 6414 via intra-peritoneal route to calculate the optimal infective dose for infection experiments. The infective dose was adding iron to a final concentration of 400 mg/kg body weight (see **Figure 4.19**).

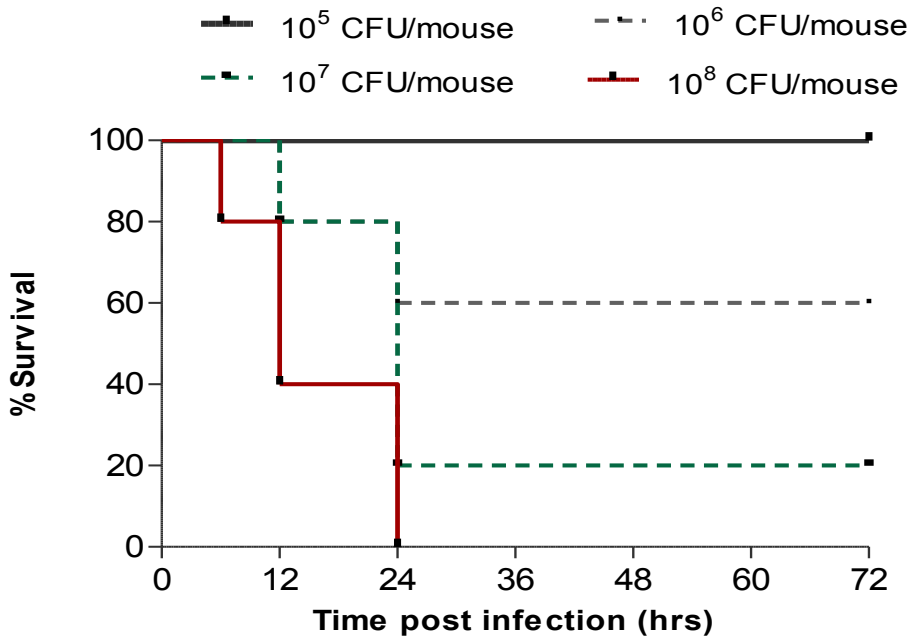

Figure 4.19: Comparison of C57BL/6 wild-type mice survival with different doses of *N.meningitidis* serogroup C strain 6414. The infective dose was adding iron to a final concentration of 400 mg/kg body weight. For evaluation of the optimal lethal dose, bacteria were injected in four different doses. Experiment included at least five mice per group.

4.1.3.1 Survival of MASP-2 sufficient wild-type and MASP-2 deficient mice (on C57BL/6 backgound) following infection with *N.meningitidis* serogroup C strain 6414

Two groups of age-matched female MASP-2 sufficient wild-type and MASP-2 deficient mice were challenged with a high dose ($3x10^6$ CFU/mouse) of *N.meningitidis* serogroup C strain 6414 via the intraperitoneal route of infection.

At 12 hrs post infection, 20% of MASP-2 sufficient mice and 10% of the MASP-2 deficient mice showed severe signs of disease and had to be euthanized. At 24 hours post-infection, 40% of MASP-2 sufficient mice approached terminal disease signs and were culled adding up to a total mortality rate of 60% for the group of wild-type mice. At that time point (24 hours) 10% of MASP-2 deficient mice had progressed to a terminal lethargic stage and had to be euthanized. The remaining 80% of this group recovered and survived the total observation period of 72 hours of the experiment. Following the course of infection revealed that there was no statistically significant difference in the survival between MASP-2 sufficient wild-type mice and MASP-2 deficient mice (see **Figure 4.20**).

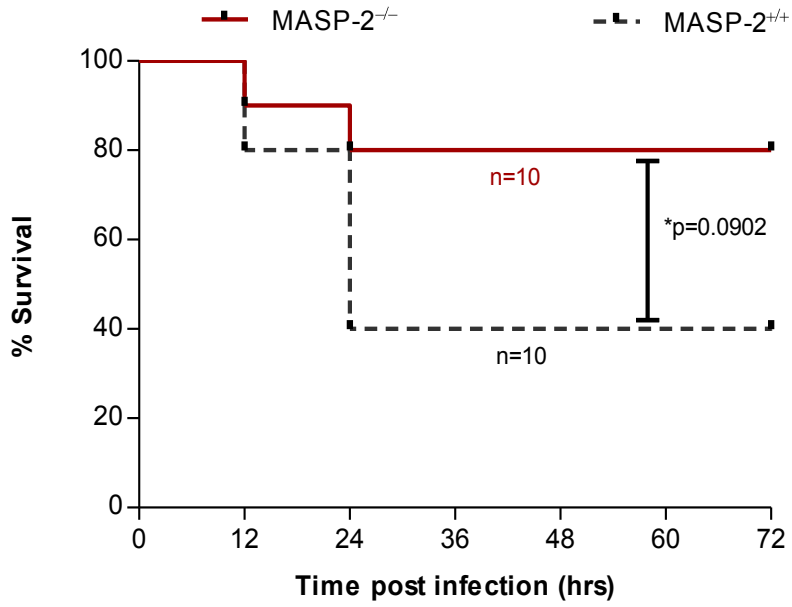

Figure 4.20: Survival of MASP-2 sufficient wild-type and MASP-2 deficient mice (on C57BL/6 backgound) following i.p. injection with a high dose ($3x10^6$ CFU/mouse) of *N.meningitidis* serogroup C strain 6414. The infective dose was adding iron to a final concentration of 400 mg/kg body weight.

4.1.3.2 Viable bacterial load of *N.meningitidis* serogroup C strain 6414 in blood and different organs of infected mice

Two groups of 8-12 weeks old female MASP-2 deficient and MASP-2 sufficient mice were challenged with a sublethal dose of $2x10^6$ CFU/mouse of *N.meningitidis* serogroup C strain 6414 via intraperitoneal injection route. At time points zero, 12 and 24 hours post-infection a number of mice, 3 at time point 0, and 5 mice from each group at time points 12 and 24 hours were culled to assess the viable bacterial load in blood and different organs.

The analysis revealed that there was a significantly higher bacterial load of *N.meningitidis* in blood of MASP-2 sufficient mice in comparison to MASP-2 deficient mice (see **Figure 4.21**).

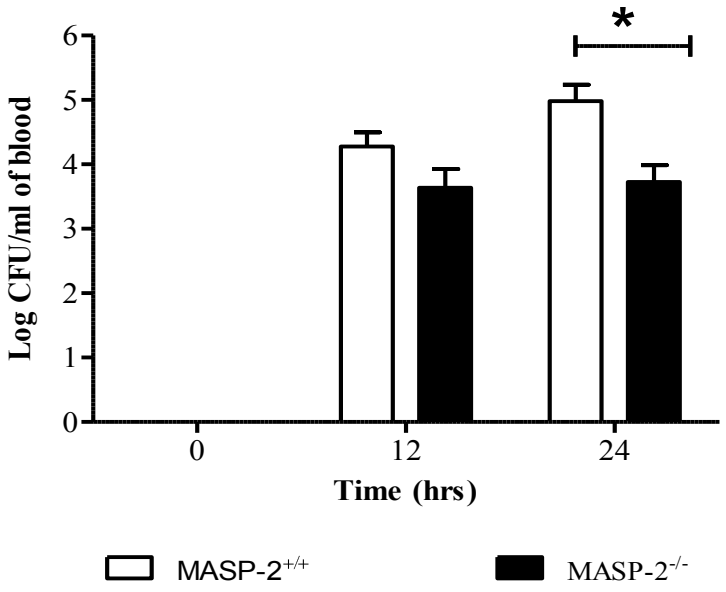

Figure 4.21: The recovery of *N.meningitidis* serogroup C strain 6414 after intraperitoneal infection with a dose $2x10^6$ CFU/mouse in blood of MASP-2 deficient wild-type and MASP-2 deficient mice at time points 0, 12 and 24 hours. The results are expressed as means±SEM. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups.

* p=<0.05

The viable meningococcal burden in the spleens of MASP-2 sufficient mice was significantly increased by 1 log difference at time point 12 hours post-infection in comparison with MASP-2 deficient mice spleens as illustrated in **Figure (4.22)**.

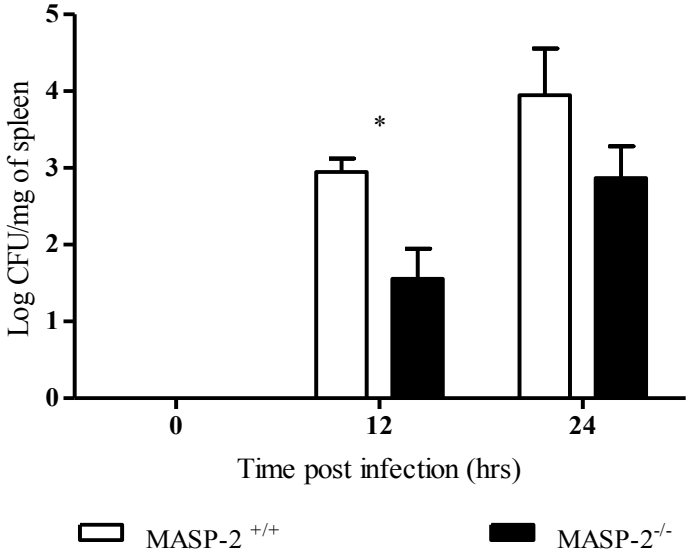

Figure 4.22: Time course of the bacterial load with *N.meningitidis* (serogroup C6414 given i.p. at a dose of $2x10^6$ CFU/mouse) in spleen tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups. * p=<0.05

Again when analysing the viable bacterial burden in the liver tissue of mice post-infection, there was a significantly higher viable bacterial load in livers of MASP-2 sufficient mice as compared to MASP-2 deficient mice at time point 24 hours post-infection (**Figure 4.23**).

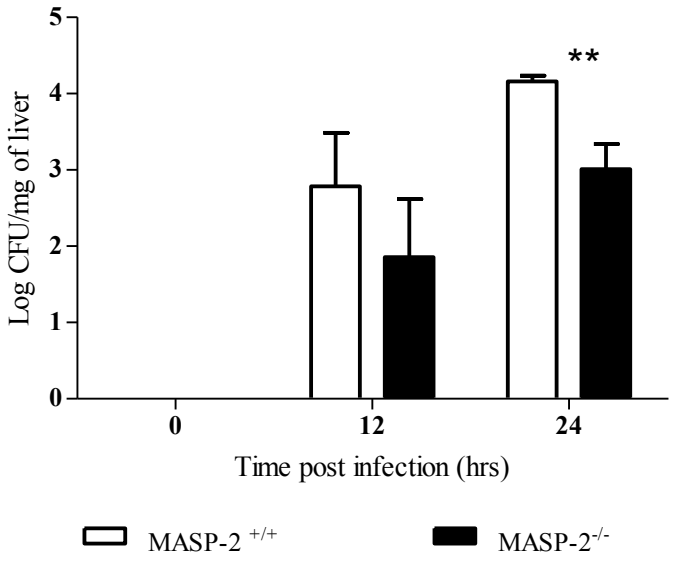

Figure 4.23: Time course of the bacterial load with *N.meningitidis* (serogroup C strain 6414 given i.p. at a dose of $2x10^6$ CFU/mouse) in liver tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups.

** p=0.0092

A significant clearance of *N.meningitidis* (serogroup C strain 6414) was observed in kidneys of infected MASP-2 deficient mice at time time point 12 hours post-infection. MASP-2 sufficient wild-type mice, on the other hand, were compromised in clearing *N.meningitidis* from kidney tissues (see **Figure 4.24**).

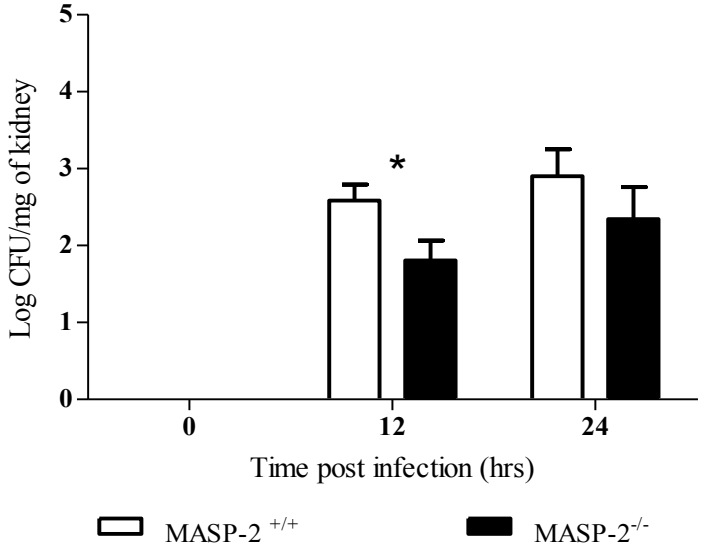

Figure 4.24: Time course of the bacterial load with *N.meningitidis* (serogroup C strain 6414 given i.p. at a dose of $2x10^6$ CFU/mouse) in kidney tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups. * p=<0.05

Monitoring the viable bacterial burden in the brain tissue of mice post-infection revealed that the MASP-2 sufficient wild-type mice showed a significantly higher bacterial burden in brain tissues as compare to MASP-2 deficient at time time point 24 hours post-infection (see **Figure 4.25**).

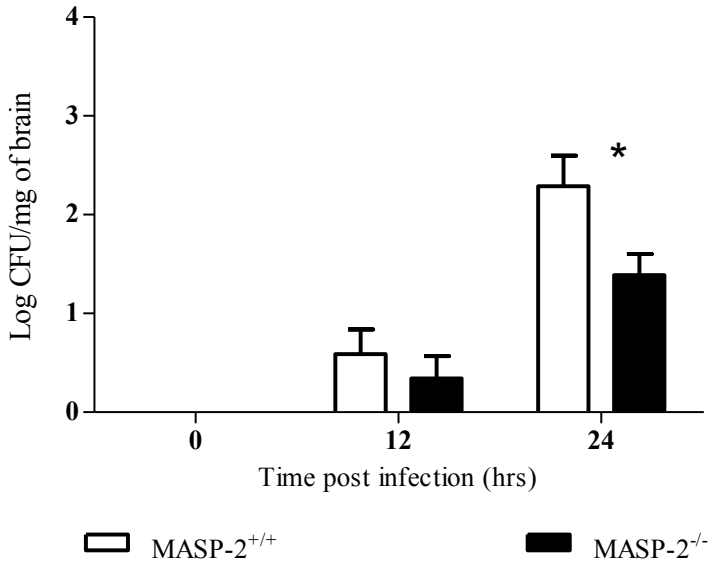

Figure 4.25: Time course of *N.meningitidis* (serogroup C strain 6414 given i.p. at a dose of $2x10^6$ CFU/mouse) viable load in brain tissue of MASP-2 deficient and MASP-2 sufficient mice at time points 0, 12 and 24 hours post infection. The results are expressed as means±SEM. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups. * p=<0.05

4.1.3.3. mRNA expression profiles of cytokines in mouse tissues post infection with *N.meningitidis* serogroup C strain 6414

The cytokine expression levels were determined in spleens of MASP-2 sufficient wild-type and MASP-2 deficient mouse groups at time points zero, 12 and 24 hours after intraperitoneal infection with *N.meningitidis* serogroup C-6414 using qRT-PCR technique. The mRNA expression levels of IFN-γ and IL-17 were significantly higher in MASP-2 deficient mice compared to MASP-2 sufficient control group at 12 hours post infection. The mRNA expression levels of TNF-α and MIP-2 were nearly identical in both MASP-2 deficient mice and MASP-2 sufficient control group at 12 and 24 hours post infection. (see **Figure 4.26**).

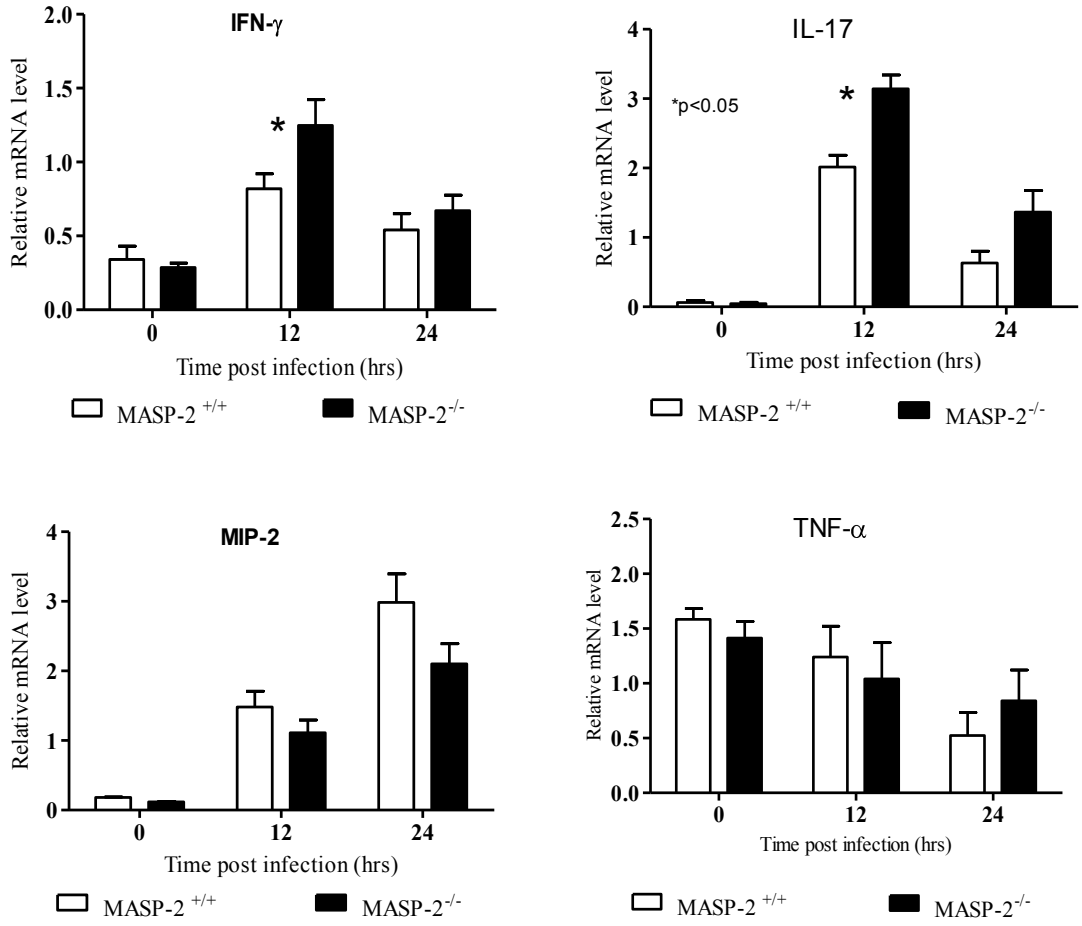

Figure 4.26: Relative cytokine mRNA expression levels in spleens of MASP-2 deficient and MASP-2 sufficient mice post *N.meningitidis* infection (serogroup C strain 6414 given i.p. at a dose of $2x10^6$ CFU/mouse) over different time points. n=3 at time 0, n=5 at time 12 and 24 hours for both mice groups. The results are expressed as means±SEM. *p<0.05

4.1.4 Experimental murine model of *N.meningitidis* serogroup B strain

MC58 infection

The optimal infective dose for infection experiments with *N.meningitidis* serogroup B strain MC58 was calculated by challenging three groups of 8 to 10 weeks old female C57BL/6 mice following intra-peritoneal route of infection. The infective dose was adding iron to a final concentration of 400 mg/kg body weight in the form of iron dextran (**Figure 4.27**).

