



Deciphering the molecular composition of two independent activation cascades of the lectin pathway of complement

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by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled “Deciphering the molecular composition of two independent activation cascades of the lectin pathway of complement” is based on work conducted by the author at the University of Leicester mainly during the period between October 2011 and September 2014.

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.

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Abstract

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Sadam Yaseen

The lectin pathway of complement activation is driven by pattern recognition molecules that direct activation of three different effector enzymes, called MASP-1, MASP-2 and MASP-3 (for Mannan binding lectin Associated Serine Protease). They drive complement activation through two independently operating effector arms. One effector arm (LEA-1) amplifies complement activation through MASP-3 dependent initiation of the alternative pathway amplification loop, while the second effector arm (LEA-2) is MASP-2 dependent and drives complement activation through the lectin pathway C3 and C5 convertases, C4bC2a and C4bC2a (C3b)_n. Recently, a residual MASP-2 dependent C4-bypass route to activate C3 has been described in C4 deficient individuals.

The first part of this thesis defines the molecular mechanism behind this C4-bypass activation route and demonstrates that MASP-2 can directly cleave native C3 to deposit C3b and iC3b on activator surfaces.

The second part of this thesis studied the natural substrates and activators of MASP-3 to elucidate the sequence of molecular events that lead to alternative pathway activation via LEA-1. My results demonstrate that MASP-3 can be activated by both MASP-1 and MASP-2 and that activated MASP-3 directly cleaves pro-FD, but not zymogen FB. While reconstitution of the deficient alternative pathway functional activity in MASP-1/-3 deficient mice could not be achieved by adding recombinant MASP-1, addition of either enzymatically active MASP-3 or injection of zymogen MASP-3 into these mice restored alternative pathway functional activity, underlining that MASP-3 is the predominant enzyme that drives LEA-1.

Finally, the important role of MASP-1 and MASP-3 in the innate immune response to infection was demonstrated through the dramatically increased susceptibility of MASP-1/-3 deficient mice to *S. pneumoniae* infection.

Notably, the defective C3b/iC3b opsonization of *S. pneumoniae* in MASP-1/-3 deficient mouse serum could be restored by reconstitution with recombinant MASP-3.

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List of abbreviation

3MC	Malpuech, Michels and Mingarelli-Carnevale syndromes
aa	Amino acid
AMD	Age-related Macular Degeneration
AP	Alkaline Phosphate
Arg	Arginine
Asp	Aspartic acid
BBS	Barbital Buffer Saline
BHI	Brain Heart Infusion
bp	base pair
BSA	Bovine Serum Albumin
C1-INH	C1-Inhibitor
C1r	Complement components 1r
C1s	complement components 1s
C3aR	C3a Receptor
C4BP	C4-bBinding Protein
C5aR	C5a Receptor
CCP	Complement Control Protein
cDNA	Complementary DNA
CFU	Colony Forming Unit
CHO	Chinese Hamster Ovary
CL-11	Collectin-11
CL-K1	Collectin Kidney 1
CL-L1	Collectin Liver
CR	Complement Receptor
SRCR	Scavenger Receptor Cysteine-Rich
CRD	Carbohydrate Recognition Domain
CRP	C-reactive protein
CUB	Complement C1r/C1s, Uegf, Bone morphogenetic protein

DAF	Decay Accelerating Factor
dATP	Deoxyadenosine Triphosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EAE	Experimental Autoimmune Encephalopathy
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol-bis-N,N,N',N'-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FB	Factor B
FBdpl	FB depleted serum
Fc	Fragment crystallizable
FD	Factor D
FDdpl	FD depleted serum
FH	Factor H
FHL-1	FH-Like Protein 1
FI	Factor I
FIM	Factor I Membrane attack complex
GalNAc	<i>N</i> -acetylgalactosamine
GlcNAc	<i>N</i> -acetylglucosamine
GVB	Gelatin Veronal Buffer
HSA	Human Serum Albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High Fidelity
His	Histidine
HIV	Human Immunodeficiency Virus
HRP	Horse Reddish Peroxidase
HSV	Herpes Simplex Virus
HUS	Hemolytic Uremic Syndrome

i.p.	Intraperitoneal
IAV	Influenza A Virus
Ig	Immunoglobulin
IGFBP-5	Insulin-Like Growth Factor Binding Protein-5
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ile	Isoleucine
IPD	Invasive Pneumococcal Disease
IPTG	Isopropyl Thiogalactoside
kDa	Kilodalton
KO	Knockout
LB	Luria-Bertani
LDL	low-density lipoprotein receptor
LEA	Lectin pathway Effector Arm
LTA	Lipoteichoic Acid
MAC	Membrane Attack Complex
MAp	MBL-Associated protein
MASP	MBL-Associated Serine Protease
MBL	Mannan Binding Lectin
MBP	Mannan Binding Protein
MCP	Membrane Cofactor Protein
MEM	Minimum Essential Medium
mL	Milliliter
mM	Millimole
MOPS	3-(N-morpholino)propanesulfonic acid
MPGN	membranoproliferative glomerulonephritis
ng	Nanogram
NHS	Normal Human Serum
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis

PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNH	Paroxysmal Nocturnal Haemoglobinuria
Pro-FD	Pro-Factor D
PspA	Pneumococcal Surface Protein A
PspC	Pneumococcal Surface Protein C
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Ser	Serine
Serpin	Serine protease inhibitor
SLE	Systemic Lupus Erythematosus
sMAP	small MBL-Associated Protein
SNP	Single Nucleotide Polymorphism
SP	Serine Protease
TAE	Tris-base/ Acetic acid/EDTA
TBE	Tris Buffered Saline with EDTA
TBS	Tris Buffered Saline
TBST	TBS Tween-20
TEMED	Tetramethylethylenediamine
Tfb	Transformation buffer
Tris/EDTA	Tris-borate/EDTA
v/v	volume/ volume
VPR-AMC	Aminomethylcoumarin conjugated Boc-Val-Pro-Arg (VPR)
vWF	von Willibrand Factor
w/v	weight/ volume
WT	Wild Type

Xaa	Any amino acid
Xgal	5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μL	Microliter

Nomenclature

The nomenclature for the complement components conforms to that adopted by the Complement Nomenclature Committee (CNC), 2014 (Kemper et al. 2014).

Chapter One: General introduction

1.2: The innate immune system

The innate immune system provides a front-line of defense against common pathogens. An effective innate immune response is composed of cellular and humoral elements that orchestrate a strong and effective protection against invaders. The cellular elements of the innate immunity include phagocytic cells and epithelial cells whereas the humoral elements involve coagulation factors, complement components, cytokines and chemokines (Takahashi 2011).

The innate arm of immunity responds instantly and non-specifically to the pathogens and altered self-cells, including damaged tissues, and promptly and properly removes pathogens and apoptotic, necrotic and infection damaged cells. The primary immune response includes identification of foreign substances or altered self-cells and then marks these (e.g. through C3b opsonization) to be removed through phagocytosis and/or to be presented to effector cells of the acquired immune system which on their own cannot provide instant responses to pathogens that have not triggered an adaptive immune response in previous exposure. It usually takes up to four days for an adaptive immune response to develop and in some cases it can take weeks or months to develop a strong and lifelong acquired immunity (Takahashi 2011).

An effective innate immune protection requires appropriate identification factors to differentiate between self and non-self patterns and recruit cellular and humoral factors that can eliminate the marked target. A major component of innate immunity is the complement system.

1.3: The complement system

The complement system was first described as a substance in serum that can kill bacteria in the late 19th century by Hans Buchner. In 1889 Jules Bordet described that this substance that Buchner called “Alexion” is composed of two components: one loses its activity after being heated (heat-labile) and another maintained the killing activity after heating (heat-stable) which represents the antibodies (Zipfel et al. 2013). The name complement for this serum defense system was coined by Paul Ehrlich in 1890 as this serum system complements the ability of antibodies and phagocytic cells to clear pathogens from an organism. The human complement system consists of seven non-enzymatic complement components (C3-C9), nine serine proteases (C1r, C1s, C2, MASP-1, MASP-2, MASP-3, FB, FD and FI), six pattern recognition molecules (C1q, MBL, M-ficolin, L-ficolin, H-ficolin, CL-11) and more than 25 complement regulators and receptors (Ricklin et al. 2010). Although the liver hepatocytes are generally considered to be the primary site for complement protein biosynthesis, extra-hepatic sources of complement synthesis have been identified; these include blood monocytes, tissue macrophages, pulmonary alveolar epithelial cells, adipose tissues, osteoblasts and skeletal myoblasts (Laufer et al. 2001). Three distinct pathways initiate the activation of the complement system namely the classical pathway, the alternative pathway, and the lectin pathway (figure 1-1).

The classical pathway is primarily an antibody-dependent complement activation route. It is strongly activated by clusters of IgG or IgM on antigen surface, which are recognized by the classical pathway pattern recognition subcomponent C1q. However, the classical pathway is also initiated by cell wall components of Gram-negative bacteria, viral envelopes, and C-reactive protein (Claus et al. 1977). The binding of C1q will subsequently lead to the activation of the C1q-associated serine proteases C1r and C1s (which are present as heterotetramers (C1s:C1r:C1r:C1s) within the C1q(C1r)₂(C1s)₂ complex. Upon activation, C1r is auto-activated and it cleaves and activates its only substrate C1s. Activated C1s cleaves C4 into C4a (9 kDa) and C4b (185 kDa). In a second

cleavage step, C1s cleaves C4b-bound C2 into C2a (60 kDa) and C2b (30 kDa). C4a and C2b are released into the microenvironment, while C4b and C2a stay together bound to the complement activator surface (immune complexes, microorganisms) to form the C3 convertase C4bC2a (Sim et al. 1993, Fujita et al. 2004, Schwaeble et al. 2011).