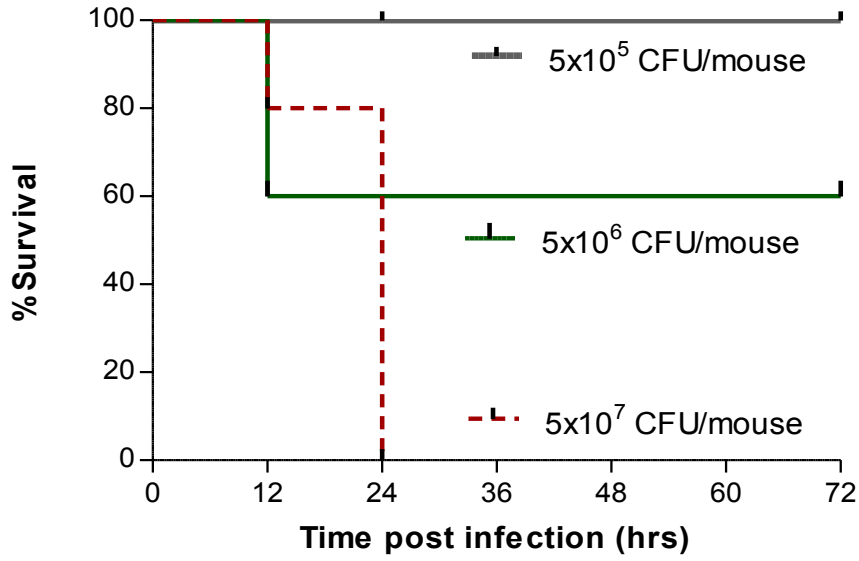

Figure 4.27: Comparison of C57BL/6 wild-type mice survival with different doses of *N.meningitidis* serogroup B strain MC58. The infective dose was adding iron to a final concentration of 400 mg/kg body weight. For evaluation of the optimal lethal dose, bacteria were injected in three different doses. Experiment included at least five mice per group.

4.1.4.1 Survival of MASP-2 sufficient wild-type and MASP-2 deficient mice following infection with *N.meningitidis* serogroup B strain MC58

In order to assess the role of the lectin activation pathway towards the host immune response to infection with *N.meningitidis* serogroup B strain MC58, groups of MASP-2 deficient and MASP-2 sufficient mouse were challenged by intraperitoneal infection with a high dose of *N.meningitidis* serogroup B strain MC58 ($8x10^6$ CFU/mouse). At 12 hrs post infection, 10% of MASP-2 sufficient mice approached terminal lethargic stage and had to be euthanized, while none of the MASP-2 deficient mice had approached to severity signs of infection that made euthanasia necessary (see **Figure 4.29**). At 24 hours post-infection, 70% of MASP-2 sufficient mice showed severe signs of disease and had to be culled adding up to a total mortality rate of 80% for MASP-2 sufficient wild-type mice. At that time point (24 hours), only 10% MASP-2 deficient mice were euthanized as they approached the terminal lethargic stage of disease.

Following the course of infection over an observation period of 72 hours post-infection, survival rates of 20% for MASP-2 sufficient mice and 90% for MASP-2 deficient mice were yielded. Using the Log-rank (Mantel-Cox) statistical analysis test revealed that MASP-2 deficient mice were significantly resistant to infection with *N.meningitidis* serogroup B strain MC58 as compared to MASP-2 sufficient wild-type mice (see **Figure 4.28**).

The scoring results of disease severity signs (see **Figure 4.29**) revealed that during the course of infection, MASP-2 deficient mice showed less severe signs of illness when compared to the disease severity scores of MASP-2 sufficient mice.

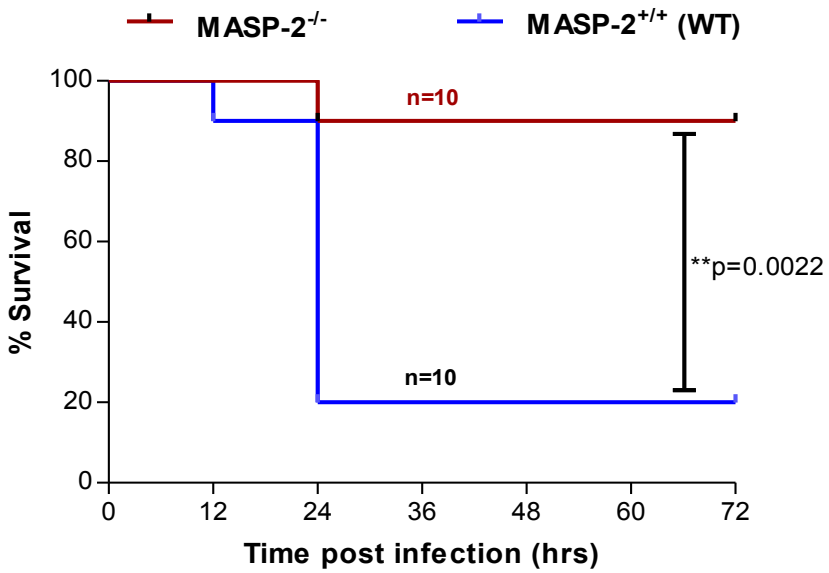

Figure 4.28: Survival of MASP-$2^{+/+}$ wild-type and MASP-$2^{-/-}$ mice (on C57BL/6 backgound) following i.p. injection with a high dose ($8x10^6$ CFU/mouse) of *N.meningitidis* serogroup B strain MC58. The infective dose was adding iron to a final concentration of 400 mg/kg body weight.

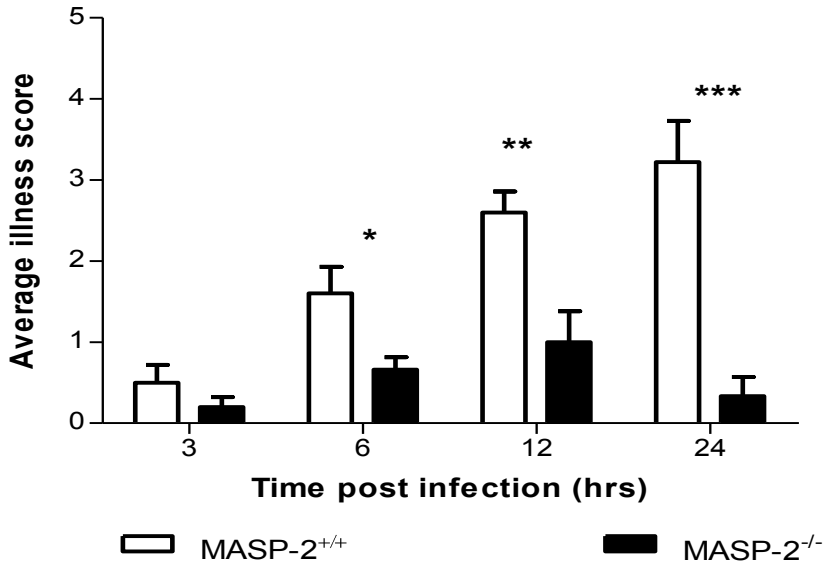

Figure 4.29: Average illness score of MASP-2 sufficient and MASP-2 deficient mice post intraperitoneal with *N.meningitidis* serogroup B strain MC58 at a high dose of $8x10^6$ CFU/mouse showing high resistance of MASP-2 deficient mice to the infection compared to wild-type mice. The results are expressed as means±SEM.

*p=.0411, **p=0.0049, ***p=0.0001

4.1.4.2 Recovery of *N.meningitidis* serogroup B strain MC58 in blood of infected mice

Two groups of age-matched female MASP-2 deficient and MASP-2 sufficient mice were challenged with an intraperitoneal dose of $8x10^6$ CFU/mouse of *N.meningitidis* serogroup B strain MC58. Blood samples were collected by tail bleed at time points 6, 12, 24 and 36 hours post-infection. When analysing the occurrence of bacterimia, the MASP-2 sufficient mice showed a significantly higher viable bacterial load at time points 6, 12 and 24 hours post-infection, when compared to MASP-2 deficient mice as illustrated in Figure (**4.30**).

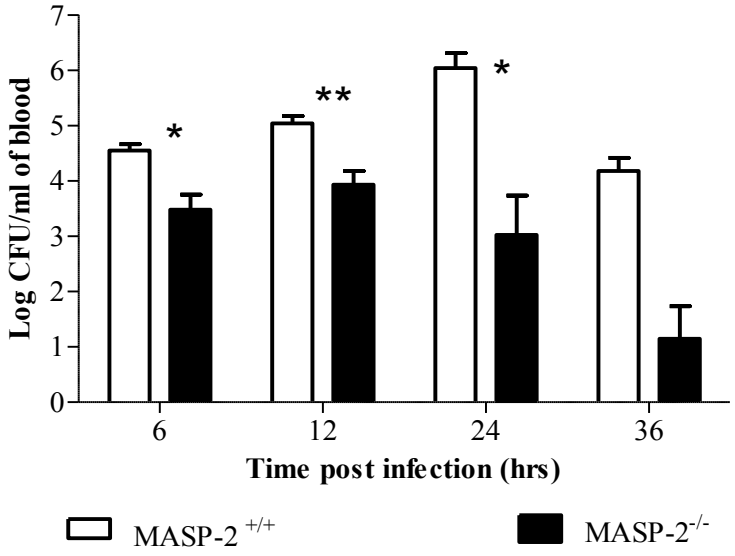

Figure 4.30: The recovery of *N.meningitidis* serogroup B strain MC58 after intraperitoneal infection with a dose $8x10^6$ CFU/mouse in blood of MASP-$2^{+/+}$ wild-type and MASP-$2^{-/-}$ mice at time points 6, 12, 24 and 36 hours. MASP-$2^{+/+}$ wild-type mice showed a significantly higher viable bacterial load in blood compared to MASP-$2^{-/-}$ mice 24 hours post infection. n=3 at different time points for both mice groups. The results are expressed as Means±SEM. *$p<0.05$ **$p=0.0043$

4.2 Discussion

The complement system plays a vital role during innate immune response towards different microbial pathogens, including *Neisseria meningitidis*. A large number of previously published reports demonstrated the vital role of complement in fighting infections caused by *N. meningitidis* (Estabrook *et al.*, 1997; Finne *et al.*, 1987; Jack *et al.,* 1998; Jarva *et al.,* 2005; Rossi *et al.*, 2001; Vogel *et al.,* 1997). Deficiencies of alternative pathway components (i.e. factor D, factor H, or properdin) or deficiencies of terminal pathway complement components (C5, C6, C7, C8, or C9) are associated with higher predispositions for *N. meningitidis* infections (Fijen *et al.,* 1995; Morgan and Walport, 1991; Spath *et al.*, 1999; Sprong *et al.,* 2006).

The aim of this work was to define the role of the lectin pathway of complement activation in the defence against *N. meningitidis* using a number of complement specific *in vitro* assays followed by *in vivo* infection experiments in a mouse model of *N. meningitidis* infection. Current study includes a series of *N. meningitidis* infection studies carried out in a unique mouse strain with a gene-targeted disruption of the murine *MASP2* gene (Schwaeble *et al.,* 2011). A number of other gene-targeted mouse strains deficient in one or more lectin pathway components were also available (including MBL-$A^{-/-}$, MBL-$C^{-/-}$, MBL-$A/C^{-/-}$, ficolin-$A^{-/-}$). However, all of them are only partially deficient of the lectin pathway functional activity. The MASP-$2^{-/-}$ mouse strain represents presently the only available mouse model with a total deficiency of the lectin pathway functional activity.

4.2.1 Lectin pathway plays an important role in activating complement on the surface of different strains of *N. meningitidis*

In order to determine which component of the lectin pathway-initating complexes is responsible for lectin pathway activation on meningococci, binding of lectin pathway specific murine carbohydrate recognition molecules (i.e. MBL-A, MBL-C, CL-11 and ficolin-A) to the surface of different strains of *N.meningitidis* was assessed. The ELISA based assays established during the current study revealed that both mouse MBL-A and MBL-C strongly bind to the surface of all of the tested clinical isolates of *N.meningitidis*. Previously, Van Emmerik *et al* (1994) had reported a very high level of binding of human MBL to unencapsulated variants of *N. meningitidis*, intermediate binding of human MBL to serogroup A meningococci while they found only weak binding of MBL with other serogroups of *N. meningitidis* (Van Emmerik *et al.,* 1994). Van Emmerik and his collegues have performed their binding experiments using radiolabelled human mannan binding protein (MBP) (now called more accurately as the mannan binding lectin) to log phase grown bacteria in their assay. In the current study, an ELISA based assays using mouse serum under physiological conditions were established. Current study also indicated that log phase grown bacteria exhibit a reduced binding capacity for MBP than bacteria grown on agar plates or in the liquid mediums to a stationary phase. Another more recent study has shown that clinical isolates of serogroups B and C meningococci grown at extremely low density can show very high binding activity to MBL (Kuipers *et al*., 2003). Another study conducted by Jack *et al* (2001) showed that MBL can bind to encapsulated strains of *N. meningitidis* serogroup C.

Ficolins are the pattern recognition molecules of the lectin pathway that selectively recognize and bind to *N*-acetylglucosamine (GlcNAc) residues on the microbial surface. Two types of ficolins have been identified in mice and rats, ficolin A and ficolin B. While ficolin A of both species is associated with MASPs and can activate complement, only ficolin B of the rat has the MASPs binding motif while mouse ficolin B appears to have lost the ability to drive lectin pathway activation due to a mutation affecting the sequence of the MASPs binding site (Girija *et al*., 2011). According to ELISA based complement activation assays, none of the tested strains of *N.meningitidis* showed any binding to murine ficolin-A.

Collectin 11 (CL-11) or collectin kidney (CL-K1) was most recently identified as one of the carbohydrate recognition molecules of the lectin pathway. CL-11 is able to selectively recognize and bind to l-fucose and d-mannose sugars on pathogen surfaces and to initiate complement activation via the lectin pathway by forming complexes with MASPs (Hansen *et al.*, 2010). In ELISA-based binding assays used during this study, a limited level of CL-11 deposition on the surface of different strains of *N.meningitidis* was observed.

Using a lectin pathway-specific C4 deposition, the results from current study clearly showed that all the clinical isolates of *N. meningitidis* activated the lectin pathway of the mouse. The C4 deposition assay was carried out under a high salt concentration to dissociate the C1 complex preventing the classical pathway to work. The findings from current study are in agreement with the findings reported by Jack *et al* (2001) who observed enhanced deposition of the complement components C4 on the surface of *N. meningitidis* leading to an increased rate of complement activation. Similarly, Sprong *et al* (2003) were

able to find a significant increase of C4bc following exposure of *N. meningitidis* to human whole blood. When anti-MBL mAbs were used, a complete inhibition of C4b and C4c formation was observed which confirmed that the C4b deposition on *N. meningitidis* was dependent on lectin pathway activation.

4.2.2 Complement activation on the surface of *N. meningitidis* requires a close cooperation between lectin and the alternative route of complement

Complement pathway specific *in vitro* assays showed that all of the tested *N. meningitidis* strains used throughout this study induced complement activation in mouse serum, mainly through the lectin and the alternative pathway. Both pathways appeared to be necessary for complement deposition. Substantial levels of C3 deposition were observed on different strains belonging to different serogroups of *N. meningitidis*. This is in agreement with previously published studies on *N. meningitidis* (Jarvis, 1994; Vogel *et al.,* 1997). Jarvis (1994) measured C3b deposition on different strains of *N. meningitidis* and reported that complement activation by *N. meningitidis* resulted in the formation of an identical repertoire of predominantly iC3b. Vogel *et al.* (1997) analysed C3 deposition on the surface of serogroup B meningococci using isogenic mutants deficient in capsule expression or sialylation of the LOS or both. Using 40% NHS for their assays, they found that C3b and iC3b were covalently linked to the surface structures of meningococci, irrespective of the surface sialic acid compounds. Vogel *et al.* (1997) further analyzed C3 deposition using

10% NHS and found that at low serum concentrations, C3 deposition occurred via the CP and was detected primarily on nonsialylated-LOS mutants.

The observed C3 deposition in the current study was reduced following incubation of different strains of *N. meningitidis* to MASP-2$^{-/-}$ serum under buffer conditions allowing classical and lectin pathway activation. This suggests an important role of the lectin pathway in activating the complement on *N. meningitidis*. To analyse this further, C3 deposition on the surface of different meningococcal strains was studied in MASP-2$^{-/-}$ serum at high serum concentrations (≥10%) and under buffer conditions allowing complement activation through all three pathways. Interestingly, a very high level of C3 deposition was observed in MASP-2$^{-/-}$ serum suggesting that the alternative pathway either provides compensation to the absence of the lectin pathway functional activity in MASP-2$^{-/-}$ mice sera or that the remaining lectin pathway enzymes MASP-1 and MASP-3 play a role in driving the alternative pathway on *N.meningitidis*.

A limited role of classical pathway activation on *N. meningitidis* is implied in the current study by the low level C1q deposition on different meningococcal strains. The minor role seen for the classical pathway might be explained by the source of serum used in these assays, which was from mice that had never been exposed to *N. meningitidis* before and therefore, have no specific antibodies that could drive effective complement activation via the classical pathway.

In the current study, C3 deposition on the surface of *N. meningitidis* serogroup A-Z2491 was also assessed using sera from different mouse strains with targeted complement deficiencies including ($C1q^{-/-}$, $MASP\text{-}2^{-/-}$, $MASP\text{-}1/3^{-/-}$). The results showed that different levels of C3 deposition were observed with all sera. A limited degree of C3 deposition on meningococci using $MASP\text{-}2^{-/-}$ serum may be attributed to classical or alternative pathway activation while the abundant C3 deposition in $C1q^{-/-}$ serum can be attributed to both lectin pathway and alternative pathway activation. The low degree of C3 deposition in $MASP\text{-}1/3^{-/-}$ sera suggests an important role of the alternative pathway of complement activation on meningococci. This conclusion is in line with and extends the findings reported by Bjerre *et al.* (2002) who studied the complement activation in whole blood model and suggested that complement activation on *N. meningitidis* is mainly dependent on the lectin and the alternative pathway. While comparing the complement activation in a whole blood model using *Neisseria meningitidis* as well as different purified bacterial components (including lipopolysaccharide (*Nm*-LPS), outer membrane vesicles (nOMVs), LPS-depleted outer membrane vesicles (dOMVs), Bjerre *et al.* (2002) measured different complement activation products, including C1rs-C1 inhibitor complexes (classical pathway), C4d (classical and lectin/MBL pathway), C3bBbP (alternative pathway), and TCC (terminal C5b-9 complement complex). It was observed that whole bacteria along with purified *Nm*-LPS and nOMVs induced a substantial complement activation by alternative and lectin pathways (as measured by increased C3bBbP, C4d and TCC) when compared with the negative control. However, no significant complement activation was observed for whole bacteria and nOMVs at C1rs-C1inh levels (Bjerre *et al.,* 2002).

4.2.3 Mice deficient of the lectin pathway effector enzyme MASP-2 show higher resistance to *N. meningitidis* infections

As part of *in vitro* analysis, sera from MASP-$2^{+/+}$ wild-type and MASP-$2^{-/-}$ mice were studied to assess the serum lytic activity of these mice against *N.meningitidis* serogroup A strain Z2491, *N.meningitidis* serogroup B strain MC58, *N.meningitidis* serogroups C strain 6414. The results have shown that all the three strains of *N.meningitidis* have significantly higher bactericidal activity in 15% MASP-$2^{-/-}$ serum when compared to MASP-$2^{+/+}$ serum at the same serum concentration. To analyse further, serum bactericidal activity of *N.meningitidis* serogroup B strain MC58 was also studied in various other complement deficient sera. A significantly impaired killing of meningococci was observed in MASP-$1/3^{-/-}$ serum as well as in MBL-A/$C^{-/-}$ serum when compared to WT serum. The same analysis was also carried out using various human sera. As seen in mouse serum before, an absent bactericidal activity against *N.meningitidis* was observed in $MBL^{-/-}$ and MASP-$3^{-/-}$ human sera. An important role of MBL in meningococcal infection has already been suggested previously by Hibberd *et al* (1999) who suggested that MBL deficiency in human is an important predisposing factor for meningococcal disease. The findings from the current study strongly suggest that both MBL and MASP-3 are essential to mediate complement dependent serum bactericidal activity against meningococci.

As reported in this PhD thesis, *in vivo* infection experiments in a mouse model of *N.meningitidis* infection in mice with gene-targeted disruption of *MASP2* gene were also carried out. At present, this mouse line provides the only mouse model with total defect of

the lectin pathway functional activity. This strain was developed in my supervisor's laboratory.

For the survival experiment, three different serogroups of *N.meningitidis* were used with a high infection dose administered through the intra-peritoneal route to induce meningococcal infection in C57BL/6 mice. The meningococcal strains and respective doses used were: Serogroup A strain Z2491 ($5x10^7$ CFU/mouse); serogroup B strain MC58 ($8x10^6$ CFU/mouse) and serogroup C strain 6414 ($3x10^6$ CFU/mouse). The use of such a high dose of infection was expected to unmask any difference in the survival times between MASP-$2^{-/-}$ and the wild-type controls even if there was only a small difference in the immune response to *N.meningitidis* infection between the two mouse groups. It is now well established that virulence of *N.meningitidis* can be critically enhanced in prior by the injection of iron compounds into the murine host (Holbein, 1980; Holbein, 1981; Perkins-Balding *et al.,* 2004). For all the infection experiments performed throughout this work, mice received iron dextran at a dose of 400 mg/kg body weight 12 hours before infection. Alternatively, infection dose was co-administered together with iron dextran where indicated.

Despite the use of high doses of infection with *N.meningitidis* serogroup A strain Z2491 (i.e. $5x10^7$ CFU/mouse) injected via intra-peritoneal route, there was a significant difference in the survival between the MASP-$2^{-/-}$ and the MASP-$2^{+/+}$ control group: MASP-$2^{-/-}$ mice proved to be resistant to meningococcal infection while 50% of MASP-$2^{+/+}$ WT control mice died. This observation is also reflected by the illness scores during the first 24

hours following infection, where MASP-$2^{+/+}$ WT mice showed significantly higher illness scores than mice of the MASP-$2^{-/-}$ group resulting in 50% of MASP-$2^{+/+}$ mice being euthanized because they approached a terminal lethargic stage of disease, while none of the MASP-$2^{-/-}$ mice had progressed to a lethargic end stage.

The presence of culturable meningococci in blood and different organs of infected mice serves as a surrogate marker for disease pattern and helps towards a better understanding of disease progression. In line with the results obtained from the survival experiment, MASP-$2^{+/+}$ mice harboured a significantly higher bacterial load in blood, spleens, kidneys, livers, brains and lungs when compared to MASP-$2^{-/-}$ at time points 12 and 24 hours following *N.meningitidis* infection. Subsequent to bacteria appearing in blood, *N.meningitidis* invaded and multiplied in the tissues of liver, spleen and kidney during early stages of infection (3 hours post infection) with spleen showing highest level of recoverable bacteria compared to kidney and liver. Viable bacterial counts in brain tissues show that meningococcal migration to the brain occurred at a later stage of infection as viable bacterial counts in brain tissues only started to appear at 6 hours post infection and reached a peak at 24 hours post infection in wild-type mice. Interestingly, viable bacterial levels in lungs started to appear at 12 hours post infection, and a high bacterial load in lungs were observed at 24 and 36 hours post infection. In general, during the course of infection, MASP-$2^{-/-}$ mice showed a significantly reduced bacterial burden indicating a better clearance of *N.meningitidis* from different organs when compared to MASP-$2^{+/+}$ mice at the same time points.

Fulminant meningococcal sepsis (FMS), is one of the most serious and fatal complications of *N.meningitidis* infection. FMS is characterized by a high intravascular concentration of endotoxin/LPS that leads to the production of high levels of proinflammatory mediators. The liberation of these inflammatory cytokines in the circulation is pivotal for the development of septic shock, which can be lethal within the first 24 hours of infection. The outcome of systemic meningococcal infection is highly dependent on LPS concentrations in plasma as well as on the degree of complement activation. In the murine model of meninogococcal infection used in the current study, the mRNA expression levels of different cytokines (IL-1β, IL-6, IL-10, MIP-2 and IFN-γ) were up-regulated in spleens, livers and kidneys of MASP-$2^{+/+}$ and MASP-$2^{-/-}$ mice group following infection with *N.meningitidis* serogroup A strain Z2491. A statistical analysis reveals that the mRNA expression levels of proinflammatory cytokines such as MIP-2, IFN-γ and IL-10 were significantly lower in spleens, livers and kidneys of MASP-$2^{-/-}$ mice compared to the MASP-$2^{+/+}$ control group.

Although the mRNA expression levels of other proinflammatory cytokines, i.e. IL-1β and TNF-α, were up-regulated in livers and kidneys of both MASP-$2^{-/-}$ and MASP-$2^{+/+}$ mouse groups at 12 hours and 24 hours post infection, no significant difference was observed between MASP-$2^{-/-}$ and MASP-$2^{+/+}$ mice. Earlier, Waage *et al* (1994) have reported high levels of proinflammatory cytokines TNF-α, IL-1, IL-6, and IL-8 in serum and cerebrospinal fluid (CSF) from patients with systemic meningococcal disease. Moreover, Sjölinder *et al* (2008) measured the levels of different cytokines in mouse serum and peritoneal cells following meningococcal infection. Using enzyme-linked immunosorbent

assays, they reported an increase in the IL-6, IL-10, IL-12, and IFN-γ levels which reached the highest levels about 6 h post challenge where as, for TNF-α, peak levels were observed 1 h post infection.

In the current study, the mRNA expression levels of IL-6 were significantly lower in spleens and livers of MASP-$2^{-/-}$ mice compared to the MASP-$2^{+/+}$ control group at 24 hours post infection. IL-6 is an interleukin that induces both a pro- and anti- inflammatory stimuli. The up-regulation of IL-6 in mice following meningococcal infection has also previously been reported by others (Sjölinder and Jonsson, 2007; Zarantonelli *et al*., 2007). Low expression levels of IL-6 is indicative of an overall decreased inflammatory response in MASP-$2^{-/-}$ mice as compared to the MASP-$2^{+/+}$ (WT) controls.

As discussed earlier, the viable meningococcal loads start to appear at a later stage of infection in brains compared to livers, spleens and kidneys of the infected mice where the pathogen can be detected much earlier. I, therefore, conclude that the cytokine response pattern is delayed as up-regulation of cytokines such as MIP-2 and TNF-α was not seen earlier than 24 hours post infection. Cytokines profiles in the brains of mice post *N.meningitidis* infection showed that the mRNA expression levels of proinflammatory cytokines such as TNF-α, IL-10 and MIP-2 were significantly lower in MASP-$2^{-/-}$ mice compared to the MASP-$2^{+/+}$ control group at the time point 24 hours post infection. Likewise, the mRNA expression profiles of anti-inflammatory cytokines, such as IL-6 and IL-10 were relatively lower in MASP-$2^{-/-}$ mice when compared to the MASP-$2^{+/+}$ mice at 24 hours post infection. IL-10 is an anti-inflammatory cytokine which negatively regulates

the expression of inflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-6, and IL-8. It has previously been reported that patients with meningococcal septic shock show elevated levels of IL-10 in their sera (Derkx *et al.,* 1995; Lehmann *et al.,* 1995). High serum IL-10 levels are present concomitantly with a high content of circulating proinflammatory cytokines like TNF-α, IL-8, and IL-6 in patients with septic shock symptoms (Lehmann *et al.,* 1995). On the other hand, the abundance of IL-17 and IFN-γ mRNA was significantly higher in MASP-$2^{-/-}$ mice compared to MASP-$2^{+/+}$ control group at 24 hours post infection. IL-17, also known as IL-17A, is a proinflammatory cytokine that is produced rapidly in response to bacterial challenge by a variety of cells (including $CD4^+$ T cells, $CD8^+$ cells and NKT cells) and promotes high levels of proinflammatory mediators and inflammation (Flierl *et al.,* 2008; Infante-Duarte *et al.,* 2001; Yao *et al.,* 1995). A number of studies have established that IL-17 contributes to antibacterial defence by the recruitment of neutrophils to the site of infection (Happel *et al.,* 2005; Liu *et al.,* 2011). Taken together, the significantly higher mRNA expression levels of IL-17 may also contribute to the enhanced bacterial clearance in MASP-$2^{-/-}$ mice besides protecting the system against over stimulation of inflammatory response by other cytokines due to the lectin pathway dysfunctional activity in MASP-$2^{-/-}$ mice.

The mRNA expression levels of complement proteins C3, C1q and properdin mRNA expression were up-regulated and significantly higher in kidneys, livers, spleens and brains of MASP-$2^{-/-}$ mice compared to MASP-$2^{+/+}$ WT mice group at 24 hours post infection. However, no significant difference in MBL-A and MBL-C mRNA expression was

observed between MASP-2$^{-/-}$ mice compared to MASP-2$^{+/+}$ mice at 24 hours post infection indicating that MBLs are not acute phase proteins.

Among the 13 clinically significant serogroups of *Neisseria meningitidis*, serogroup B meningococcus is of particular interest due to the challenge it presents in developing effective vaccines. The polysaccharide capsule of serogroup B meningococci is composed of α (2–8) *N*-acetylneuraminic acid, which is structurally identical to the polysialic acid present in many human glycoproteins. This antigenic mimicry rules out the possibility of developing polysaccharide vaccines with long-term protection against serogroups B meningococci (Zhu *et al*., 2005). As impairment of MASP-2 dependent lectin pathway functional activity provides protection against infections with serogroup A meningococci, the role of MASP-2 deficiency was also studied in an experiment murine model of *N.meningitidis* serogroup B infection using MASP-2$^{+/+}$ WT mice and MASP-2$^{-/-}$ mice.

In the survival experiment, intra-peritoneal application of *N.meningitidis* serogroup B strain MC58 at a dose of $8x10^6$ CFU/mouse induced systemic meningococcal infection. Following the course of infection, MASP-2$^{-/-}$ mice group was found to be significantly advantaged in fighting infection with *N.meningitidis* serogroup B when compared to MASP-2$^{+/+}$ WT mice group. MASP-2$^{-/-}$ mice showed less severe disease illness scores with significantly better survival rates (90%). This contrasts from the outcome using MASP-2$^{+/+}$ mice which showed significantly higher disease illness scores associated with very low survival rates (20%). In line with the severe course of infectious disease, a significantly

higher meningococcal burden was observed in blood of MASP-$2^{+/+}$ mice as compared to MASP-$2^{-/-}$ mice at all times points i.e. 6, 12 and 24 hours post infection.

The role of the lectin pathway of complement activation was also assessed in an experimental mouse model of *N.meningitidis* serogroup C infection comparing the phenotype of MASP-$2^{+/+}$ WT mice with MASP-$2^{-/-}$ mice. When using serogroup C meningococci (strain 6414 as the infectious agent), the presence or absence of MASP-2 functional activity made a difference in terms of survival with survival rates of 80% for MASP-$2^{-/-}$ mice and 40% for MASP-$2^{+/+}$ WT mice. However, the statistical analysis did not reach significant value (p= 0.0902) and larger animal groups may need to be used in later experiments. The viable bacterial load in blood and different organs (livers, spleens, kidneys and brains) however, revealed that there was a significantly higher meningococcal load in blood and in different organs of MASP-$2^{+/+}$ mice in comparison to MASP-$2^{-/-}$ mice.

While studying the molecular basis of complement pathways using rMASP-3 in mice models deficient in different MASPs (MASP-$1/3^{-/-}$, MASP-$2^{-/-}$ and MASP$1/2/3^{-/-}$), Iwaki *et al.* (2011) have recently reported that following incubation with surface preparations of *Staphylococcus aureus,* MASP-3 complexed with MBL is able to trigger the activation of the alternative pathway of complement activation by cleaving C3(H_2O)B or C3bB and convert these proenzymes into their enzymatically active form. Furthermore, *in vivo* phagocytic activity of *Staphylococcus aureus* with different mouse sera suggested that MASP-1 and/or MASP-3 play a more important role than MASP-2 in the phagocytosis and clearance of these bacteria (Iwaki *et al*., 2011).

Strong experimental evidence from recent published work from our laboratory (Schwaeble *et al*., 2011) along with the findings from the current study have pointed to the conclusion that lectin pathway has two effector arms:

i) A MASP-2 driven formation of the lectin pathway C3 and C5 convertase complexes (Schwaeble *et al*., 2011) and

ii) A novel MASP-3 driven activation route that initiates complement activation via the alternative pathway by generating alternative pathway C3 convertase (C3bBb) through cleavage of C3b-bound factor B on the activator surfaces.

The lectin pathway dependent MASP-3 driven activation of the alternative pathway appears to be required in addition to the well-established factor D mediated cleavage of C3b-bound factor B to achieve optimal activation rates for complement-dependent lysis through the terminal activation cascade to lyse bacteria through the formation of C5b-9 membrance attack complex (MAC) on the cellular surface.

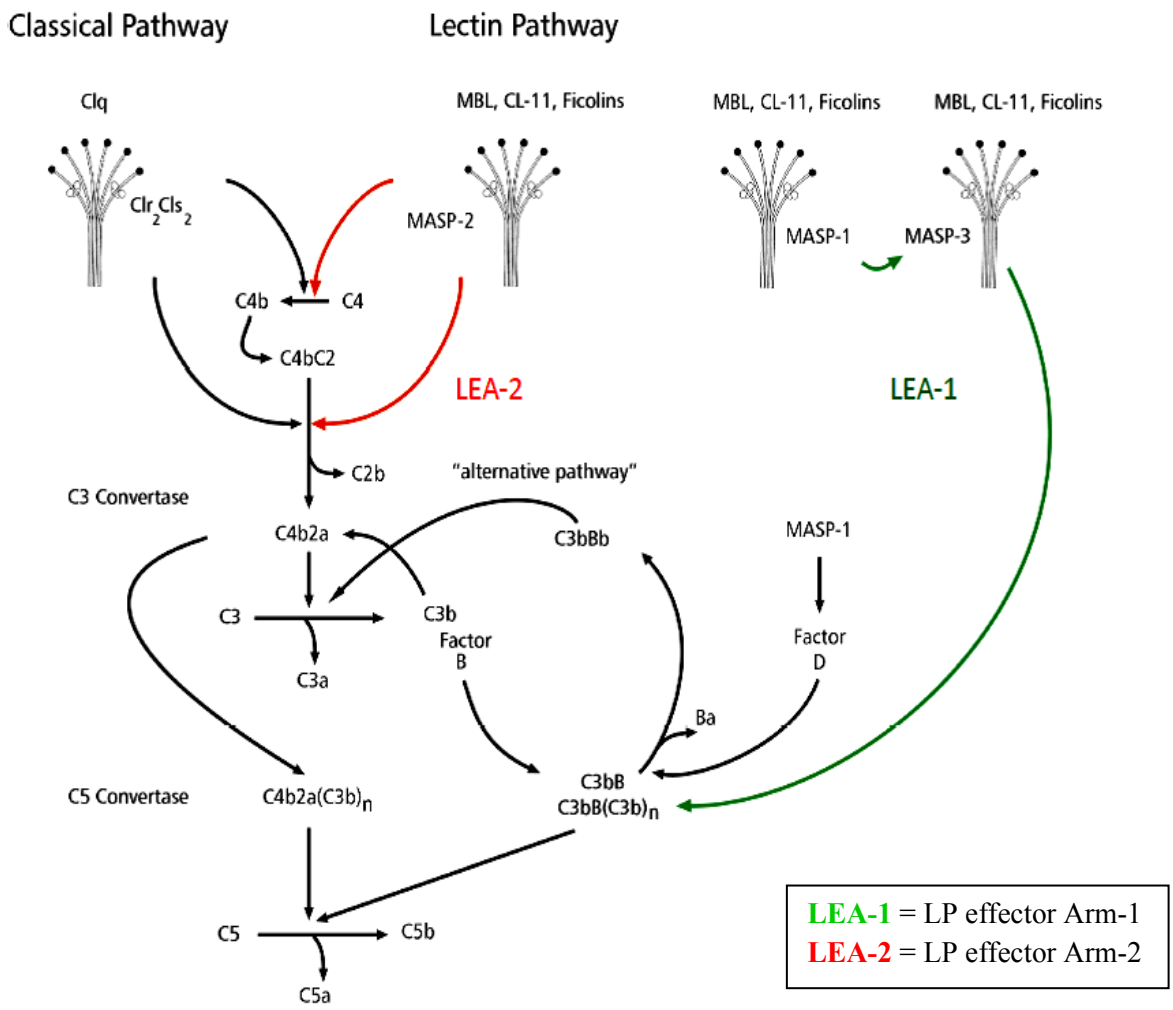

Figure 4.31: Two effector arms of the lectin pathway of complement activation (Figure courtesy of Professor W Schwaeble, University of Leicester UK).

In the current study, a high level of MBL binding to different strains of *N. meningitidis* was observed which was followed by high levels of C3 deposition on several meningococcal strains when incubated with MASP-2 sufficient WT mouse serum. The lectin pathway-mediated C3 deposition was significantly decreased when meningococcal strains were incubated in MASP-$2^{-/-}$ serum at low serum concentration (≤10%). At high serum

concentrations (≥10%), however, the relative amount of C3 deposition was higher in MASP-2 deficient sera than in their MASP-2 sufficient wild type controls. These assays were run under buffer conditions allowing complement activation through all three pathways.

MASP-2 deficient mice showed significantly higher serum bactericidal activity *in vitro* when compared to MASP-2 sufficient mice (see **Chapter 3**: **Figure 3.31)**. Following *in vivo* infection experiments, the viable meningococcal load in blood and different tissues (i.e. livers, spleens, kidneys and brains) of infected mice revealed that MASP-2 deficient mice showed a significantly better clearance of meningococci from blood and different organs when compared to MASP-2 sufficient WT mice (see **Chapter 4**: **Figure 4.5-4.10**). Furthermore, the mRNA expression levels of murine complement factor properdin were significantly up-regulated in different tissues of MASP-2 deficient mice when compared to MASP-2 sufficient mice following infection with *N.meningitidis* (see **Chapter 4**: **Figure 4.11-4.14**). In addition, a significantly higher SBA was observed following incubation of *N.meningitidis* serogroup B strain MC58 with normal human/mouse serum added with recombinant properdin when compared to normal human/mouse serum alone (see **Chapter 3**: **Figure 3.31 & Figure 3.32**). The same effect was also observed *in vivo* (see **Chapter 5**: **Figure 5.4)**.

Properdin is the only positive regulator of AP of complement activation. The AP-C3 convertase (C3bBb) has a half-life of only 900 seconds. Once properdin binds to surface-bound C3 convertase deposited on immune complexes or foreign surfaces, it stabilizes the

labile convertase and increases its half-life 5- to 10-fold (Farries *et al*., 1988; Spitzer *et al*., 2007; Agarwal *et al.,* 2010). The important role of properdin in meningococcal disease is highlighted by the evidences which suggest that properdin-deficient patients are selectively predisposed to lethal meningococcal infection (Linton and Morgan, 1999; Sprong *et al.,* 2006).

The observed C3 deposition in MASP-2 deficient serum along with significantly higher properdin mRNA expression levels as well as better meningococcal clearance in MASP-2 deficient mice following i.p. infection of *N.meningitidis* point to a very critical role of properdin against *N.meningitidis*. The current study suggests that following recognition and binding of MBL to *N.meningitidis,* the deficiency of MASP-2 (in MASP-2 deficient mice) allows the available serine proteases i.e. MASP-1 and MASP-3 to switch the LP-mediated complement activity to AP-mediated complement activation. The MASP-3 alongwith MBL drives the complement activation via the alternative pathway by generating alternative pathway C3 convertase (C3bBb) through cleavage of C3b-bound factor B on the meningococcal surface. The upregulation of properdin in MASP-2 deficient mice leads to a significantly higher stabilization effect on AP-C3 convertase in MASP-2 deficient mice when compared to MASP-2 sufficient wild type mice. The enhanced stabilization then allows the amplification of more and more C3bBb formation in competition with the facor I mediated catabolism of C3b (which uses factor H as a cofactor). The local amplification leads to the formation of AP-C5 convertase (C3bBb3b) which then results in formation of MAC on *N.meningitidis* eventually leading to the lysis of meingococci.

In addition, when studying the phenotype of MASP-2 deficiency and MASP-2 inhibition in an experimental mouse models of *N.meningitidis* infection, MASP-2 deficient mice and wild-type mice treated with antibody-based MASP-2 inhibitors (see **Chapter 5**) showed high survival rates with better clearance of bacteria. This extremely high degree of resistance was reflected in a significant increase of serum bactericidal activity in MASP-2 deficient or MASP-2 depleted mouse serum. Interestingly, mouse sera deficient of MBL-A and MBL-C as well as mouse sera deficient of the lectin pathway associated serine proteases MASP-1 and MASP-3 showed no bacteriolytic activity towards *N.meningitidis.*

A recent paper by Teizo Fujita's group identified that that MASP-1 is needed to convert zymogen form of factor D into its enzymatically active form and mice deficient in lectin pathway serine protease MASP-1 and MASP-3 lack a fully functional alternative pathway activity (Takahashi *et al*., 2010). However, when testing human serum from patient with a rare autosomal recessive disorder called Carnevale, Mingarelli, Malpuech and Michels (3MC) syndrome (Rooryck *et al*., 2011) with mutations that render serine protease domain of MASP-3 dysfunctional (these sera have MASP-1 and factor D, but no MASP-3), no bactericidal activity against *N.meningitidis* was detectable. The hypothesis that human serum also requires lectin pathway mediated MASP-3 dependent activity to develop bactericidal activity is further supported by the observation that MBL deficient human sera also fail to lyse *N.meningitidis* (Since MBL is the key carbohydrate recognition molecule that binds and activate the lectin pathway on meningococci). These findings suggest for the first time that following high binding of MBL to meningococcal strains, C3 deposition is shifted to alternative pathway-mediated C3 deposition, a mechanism which is more

pronounced in MASP-2$^{-/-}$ mice. The C3 deposition is dependent on MBL-MASP-3 complex and factor B (Bf) as shown by high alternative pathway-mediated C3 deposition on the surface of different strains of *N.meningitidis* observed when using at high serum concentrations.

In addition, a significantly higher SBA was observed following incubation of *N.meningitidis* with mouse or human serum added with recombinant properdin when compared to the SBA of normal serum alone. Further evidence of the importance of the augmentation of the alternative pathway in a murine model of meningococcal infection has been provided during *in vivo* experiments, which revealed that mouse groups injected with recombinant properdin showed a significant increase in survival when compared to saline injected control mice (see **Chapter 5**).

Taken together, the current study suggests that the alternative pathway has a major impact on the bactericidal activity against *N.meningitidis*, which is significantly higher in MASP-2$^{-/-}$ sera since MASP-3 (which drives AP activation through direct cleavage of C3bBb (Iwaki *et al.,* 2011)) requires to be activated by MASP-1 (a step that also requires MASP-1 and MASP-3 to be associated with lectin pathway recognition complexes binding in close proximity to a microbial surface to achive MASP-3 activation). Since all lectin pathway activation complexes will be loaded with either MASP-1 or MASP-3 in MASP-2 deficient or MASP-2 depleted serum, the MASP-1/3 activation axis shows a stronger disposition to drive a critical degree of AP activation required for the rate limiting events leading to bacteriolysis in MASP-2$^{-/-}$ serum.

Using MASP-2$^{-/-}$ mice in a model of systemic meningococcal infection, the current work has also demonstrated that the lectin pathway is an essential part of the inflammatory response following *N.meningitidis* infection which is in part, responsible for the high morbidity and mortality associated with potentially devastating manifestations of meningococcal disease like septicaemia and fulminant meningococcal septic shock. The alternative pathway of complement activation provides a critical immune defence against *N.meningitidis* infection by promoting an optimal rate of complement activation to occur on the bacterial surface which in turn allows the terminal activation cascade to be activated at a rate required to ensure bacteriolysis. The results from current study strongly support and underline the most recently published hypothesis that activation of MASP-3 within lectin pathway activation complexes essentially facilitates the formation of the alternative pathway C3 and C5 convertases through a MASP-3/lectin pathway dependent conversion of the zymogen complexes C3B(H_2O)B or C3bB into its enzymatically active form (C3bBb). The prospect of inhibiting MASP-3 dependent lectin pathway activities mediated by the alternative pathway opens a totally new avenue to treat inflammatory pathologies, while augmentation of the MASP-1/MASP-3 dependent route through the MASP-2 inhibition might provide a novel therapeutic opportunity in the treatment of meningococcal disease.

Chapter 5. Results

5.1 Effect of properdin and inhibitory MASP-2 antibody treatment on mortality in a mouse model of meningococcal infection

Current study of meningococcal infection using a murine model deficient in MASP-2 implied that MASP-2 deficient mice were significantly resistant to *N.meningitidis* infection as compared to MASP-2 sufficient wild-type mice. Cytokine profiles in different organs also confirmed that there was a significantly reduced expression of different inflammatory mediators in MASP-2 deficient mice as compared to MASP-2 sufficient WT mice.

5.1.1 Effect of inhibitory MASP-2 antibody treatment on mortality in a mouse model of meningococcal infection

The therapeutic benefits of using complement inhibitors to limit myocardial ischemia/reperfusion injury (MIRI) were demonstrated in a model of myocardial infarction using recombinant complement receptor type 1 (sCR1) (Weisman *et al*., 1990). It was shown that the intravenous injection of a soluble truncated derivative of sCR1 to rats can reduce the infarct volume by more than 40%. In order to investigate the potential risks and benefits of blocking the lectin pathway of complement using MASP-2 specific inhibitory antibodies, wild-type mice were infected with *N.meningitidis* which were rendered lectin pathway deficient by i.p. infection with specific MASP-2 inhibitory antibodies. Antibodies

used in this study were kindly provided by our commercial partners, Omeros Corporation (Seattle, USA).

To assess the potential risks and benefits of blocking the lectin pathway of complement activation, two groups of age- and sex-matched wild-type C57BL/6 (Charles River) mice were treated with a recombinant anti MASP-2 antibody inhibiting murine MASP-2 (1 mg/kg i.p. administered dose) or an identical dose of an irrelevant isotype control antibody 6 hours before the i.p. injection of a high dose ($5x10^6$ CFU/mouse) of *N.meningitidis* serogroup B strain MC58. In addition, mice in both groups received iron dextran (400 mg/kg body weight) 12 hours before infection. At 12 hrs post infection, 10% of the mice treated with the control isotype antibody showed severe signs of illness and had to be euthanized, while none of the mice administered with the inhibitory MASP-2 antibody had progressed to show severe signs of infection. At 24 hours post-infection, 40% of the mice treated with the control isotype antibody approached the terminal illness state and had to be culled where as only 10% of mice administered with the inhibitory MASP-2 antibody approached the terminal lethargic stage of disease and had to be euthanized.

The course of experimental infection was monitored over 72 hours. After 72 hours, a survival rate of 50% was observed in the mouse group administered with the control isotype antibody while in the mouse group administered with the inhibitory MASP-2 antibody, the survival rate was 91.67%.

Statistical significance was established using the Mantel-Cox log rank analysis and showed a significant increase in survival in mice receiving inhibitory MASP-2 antibody when compared to the mouse group administered with control isotype antibody (see **Figure 5.1**). Blood samples were also taken during the course of infection at various time points in order to assess the viable bacterial count. However, no significant difference was seen in bacterial burden within blood samples of different infection groups (**Figure 5.2**).

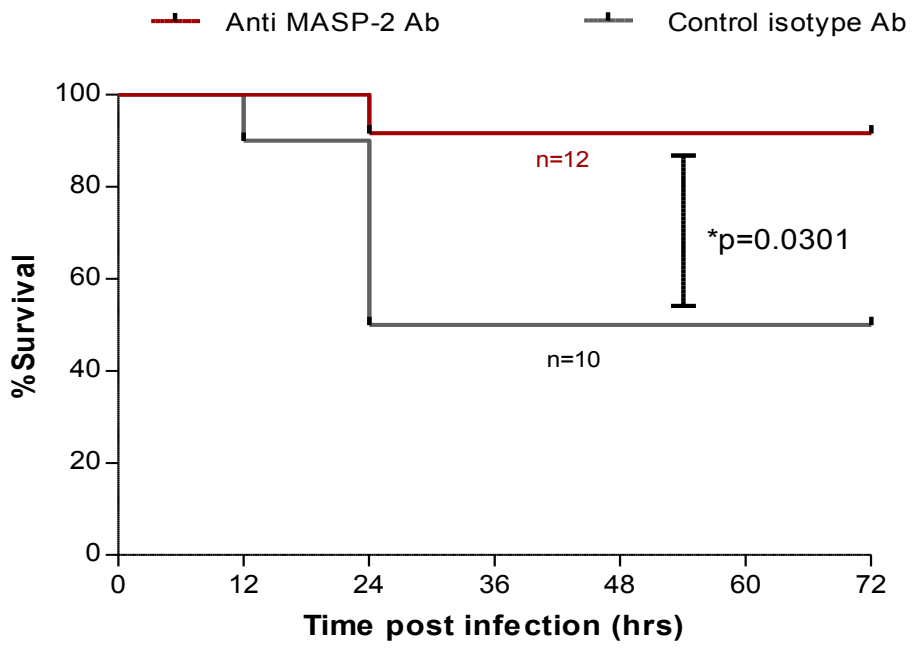

Figure 5.1: Application of a therapeutic MASP-2 inhibitor significantly reduces mortality in a mouse model of experimental *Neisseria meningitidis* infection: 9 weeks old C57BL/6 Charles River mice were treated with inhibitory MASP-2 antibody (1mg/kg body weight) and an irrelevant isotype control antibody following i.p. injection with a high dose ($5x10^6$ CFU/mouse) of *N.meningitidis* serogroup B strain MC58. The infective dose was adding iron to a final concentration of 400 mg/kg body weight.

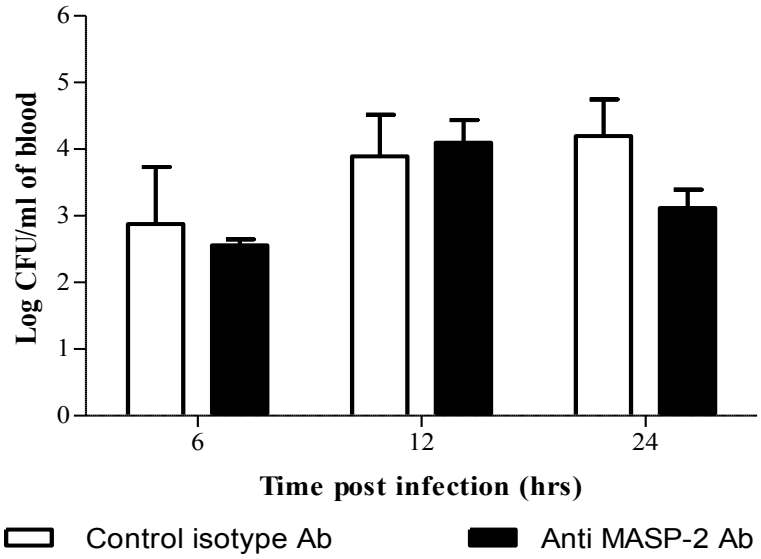

Figure 5.2: Time course showing *N.meningitidis* (serogroup B strain MC58 given i.p. at a dose of $5x10^6$ CFU/mouse) viable load in blood of anti MASP-2 antibody and isotype control antibody treated mice at time points 6, 12, and 24 hours post infection. The results are expressed as means±SEM. n=5 at different time points for both the mouse groups.

5.1.2 Effects of recombinant properdin administration on mortality using a mouse model of meningococcal infection

Properdin is a positive regulator of AP of complement activation. It binds and stabilizes surface-bound C3 convertase C3bBb, significantly extending its half-life. A very important role of properdin in an *in vivo* model of polymicrobial septic peritonitis has been suggested where properdin-deficient mice were significantly susceptible to polymicrobial septic peritonitis when compared to their wild-type littermates (Stover *et al.,* 2008). Therapeutic targeting of properdin as a potential treatment strategy for complement-dependent human diseases has also been studied in the past. It has previously been shown that a very high C3 deposition was observed on the surface of *N.meningitidis* when pre-incubated with

unfractionated properdin, followed by the addition of properdin-depleted serum. In comparison, the level of C3 deposition was much less when bacteria were incubated with properdin-depleted serum alone (Agarwal *et al.,* 2010).

Interestingly, mRNA profiling of analysis shown in **Figures 4.11-4.14** revealed that properdin mRNA expression was significantly increased in MASP-2 deficient mice in different organs following *N.meningitidis* infection when compared to MASP-2 sufficient wild-type control mice. The higher expression levels of properdin mRNA could be attributed to a significantly enhanced clearance of meningococci from blood and different organs of MASP-2 deficient mice as compared to MASP-2 sufficient mice.

In order to determine the potential additional benefits of properdin by stabilizing the alternative pathway complement activation loop to enhance complement mediated bactericidal clearance of the pathogen, mice were injected with recombinant murine properdin before and after *N.meningitidis* infection. The possibility that administration of properdin may lead to an over stimulation of inflammatory response was also considered. The combined therapeutic benefit of administering the anti MASP-2 antibody and recombinant properdin was, therefore, assessed.

To evaluate the therapeutic benefits of the administration of recombinant properdin in a murine model of *N.meningitidis* infection, age-matched wild-type female C57BL/6 (breeder Charles River) mice were tested in three groups. Mice in first group were given an i.p. administration of recombinant properdin 3 hours before infection, mice in a second group

were treated with recombinant properdin 3 hours post infection, and the third group of mice was injected with saline only prior to infection. Mice in all the groups were infected via i.p. injection with a high dose ($8x10^6$ CFU/mouse) of *N.meningitidis* serogroup B strain MC58. Mice in all the groups received iron dextran (400 mg/kg body weight) 12 hours before infection. At 12 hrs post infection, 20% of the mice in the infected untreated control group progressed to severe signs of infectious disease and had to be euthanised, while none of the mice treated with recombinant properdin (100 μg/mouse) had approached the terminal stage of infectious disease (see **Figure 5.3)**. At 18 hours post-infection, 14.29% of mice treated with recombinant properdin 3 hours before infection, 10% of mice treated with recombinant properdin 3 hours post infection and 40% mice from infected control group had approached terminal illness state and had to be culled. At 22 hours post infection, another 30% of mice treated with recombinant properdin 3 hours post infection and 20% mice from infected control group had to be culled as they approached the terminal stage of infectious disease. At 30 hours post infection, 40% of mice treated with recombinant murine properdin 3 hours post infection showed severe signs of disease and were culled while all of mice in rest of the groups recovered at 30 hours post infection.

The mice were monitored for survival over a period of 72 hours post-infection, after which mice treated with recombinant properdin 3 hours before infection showed survival rates of 85.71%, while mice from the infected control group and the mice treated with recombinant properdin 3 hours post infection showed survival rates of only 20% each (see **Figure 5.3**). Survival proportions were analyzed by using the Mantel-Cox log rank analysis and Gehan-Breslow-Wilcoxon Test. Using the Mantel-Cox log rank analysis, the mouse group treated

with recombinant properdin 3 hours before infection showed a significant increase in survival when compared to the mice treated with recombinant properdin 3 hours post infection. The Mantel-Cox log rank analysis did not show any significant difference between second (mice with recombinant murine properdin 3 hours post infection) and third group (infected untreated control mice) of mice. However, when survival curves were compared using Gehan-Breslow-Wilcoxon Test (which gives more weight to deaths at early time points), a significant difference was observed between mice with recombinant murine properdin 3 hours post infection and the infected control group.

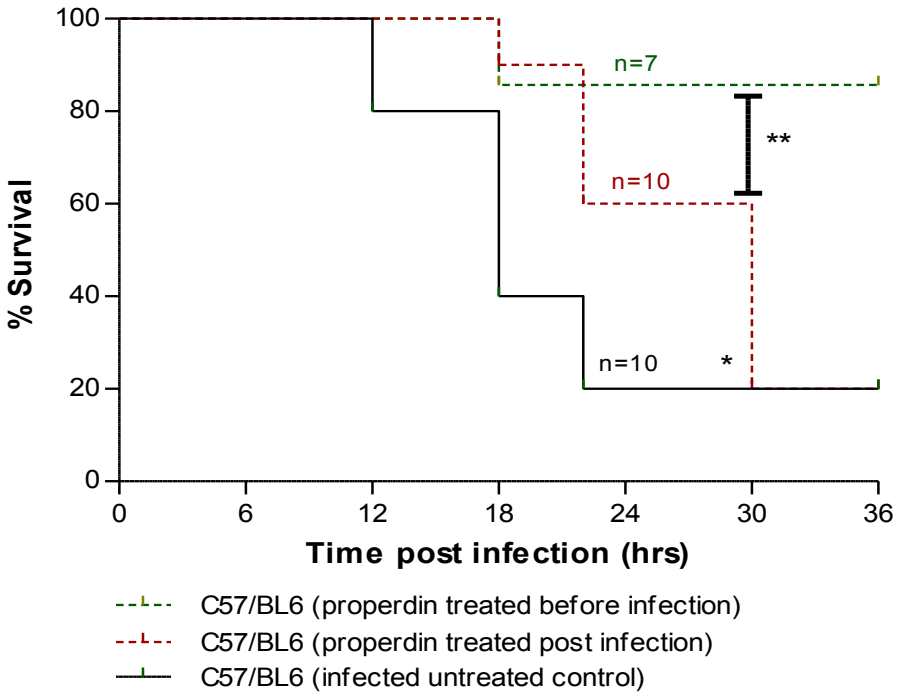

Figure 5.3: Survival of mice WT C57BL/6 and C57BL/6 treated with recombinant murine properdin (100 µg/mouse) 3 hours before and after injection with a high dose ($8x10^6$ CFU/mouse) of *N.meningitidis* serogroup B-MC58. The infective dose was adding iron to a final concentration of 400 mg/kg body weight.

**p=0.0090 (Mantel-Cox log rank test) *p=0.0486 (Gehan-Breslow-Wilcoxon Test)

5.1.3 Effects of anti MASP-2 antibody and recombinant properdin administration on mortality in a mouse model of *N.meningitidis* infection

In order to assess the combined effects of MASP-2 inhibitory antibody and recombinant properdin in a murine model of *N.meningitidis* infection, age-matched female WT C57BL/6 (Charles River) mice were distributed into 5 groups containing at least ten mice per group; Group A received recombinant properdin (100 µg/mouse) 3 hour before infection; Group B received recombinant properdin (100 µg/mouse) and anti MASP-2 antibody (1mg/kg body weight) 3 hour before infection; Group C received recombinant properdin (100 µg/mouse) 1 hour post infection; Group D received recombinant properdin (100 µg/mouse) and inhibitory MASP-2 antibody (1 mg/kg body weight) 1 hour post infection and Group E was the infected untreated control group. A negative control group of five sham-infected mice (treated with PBS) was also included in the study. Mice in all the groups received iron dextran (400 mg/kg body weight) 12 hours before infection. A high infection dose ($7x10^6$ CFU/mouse) of *N.meningitidis* serogroup B-MC58 was injected into each mouse via intra-peritoneal route.

At 12 hrs post infection, 10% of mice from group A and group C while 30% mice from group E reached terminal stage of illness and were culled. At 24 hours post-infection, 20% of mice from group A, 10% of mice from group B, 30% of mice from group C, 20% of mice from group D and 40% of mice from group E approached terminal disease signs and had to be euthanized. At the end of experiment, the total survival rates for each group were; Group A 70%; Group B 90%; Group C 60%; Group D 80% and Group E 30%. None of the

animal in sham treated group showed any signs of illness during the course of infection (see **Figure 5.4**). Data analyzed between different mouse groups by one-way ANOVA with Dunnett's test revealed that there was a significant difference between survivals of all the groups when compared to the control/infection group (Group E).

The scoring results of disease severity signs (**Figure 5.5**) showed that during the course of infection, mice groups which received both recombinant properdin and inhibitory MASP-2 antibody (Group B and Group D) showed less sever signs of illness when compared to the disease severity scores of control infected mouse group or mouse groups which only received recombinant properdin (Group A and Group C). Blood samples were collected by tail bleed at time point 12 hours post-infection. No significant difference was observed in the occurrence of bacteraemia amongst different mouse groups at time point 12 hours post-infection (see **Figure 5.6**).

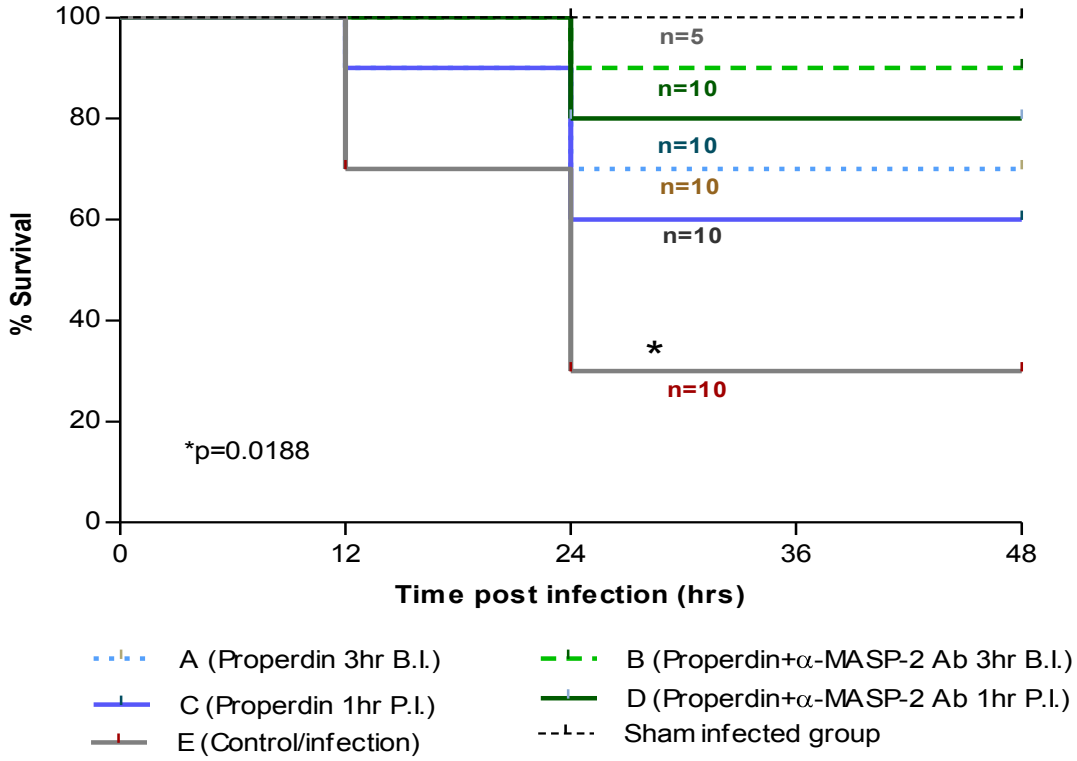

Figure 5.4: Survival of WT C57BL/6 mice and C57BL/6 mice treated with recombinant properdin (100 µg/mouse) and inhibitory MASP-2 antibody (1 mg/kg body weight) before and after injection with a high dose ($7x10^6$ CFU/mouse) of *N.meningitidis* serogroup B-MC58. The infective dose was adding iron to a final concentration of 400 mg/kg body weight. Data was analysed by one way ANOVA followed by Dunnet's test.

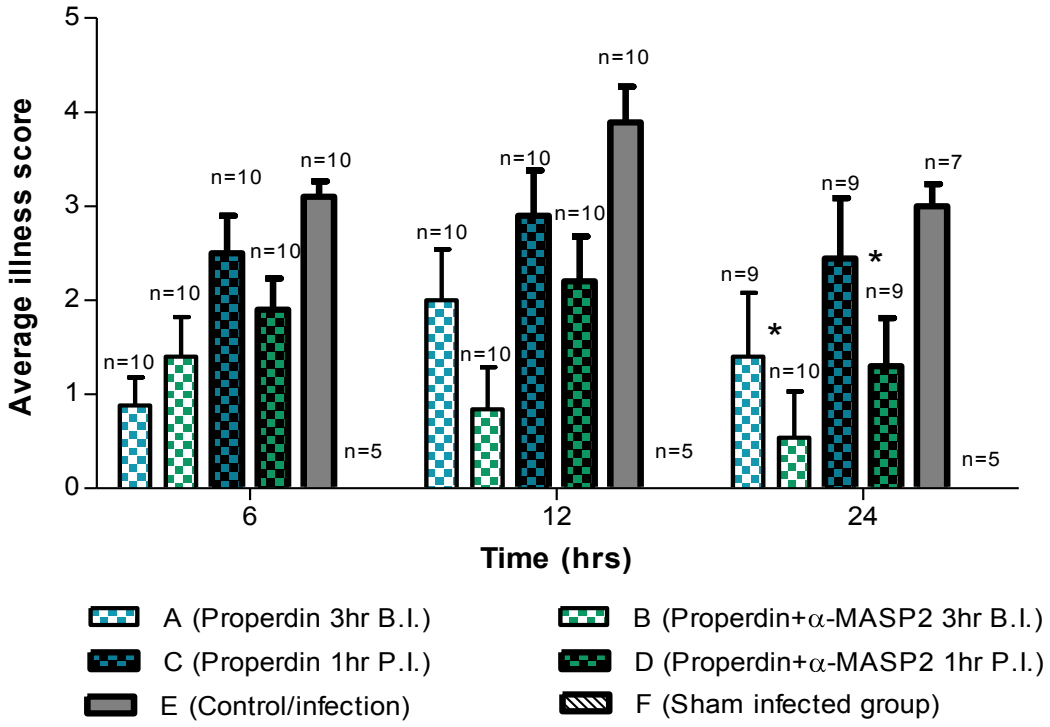

Figure 5.5: Average illness score of WT C57BL/6 and C57BL/6 treated with properdin (100 µg/mouse) and inhibitory MASP-2 antibody (1 mg/kg body weight) before and after injection with a high dose ($7x10^6$ CFU/mouse) of *N.meningitidis* serogroup B-MC58. The results are expressed as means±SEM. Experiment included at least ten mice per group except for sham infected mice (n=5).

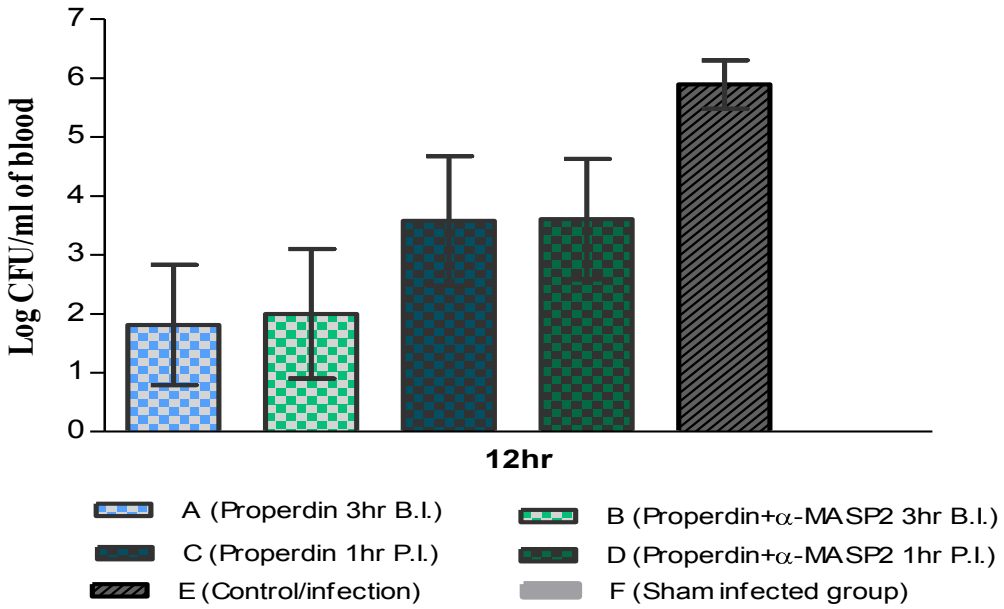

Figure 5.6: Time course of *N.meningitidis* (serogroup B-MC58 given i.p. at a dose of $7x10^6$ CFU/mouse) viable load in blood of WT C57BL/6 and C57BL/6 treated with properdin (100 µg/mouse) and inhibitory MASP-2 antibody (1 mg/kg mouse) at time point 12 hours post infection (n=5). The results are expressed as means±SEM.

5.2 Discussion

5.2.1 Application of specific anti-MASP-2 antibody provides protection in murine model of meningococcal infection

Complement can influence the pathogenesis of meningococcal infections in two ways: on one hand, the deficiency of one or more complement components predisposes to meningococcal infection, and on the other hand, excessive or in appropriate complement activation (as in FMS) is correlated with severe disease signs and a poor outcome for the patient. The development of complement inhibitors is therefore, an attractive approach for possible therapeutic applications. The complement inhibition strategy to achieve therapeutic benefits during meningococcal infection should be aimed at inhibiting the negative effects of inflammatory activation products without impairing the bactericidal activity of this system in order to eliminate the pathogen. Evidence has been provided by previous studies which suggest that a therapeutic anti-C5a antibody or C5a receptor antagonists (that inhibit C5a-mediated inflammatory effects but preserve bactericidal activity) showed beneficial effects on the survival in several different animal models of using septic shock gram-negative bacteria (Czermak *et al.,* 1999; Strachan *et al.,* 2000; Sprong *et al.,* 2003). Recombinant complement receptor type 1 (sCR1) have previously been used in a model of myocardial infarction to limit myocardial ischemia/reperfusion injury (MIRI) (Weisman *et al*., 1990). Results show that intravenous injection of a soluble truncated derivative of sCR1 to rats can reduce the infarct volume by more than 40%.

Recent work in Prof. Wilhelm Schwaeble's laboratory demonstrated that MASP-2$^{-/-}$ mice are significantly protected against ischemia/reperfusion (I/R) injury in an experimental murine model of myocardial infarction and models of intestinal ischemia comparing MASP-2$^{-/-}$ mice with their WT littermates (Schwaeble *et al.,* 2011). In addition, use of specific monoclonal antibody inhibitors against MASP-2 to block the lectin pathway functional activity as a therapeutic approach to limit I/R injury was shown to be highly effective in mouse models of I/R injury.

The results from current study have demonstrated that mice with a deficiency of MASP-2 acquired resistance against a lethal infection with *N. meningitidis.* To determine whether an inhibitory monoclonal antibody against MASP-2 could prevent a fatal outcome in a murine model of meningococcal infection, mice were injected intraperitoneally with specific recombinant MASP-2 inhibitory antibodies prior to infection with *N. meningitidis*. The results clearly show that the use of anti MASP-2 antibodies provided a significant degree of protection against meningococcal infection. Mice treated with the inhibitory MASP-2 antibody showed survival rates of 91.67% while the infected control mice group (injected with an irrelevant isotype antibody) showing survival rates of only 50%. Surprisingly, no significant difference in viable bacterial counts in blood was observed between the two mice groups.

5.2.2 Application of recombinant properdin enhances the serum bactericidal activity against *N.meningitidis* resulting in reduced mortality in an experimental murine model of meningococcal infection

Properdin is a positive regulator of the alternative pathway complement activation which amplifies the ongoing complement activation by stabilizing and extending the half-life of surface-bound C3 convertase C3bBb. More recent work suggests that properdin is able to initiate the alternative pathway of complement system by two possible model mechanisms: According to the most widely accepted hypothesis, properdin binds to the pre-formed C3bBb complex on the surface of pathogen in a non-specific manner. Another more recent and not very likely hypothesis suggests that properdin initiates activation of the alternative pathway through direct binding to the target alternative pathway activator surfaces and forms a catalyst for the formation of C3b and Bb complexes (Spitzer *et al*., 2007).

In an *in vivo* model of polymicrobial septic peritonitis induced by cecal ligation and puncture (CLP), properdin deficient mice were significantly impaired in their survival when compared to the survival of their wild-type littermates (Stover *et al.,* 2008). Therapeutic targeting of properdin as a potential treatment strategy for complement-dependent human diseases is highlighted by a study in which, *N.meningitidis* following pre-incubation with unfractionated purified properdin, showing high levels of C3 deposition after addition of properdin-depleted serum. In line with this, C3 deposition on meningococci was comparatively low following incubation with properdin-depleted serum alone (Agarwal *et al.,* 2010).

A critical role of the alternative pathway of complement activation is suggested in the present study. A high level of the alternative pathway mediated C3 deposition and enhanced bactericidal activity was observed in MASP-$2^{-/-}$ serum, suggesting that components of lectin activation pathway other than MASP-2 (i.e. MBL and MASP-3) are enhancing alternative pathway activation and thus provide protection against *N.meningitidis* infection.

The serum bactericidal activity of WT mouse serum against *N.meningitidis* was significantly enhanced following addition of recombinant murine properdin. Likewise, the mRNA expression levels of properdin mRNA were up-regulated in different organs of infected mice following infection with a high dose of *N.meningitidis*. MASP-$2^{-/-}$ mice showed significantly higher mRNA expression levels for properdin when compared to MASP-$2^{+/+}$ wild-type mice, which may implicate the significantly enhanced clearance of meningococci from blood and different organs of MASP-$2^{-/-}$ mice as compared to MASP-$2^{+/+}$ WT mice. To further investigate the potential benefits of properdin administration to limit systemic disease, mice infected with *N.meningitidis* were given an i.p. dose of recombinant murine properdin. The current study revealed that mice injected with recombinant properdin showed a significant increase in survival when compared to the infection control mice given i.p. injection of buffer only. To evaluate this further, the survival rates of mice following administration of recombinant murine properdin before and after infection with a high dose of *N.meningitidis* infection were studied. The survival curves generated by the infection experiments show that the mouse group injected with recombinant properdin 3 hours before infection displayed a significant increase in survival

(showing survival rates of 85.71%) when compared to the mice treated with recombinant properdin 3 hours post infection (showing survival rates of 20% only).

When developing a potential therapeutic candidate to modulate complement activation, it is very important to consider that a balance needs to be maintained between antibactericidal properties of complement and possible side effects due to over stimulation of inflammatory response. In order to achieve this, combined therapeutic benefits of administering the inhibitory MASP-2 antibody (to limit the inflammatory response and to enhance MASP-1/MASP-3 mediated activation of the alternative pathway) and recombinant properdin (to limit bacterial multiplication by stabilising the alternative pathway C3 and C5 convertase) was tested in a murine model of meningococcal infection. In this *N.meningitidis* infection experiment, mouse groups received recombinant murine properdin combined with inhibitory MASP-2 antibody 3 hours before or 1 hour after infection. The group receiving properdin and inhibitory MASP-2 antibody 3 hours prior to infection showed a survival rate of 90%, while the mouse group given properdin and inhibitory MASP-2 antibody 1 hour post infection achieved a survival of 80% while the non treated control/infection group showed survival rates of 30% only.

In addition, it was observed that during the course of infection, the mouse groups which received both recombinant properdin and inhibitory MASP-2 antibody showed lower disease severity scores when compared to control/infection mice group or mice groups which received recombinant properdin or inhibitory MASP-2 antibody alone.

In conclusion, this study demonstrates that a transient blockage of the lectin pathway (achieved *in vivo* by application of inhibitory MASP-2–specific monoclonal antibodies) does augment alternative pathway activity. An additive, synergisitic effect is achieved *in vivo* by administration of recombinant murine properdin. The combined use of inhibitory MASP-2 antibodies along with the application of murine recombinant properdin will be a very effective way forward to develop a therapeutic approach to limit morbidity and mortality in meningococcal disease.

Chapter 6. Summary

Previous investigations have studied the different roles of different complement components in fighting *N. meningitidis* infections. The results of previous studies suggested the general importance of the complement system in the immune response (Estabrook *et al.*, 1997; Finne *et al.*, 1987; Jack *et al.,* 1998; *Jarva et al.,* 2005; Rossi *et al.*, 2001; Spath *et al.*, 1999; Vogel *et al.,* 1997). Complement activation is mediated via three pathways, the classical (CP), the alternative (AP) and the lectin pathway (LP). Regardless of the pathway involved, complement once activated, generates effector molecules which mediate an instant host response by orchestrating the initial phases of immune defence. The main consequences of complement activation are the opsonization of the pathogen, recruitment and activation of inflammatory and immunocompetent cells, and the direct killing of serum sensitive pathogens.

Neisseria meningitidis is an obligate human pathogen. *N. meningitidis* infections are the leading cause of bacterial meningitis and sepsis world-wide. The bacterium colonizes and lives as a commensal in the nasopharynx of about 10-40% of healthy human subjects. Under exceptional circumstances, the bacterium penetrates the mucosal epithelium and enters the bloodstream to cause meningococcal septicaemia followed by the invasion of CSF and meninges leading to fatal meningitis.

The aim of this thesis was to evaluate the role of the complement system with a particular focus on the lectin activation pathway in the host immune response against *N. meningitidis*.

The experimental approach used in current study involved a combination of complement specific *in vitro* assays along with *in vivo* infection experiments using a unique mouse model of total LP functional deficiency, a mouse line deficient of the lectin pathway effector enzyme MASP-2. Furthermore, the therapeutic utility of properdin, a key regulator of the AP of complement activation, was also evaluated in a murine model of meningococcal infection since there is prior published evidence for an essential role of properdin in an effective immune response to *N. meningitidis* infection.

6.1 Complement activation on the surface of *N. meningitidis* requires a close cooperation between the lectin and the alternative route of complement.

In the first part of this study, complement activation on different strains of *N. meningitidis* was evaluated by studying the deposition of the different complement activation products (C3b and C4b) as well as the deposition of carbohydrate recognition molecules of the lectin pathway in mouse serum (i.e. MBL-A, MBL-C, CL-11 and ficolin-A) on different strains of *N. meningitidis*. Lectin pathway activation on all of the tested meningococcal strains appears to be exclusively mediated by the lectin pathway recognition molecules, MBL-A and MBL-C since no binding of ficolin-A to *N. meningitidis* was observed. However, CL-11 showed a limited binding to *N. meningitidis.*

Similarly, significant levels of C3 deposition on different meningococcal strains when using alternative pathway specific conditions (i.e. high serum concentration and Mg^{2+} EGTA buffer) suggested an important role of the alternative pathway of complement

activation on *N. meningitidis*. In contrast, a low level C1q deposition on different meningococcal strains implied a limited role of the classical pathway activation on *N. meningitidis*. The results clearly demonstrated that all the tested strains of *N. meningitidis* were able to activate complement mainly through lectin pathway activation complexes as well as through the alternative pathway. Interestingly, in MASP-$2^{-/-}$ serum, a higher amount of C3 deposition observed on *N.meningitidis* following incubation with MASP-$2^{-/-}$ serum suggests that the alternative pathway either provides compensation to the absence of lectin pathway functional activity in MASP-2 deficient mice sera or that the remaining lectin pathway enzymes (i.e. MASP-1 and MASP-3) play a role in driving the alternative pathway on *N.meningitidis*.

6.2 Mice deficient of the lectin pathway effector enzyme MASP-2 show higher resistance to *N. meningitidis* infections

As part of *in vitro* assays, the role of the lectin pathway in killing of *N.meningitidis* was studied in mouse serum. It was found that *N.meningitidis* were killed faster in MASP-$2^{-/-}$ serum than in MASP-$2^{+/+}$ serum at the same serum concentration. The serum bactericidal activity (SBA) lysing *N.meningitidis* was further assessed following incubation of serogroup B *N.meningitidis* (strain MC58) with various complement deficient sera. A defective SBA against meningococci was observed following incubation with MASP-1/3 deficient serum. A recent study has demonstrated an essential role of mannose-binding lectin-associated serine proteases-1/3 (MASP-1/3) in activating the alternative pathway (AP) of the complement system (Banda *et al.,* 2011). In addition, this group has suggested

that MASP-3 complexed with MBL promotes alternative pathway activation by cleaving C3b bound factor B on the bacterial surface (Iwaki *et al.,* 2011). In the current study, a totally impaired SBA was observed when *N.meningitidis* was incubated with MBL-A/C$^{-/-}$ mouse serum. The SBA against *N.meningitidis* was also tested in various complement deficient human sera. A defective bactericidal activity of human MBL$^{-/-}$ as well as MASP-3$^{-/-}$ (but MASP-1 sufficient) serum against *N.meningitidis* was observed which identified for the first time that both MBL and MASP-3 are essential for driving SBA on meningococci.

In support of the results from *in vitro* studies, the role of the lectin pathway of complement activation was assessed in a murine model of meningococcal infection using MASP-2$^{-/-}$ mice and MASP-2$^{+/+}$ controls. *In vivo* studies demonstrated that following intraperitoneal challenge with a high dose (5x10^{7} CFU) of *N.meningitidis* serogroup A strain Z2491*,* MASP-2$^{+/+}$ (WT) mice showed an increased rate of mortality after 72 hrs of infection (50%) when compared to MASP-2$^{-/-}$ mice throughout (which all survived the infection, i.e. showed 0% mortality). In accordance, MASP-2$^{+/+}$ mice presented with significantly higher disease illness scores than MASP-2$^{-/-}$ mice. In support of the results from the survival experiment, MASP-2$^{+/+}$ mice showed higher bacterial loads in blood as well as in liver, spleen, kidney, lung and brain tissues compared to MASP-2$^{-/-}$ mice.

Studying the mRNA expression profiles for various cytokines during the course of *N.meningitidis* infection showed that MASP2$^{-/-}$ mice expressed significant lower levels of inflammatory cytokines (MIP-2, IFN-γ and IL-10) in spleens, livers and kidneys when

compared to the MASP-$2^{+/+}$ control group. The mRNA expression levels for different complement components, including C3, C1q and properdin were up-regulated and significantly higher in MASP-$2^{-/-}$ mice (kidneys, livers, spleen and brains) when compared to MASP-$2^{+/+}$ (WT) mice group following *N.meningitidis* infection.

Following intraperitoneal challenge of mice with *N.meningitidis* serogroup B strain MC58 with a high dose ($8x10^6$ CFU/mouse), MASP-$2^{+/+}$ mice group (80% mortality rate) were found to be significantly compromised against meningococcal infection showing higher bacteraemia with severe disease illness scores when compared to MASP-$2^{-/-}$ mice group (10% mortality rate).

No significant differences in survival rates were observed following intraperitoneal challenge of mice with *N.meningitidis* serogroup C (strain 6414) with MASP-$2^{-/-}$ mice showing survival rates of 80% compared to 40% survival rates for MASP-$2^{+/+}$ WT mice. However, a significantly higher bacterial load in blood, spleens, livers, kidneys and brains of infected MASP-$2^{+/+}$ mice was observed when compared to MASP-$2^{-/-}$ mice.

The second part of current thesis focused on assessment of the therapeutic benefits of lectin pathway inhibition in a murine model of meningococcal infection. Following infection of mice with a high dose of *N.meningitidis* via the intraperitoneal administration route, an application of specific anti MASP-2 antibodies was carried out in order to investigate the therapeutic benefits of a transient blockage of MASP-2 mediated LP-functional activity or a therapeutic depletion of MASP-2. Following the course of the infection experiments,

mice administered with an inhibitory MASP-2 antibody showed significantly improved survival (survival rate of 91.67%) when compared to the control mice group injected with an irrelevant isotype control antibody (survival rate of 50%).

6.3 Application of recombinant properdin enhances the SBA against *N.meningitidis* resulting in reduced mortality in an experimental murine model of meningococcal infection

In order to investigate the potential benefits of properdin administration to limit *N.meningitidis* infection, mice were injected before and after meningococcal infection with recombinant murine properdin. It was found that following infection with a high dose of *N.meningitidis*, mice injected with murine recombinant properdin showed a significant increase in survival when compared to the untreated infection control mice. Furthermore, the administration of recombinant properdin 3 hours before infection yielded significantly better survival rates (85.71%) when compared to mice treated 3 hours post infection (survival rates of 20%).

In addition, infected mice were also subjected to a combined administration of the inhibitory MASP-2 antibody and recombinant murine properdin in order to study a possible additive effect of both therapeutic approaches in limiting mortality and bacterial multiplication respectively. Following the course of infection, it was observed that mouse groups which received both properdin and the inhibitory MASP-2 antibody 3 hours before (90%) and 1 hour after (80%) the application of the infection dose showed significantly

better survival rates when compared to the control infection group (30% survival rates). Likewise, disease severity scores were significantly lower in mice groups receiving both properdin and inhibitory MASP-2 antibody when compared to control infection mice group or mice groups which received properdin or inhibitory MASP-2 antibody alone.

6.4 Final conclusion

The current study shows for the first time the importance of the co-operation between the lectin pathway-activation complexes and the alternative pathway in the complement mediated clearance of *N.meningitidis*. *N.meningitidis* is recognized by the lectin pathway specific recognition molecules (MBL-A and MBL-C), followed by the deposition (C4b and C3b) and activation of downstream complement components leading to opsonophagocytosis of the bacteria by immune cells or direct lysis of meningococci by the assembly of the MAC in the cell wall of bacteria.

Meningococcal components (such as LOS and peptidoglycan) released during the course of systemic infection are suggested to be responsible for the induction of a potentially devastating inflammatory response which is mediated by the effector molecules of the lectin pathway as well as the alternative pathway of complement activation. However, absence of MASP-2 in MASP-2 deficient mice leads to a favourable outcome following meningococcal infection. MASP-$2^{-/-}$ mice showed higher serum bactericidal activity *in vitro* which is likely to account for the enhanced clearing of meningococci *in vivo*. A totally impaired bactericidal activity of human $MBL^{-/-}$ as well as MASP-$3^{-/-}$ serum at a higher serum concentration against *N.meningitidis* suggested that MASP-3 cooperates with MBL

in the generation of the alternative pathway C3 convertase (C3bBb) on the bacterial surface. The therapeutic interventions targeting lectin pathway (achieved *in vivo* by application of inhibitory MASP-2–specific monoclonal antibodies) leading to enhanced alternative pathway activity (achieved *in vivo* by administration of mouse complement factor properdin) show better survival in a murine model of meningococcal infection.

In conclusion, results from *in vitro* experiments as well *in vivo* infection experiments suggested an important MASP-2 independent role of the lectin pathway specific enzyme MASP-3 in driving the alternative pathway mediated complement activation to fight *N.meningitidis* infections.

6.5 Future work

6. 5.1 Assessment of recombinant properdin and anti MASP-2 antibody treatment in other pathological conditions

The present study underlines the previously described critical role of properdin in driving the alternative pathway to promote bacteriolytic activity of plasma/serum complement to fight and prevent *N.meningtidis* infections. A beneficial therapeutic application administering recombinant properdin has been established in current models of *N.meningtidis* infections. Furthermore, the combined administration of recombinant properdin and an antibody based MASP-2 inhibitor will be analysed under varying physiological conditions.

6.5.2 The role of phagocytic cells in killing of *N. meningitidis*

A critical role of complement-mediated serum bactericidal activity against *N.meningtidis* is highlighted in this study. It will be interesting to study the interdependence and the cross-talk between complement-mediated opsonization and bacteriolysis of *N.meningtidis* with the activity of the phagocytic elimination of this pathogen. This can be accomplished by comparing killing of *N. meningitidis* following opsonization with normal human serum with sera from individuals with deficiencies of terminal complement components such as C5, C6 or C8 deficient sera.

6.5.3 Role of MASP-1/3 in murine model of meningococcal infection

A total loss of complement mediated killing of *N.meningtidis* was observed in MASP-1/3$^{-/-}$ serum. The susceptibility of MASP-1/3$^{-/-}$ mice in murine model of meningococcal infection should be tested in more detail to further understand the physiological role of the MASP-1/MASP-3 effector novel arm of the lectin pathway.

6.5.4 Meningococcal study in properdin deficient mice

The susceptibility of properdin deficient mice against *N.meningtidis* in a mouse model of meningococcal infection can also be tested. Application of recombinant murine properdin to reconstitute properdin deficient mice will be analysed following infection of mice with *N.meningtidis* to assess the efficacy of properdin replacement therapy in properdin deficient individuals.

Chapter 7. Appendices

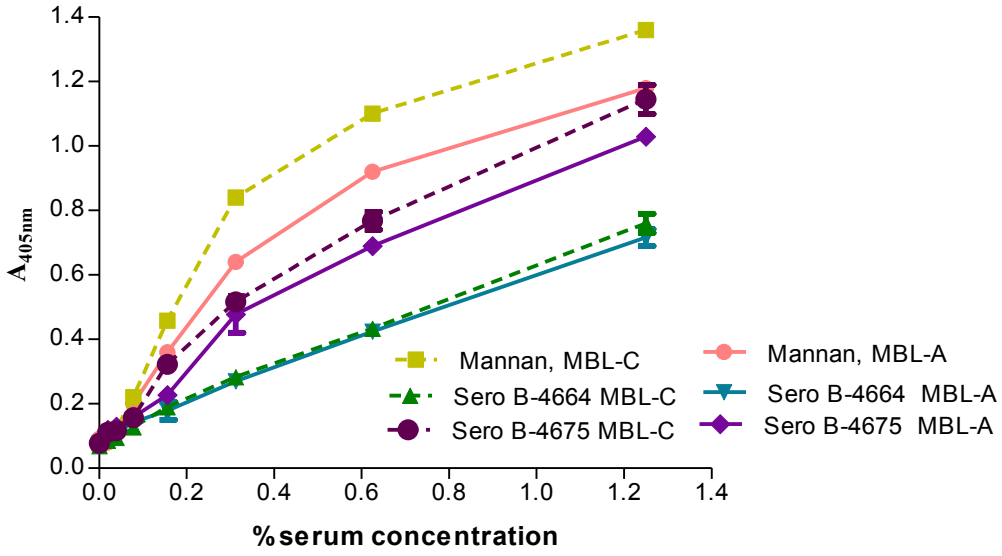

Figure 7.1: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving buffer instead of serum were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

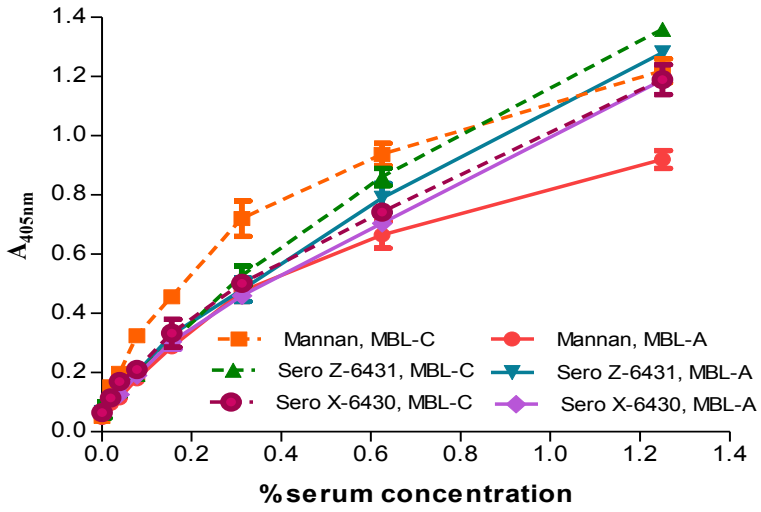

Figure 7.2: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

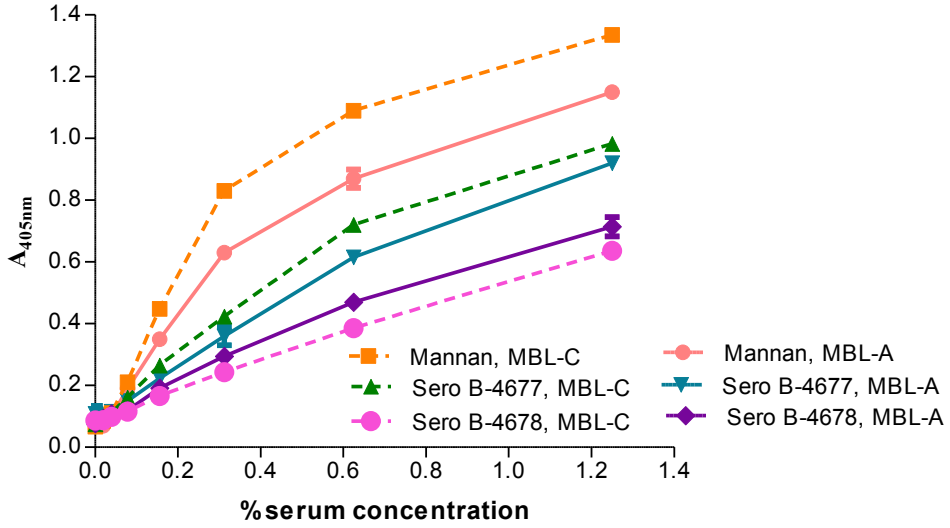

Figure 7.3: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

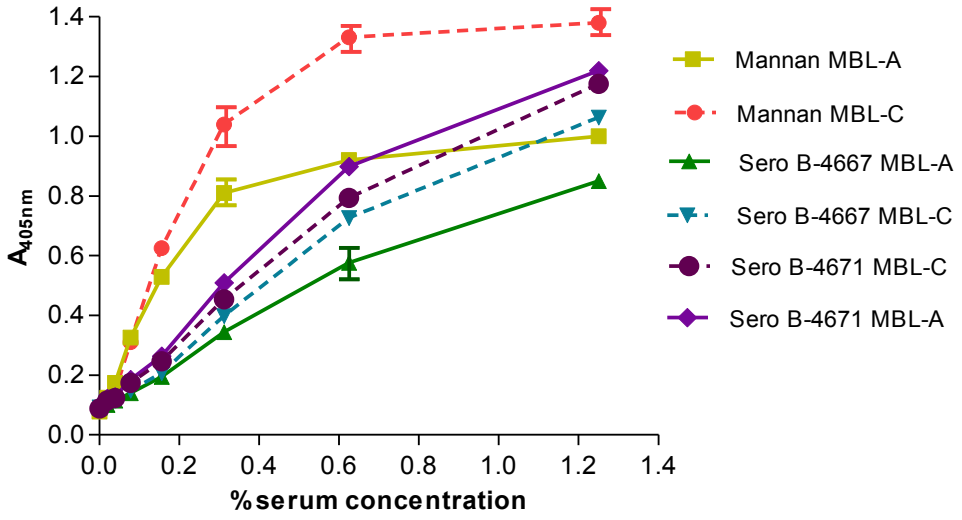

Figure 7.4: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

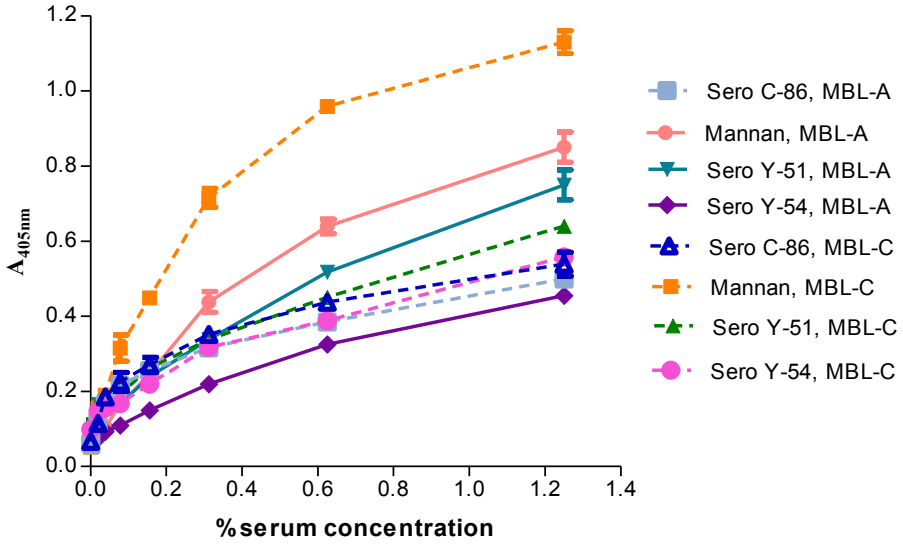

Figure 7.5: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

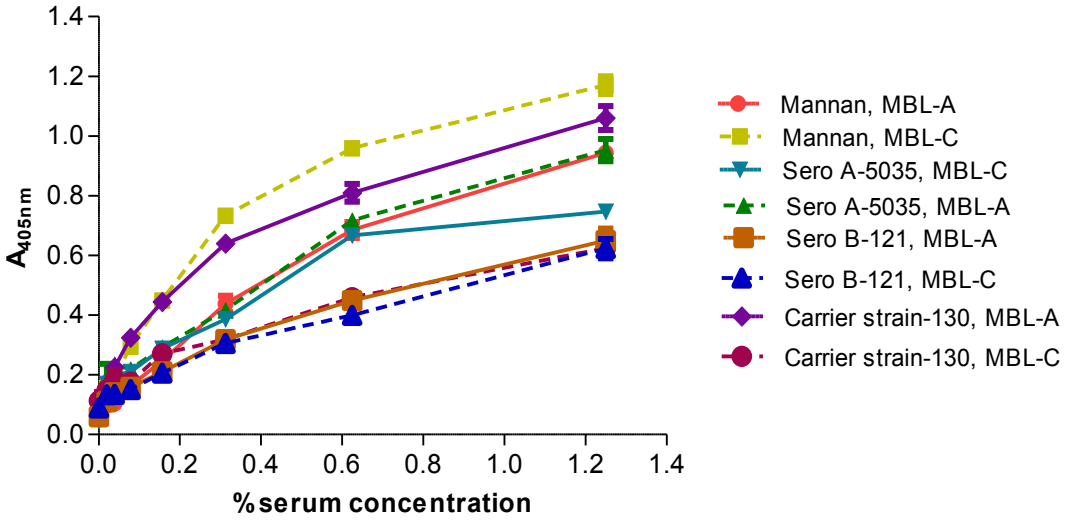

Figure 7.6: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

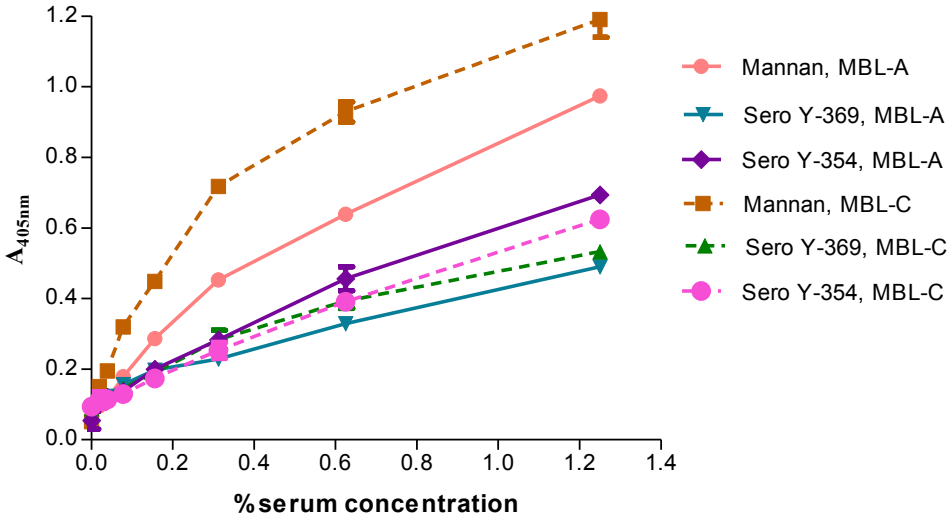

Figure 7.7: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

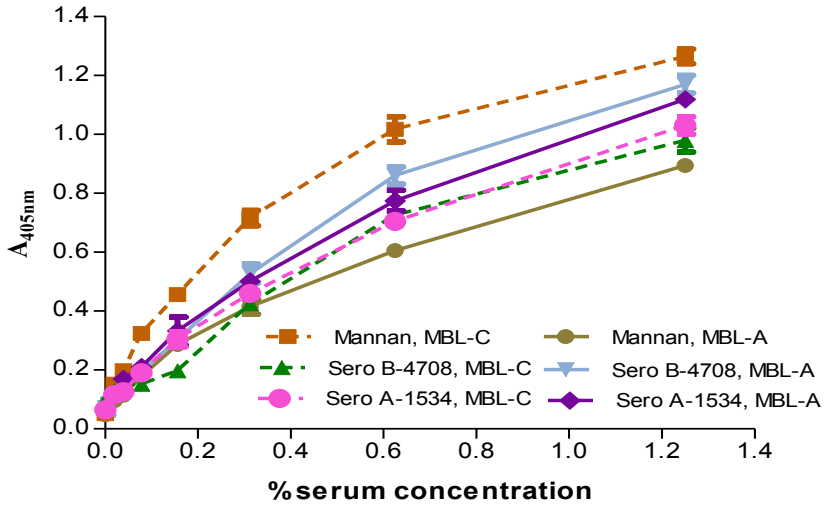

Figure 7.8: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

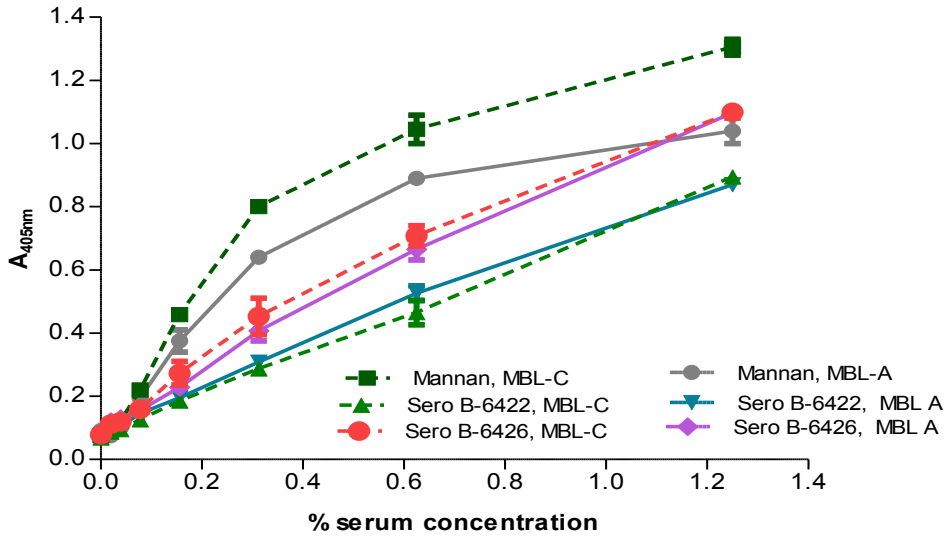

Figure 7.9: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. Wells receiving only buffer were used as negative controls. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM

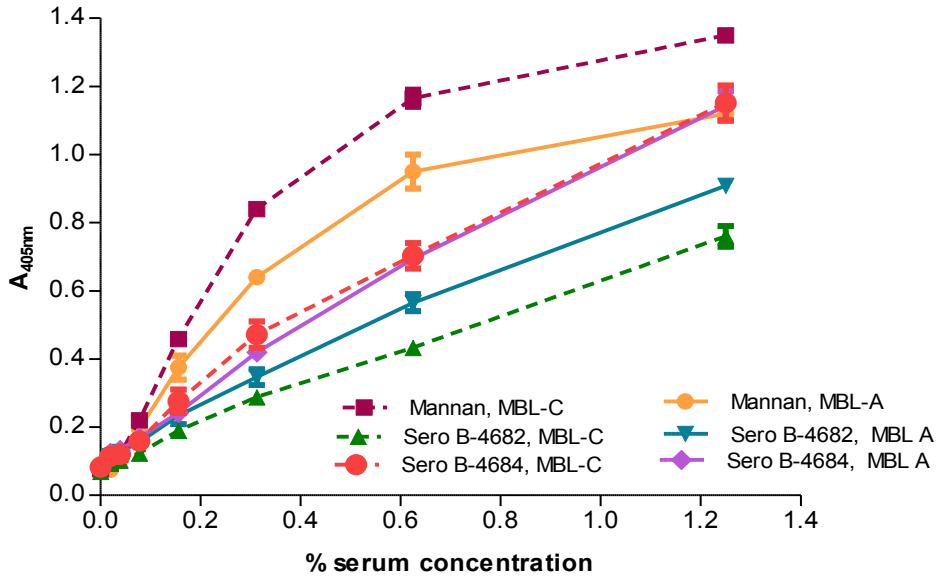

Figure 7.10: MBL-A and MBL-C deposition on the surface of different strains of *N.meningitidis* using wild-type mouse serum. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

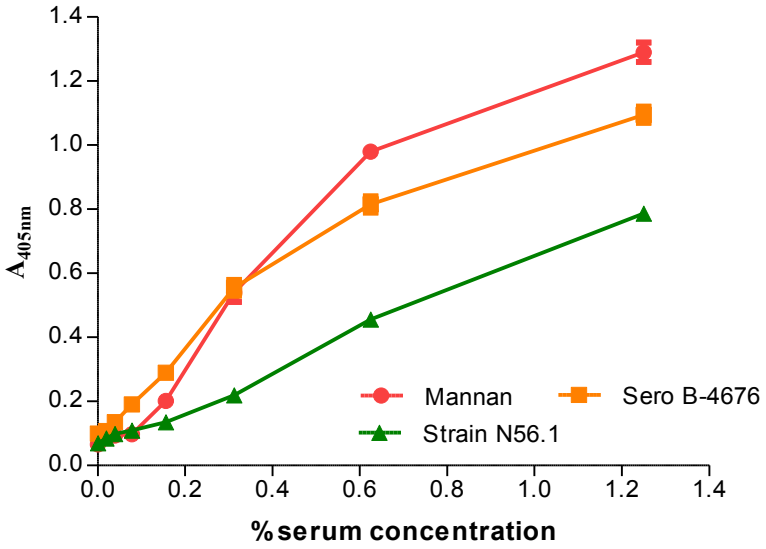

Figure 7.11: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

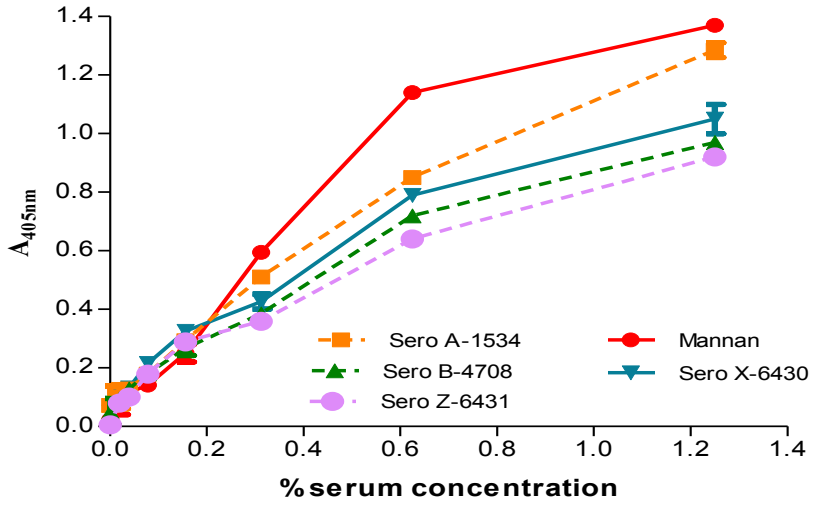

Figure 7.12: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

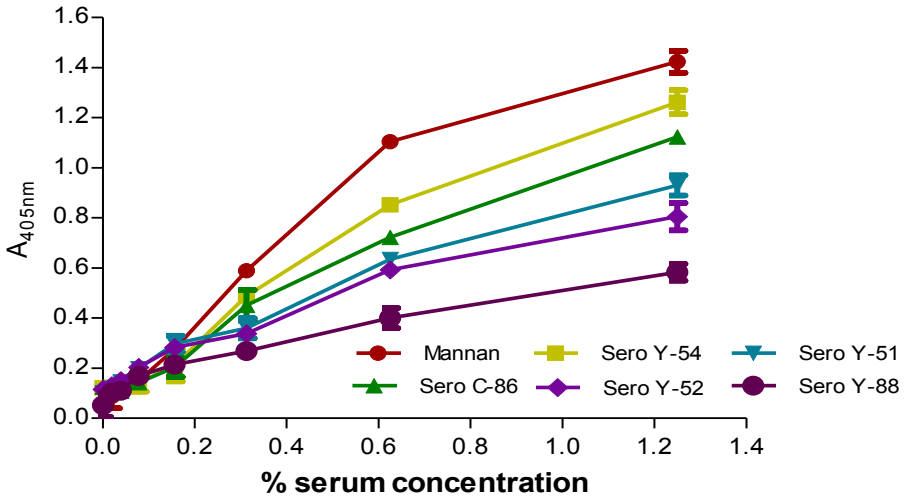

Figure 7.13: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

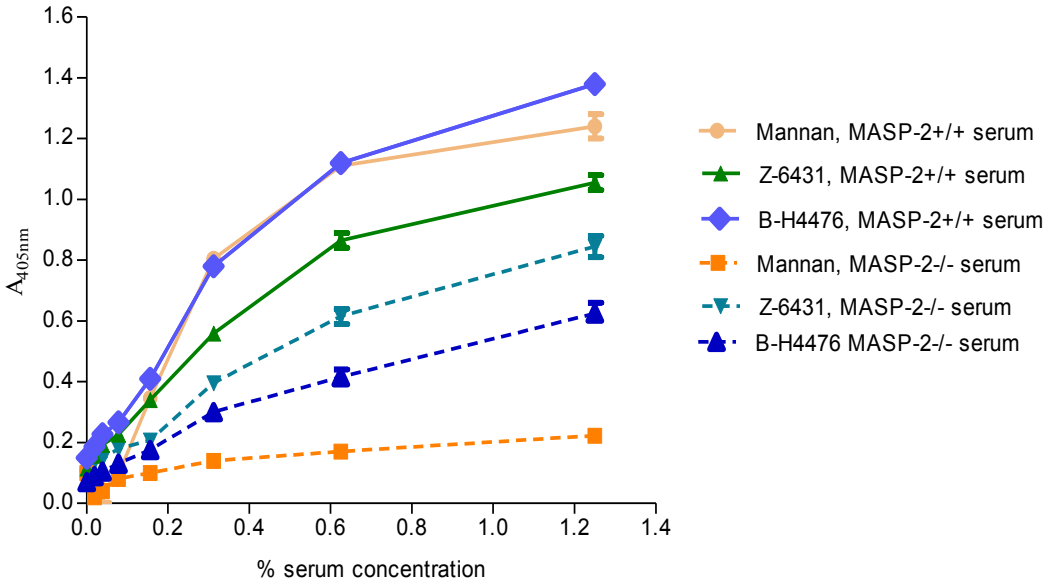

Figure 7.14: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

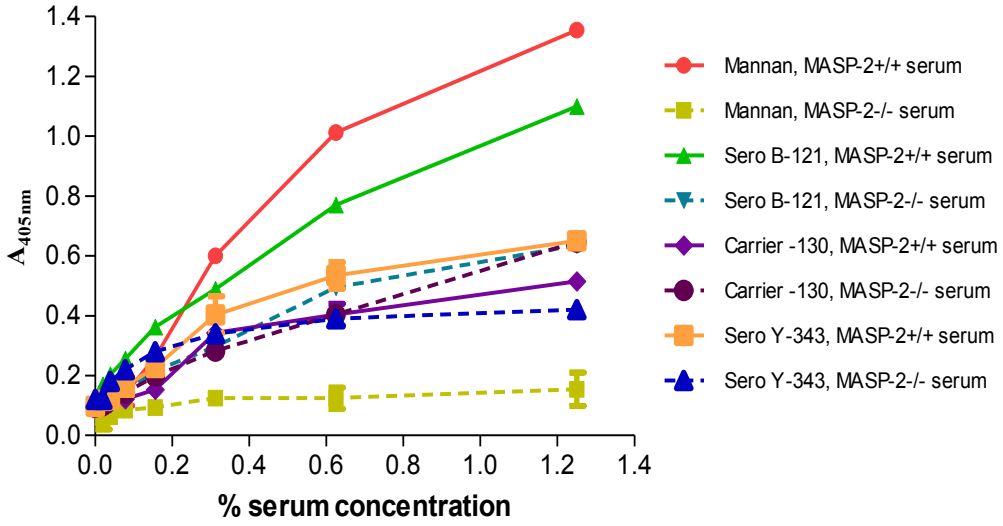

Figure 7.15: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

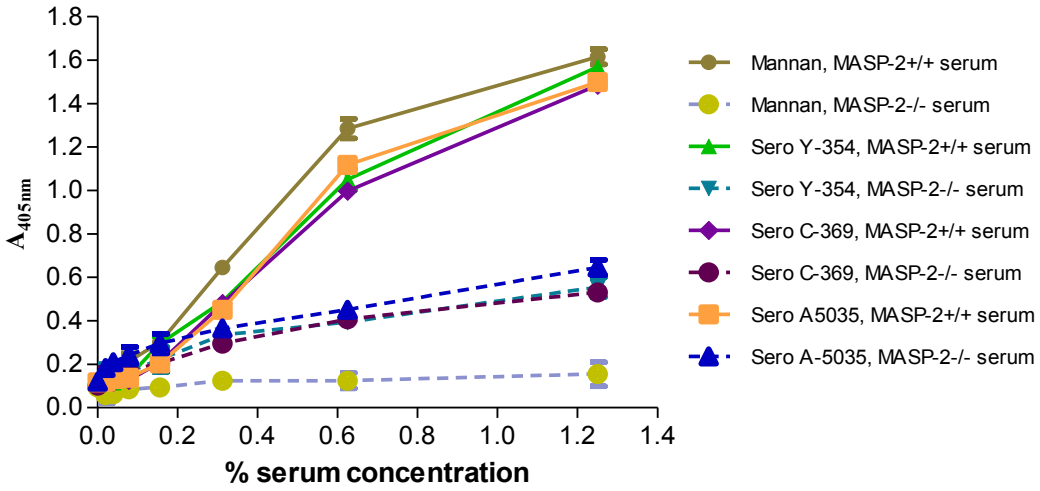

Figure 7.16: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

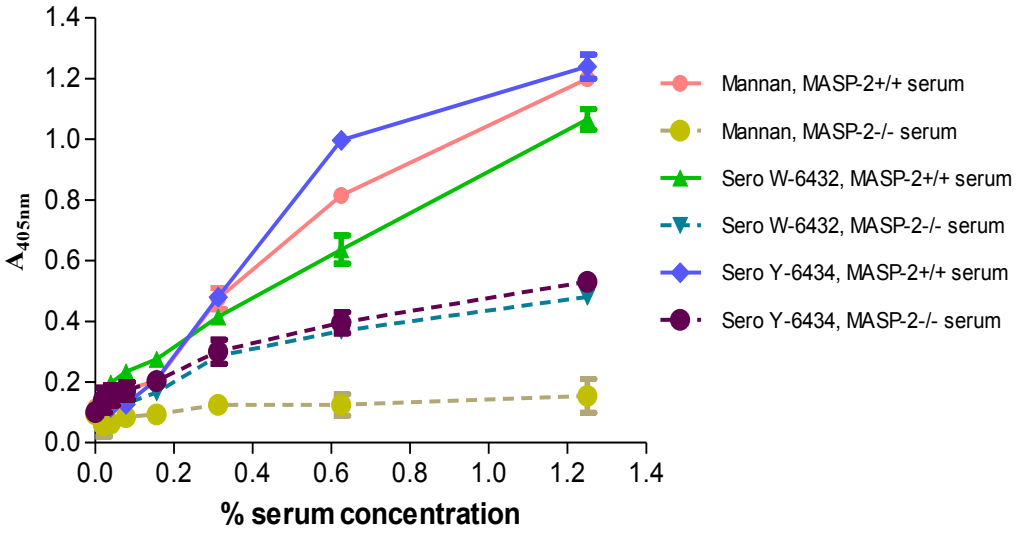

Figure 7.17: C3c deposition on the surface of different strains of *N.meningitidis* using mouse serum under buffer conditions allowing activation via the classical and the lectin pathway. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

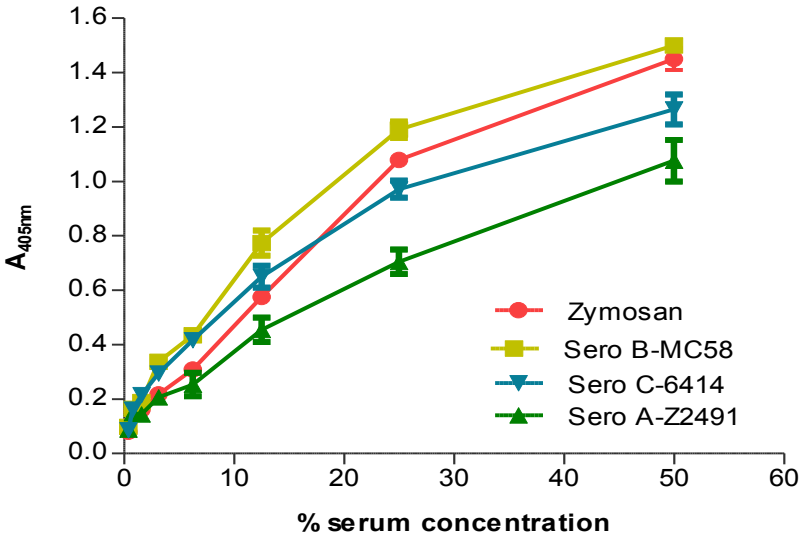

Figure 7.18: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of human serum under conditions that selectively allow activation via the alternative pathway. Zymosan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

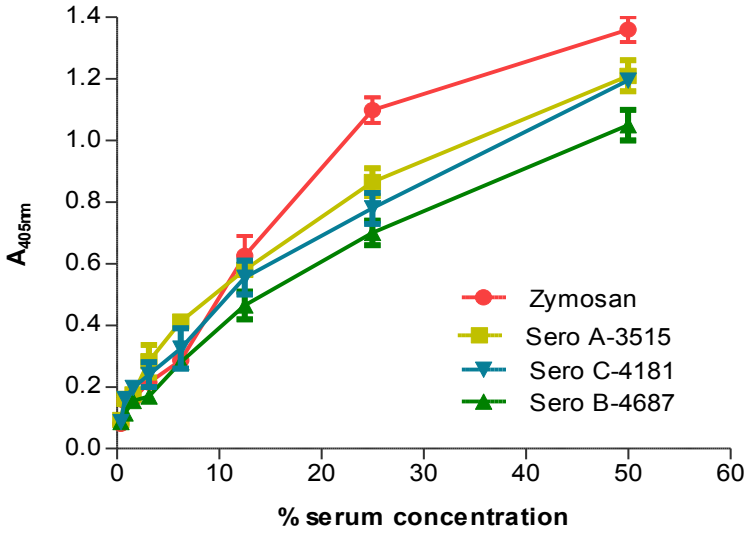

Figure 7.19: C3c deposition on the surface of different strains of *N.meningitidis* using dilutions of human serum under conditions that selectively allow activation via the alternative pathway. Zymosan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

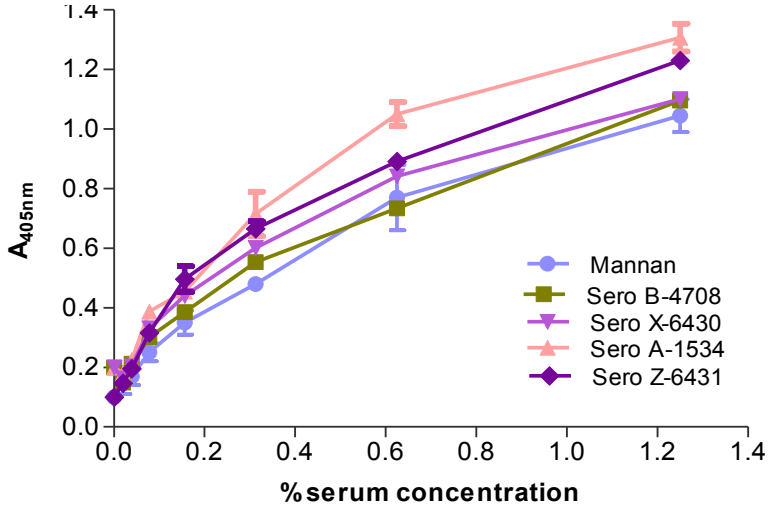

Figure 7.20: C4c deposition on the surface of different strains of *N.meningitidis* exposed to mouse serum diluted in high salt buffer i.e. conditions allowing only the lectin pathway specific activation. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

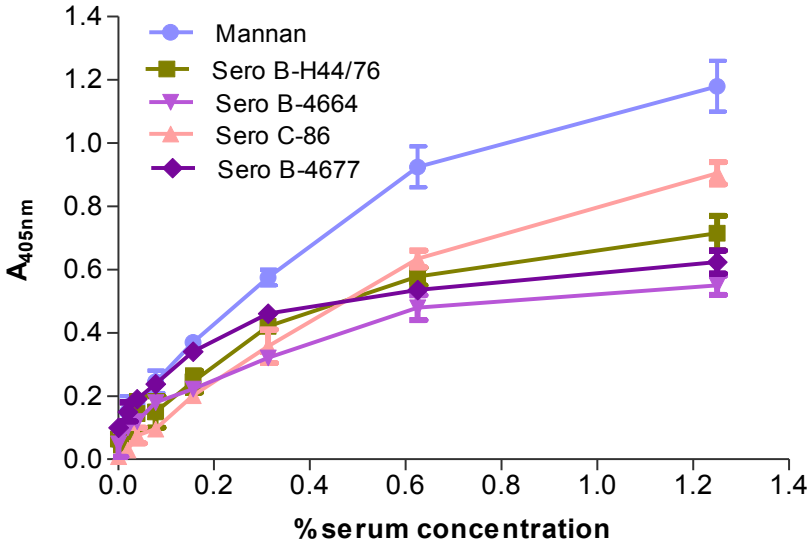

Figure 7.21: C4c deposition on the surface of different strains of *N.meningitidis* exposed to mouse serum diluted in high salt buffer i.e. conditions allowing only the lectin pathway specific activation. Mannan coating was used as a positive control. This is a typical example representing a series of identical experiments. The results represent duplicate experiment for each time point on the serum concentration scale and are expressed as means±SEM.

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