The other routes of complement activation, the lectin and the alternative activation pathways, are primarily antibody-independent. The lectin pathway is initiated by the binding of the carbohydrate recognition molecules i.e. Mannose Binding Lectin (MBL), Collectin-11 (CL-11) and ficolins (M-ficolin, L-ficolin, H-ficolin) to pathogen surfaces. The binding of the carbohydrate-recognition subcomponent initiates the activation of the three-lectin pathway-specific serine proteases called mannose binding lectin associated serine proteases i.e. MASP-1 (Matsushita and Fujita 1992), MASP-2 (Thiel et al. 1997), and MASP-3 (Dahl et al. 2001). Over the recent years, it emerged that the lectin pathway drives complement through two independently operating effector arms, termed LEA-1 and LEA-2 (for lectin pathway effector arm-1 and -2). The first evidence of the existence of the lectin pathway effector arm LEA-1 was provided by Teizo Fujita's group showing that MASP-1/-3 deficient mice have severely compromised alternative pathway functional activity (Takahashi et al. 2010). It was suggested that MASP-1 cleaves the complement pro-FD, which in turn activates C3b-bound complement zymogen FB resulting in activation of the alternative pathway. A subsequent paper by the same research group also suggested that MASP-3 may cleave FB in C3bB complex directly (Iwaki et al. 2011). In my thesis, I have assessed the different possibilities using recombinant and purified single components in protein-biochemical assays.

My results led to the conclusion that LEA-1 is driving complement activation in a lectin pathway dependent fashion through the effector enzyme MASP-3 which essentially converts zymogen pro-FD into its active form. This allows FD to convert the alternative pathway pro-enzymatic complex C3bB into its enzymatically active form. The results presented in this thesis show that for the

conversion of pro-FD, MASP-3 needs to be cleaved and activated by either MASP-1 or MASP-2 in a lectin pathway specific manner.

The second effector arm of the lectin pathway, LEA-2, is MASP-2 dependent and drives complement activation through the formation of the lectin pathway C3 and C5 convertases C4bC2a and C4bC2a(C3b)_n, respectively. Like the classical pathway effector enzyme C1s, MASP-2 cleaves C4 into C4a and C4b. In a second cleavage step, MASP-2 cleaves C4b-bound C2 into C2a and C2b generating C4bC2a the C3 convertase of the lectin pathway.

The C3 convertase generated by the classical pathway or by the lectin pathway cleaves C3 into C3a (9 kDa) and C3b (171 kDa), and the end product C3b binds in turn in close proximity to the C3 convertase to form a C5 convertase, C4bC2a(C3b)_n (Matsushita et al. 2000a, Fujita et al. 2004).

The alternative pathway accounts for 80-90% of complement activation (Ricklin et al. 2010). C3b resulting from the classical or the lectin pathways can initiate the alternative pathway. However, C3b must bind to the complement activator surface to initiate the alternative pathway. Factor B (FB) binds to a C3b-bound activator surface and undergoes conformational changes exposing a new cleavage site for factor D (FD). FD cleaves C3bB to form the alternative pathway C3 convertase C3bBb (Sim et al. 1993). However, another route of alternative pathway activation occurs at low constant rate in the blood by spontaneous hydrolysis (tick-over) of the thioester bond in native C3 to produce C3(H₂O), which exposes new binding sites for FB which in turn binds to C3(H₂O) to form C3(H₂O)B complex. FD cleaves FB in the C3(H₂O)B complex forming C3(H₂O)Bb, another form of alternative pathway C3 convertase (Ricklin et al. 2010).

Prior to the discovery of the lectin pathway dependent involvement of MASP-3 in the activation of the alternative pathway zymogen pro-FD no specific pattern recognition mechanism has been described to initiate alternative pathway activation. Recent work proposed that properdin could act as a pattern recognition molecule that binds to microbial pattern of pathogens and initiates

alternative pathway activation (Ricklin et al. 2010). However, it had become clear that properdin does not bind to the bacterial surface in absence of C3, indicating that properdin binding to pathogen surfaces is secondary and only occurs in conjunction with pathogen bound alternative pathway C3 convertase complexes (Harboe and Mollnes 2008).

As noted above, the C3b and FB form C3 convertase of the alternative pathway whereas C4b and C2a form the convertase of the classical and the lectin pathways. The convertases cleave C3 into C3a and C3b. C4b and C3b in the C3 convertase complex provide a binding site for complement C5 and facilitate its cleavage by C2a (Sim et al. 1993). The accumulation of multiple C3b cleavage products in close proximity of the C3 convertase allows the component C5 to be cleaved into its fragments C5a (11 kDa) and C5b (180 kDa). C5b has no thioester bond so that it does not bind covalently to the surface, but it undergoes conformational changes leading to the exposition of a binding site for C6. The subsequent binding of C6, C7 and C8 forms the C5b-8 complex which facilitates polymerization of C9 and insertion of poly C9 into cellular membranes and pathogen surfaces which may initiate their osmotic lysis through the pore formed by poly C9 and even nucleated cells can be lysed via a multi-hit process (Podack et al. 1984, Sim et al. 1993).

In addition to the three pathways of complement activation described above, new possible routes of complement activation have been suggested several years ago. It was proposed that certain coagulation proteases such as thrombin and plasmin can activate C3 directly (Markiewski et al. 2007). Moreover, it was proposed that thrombin may directly cleave C5 generating C5a and C5b also in absence of C3 (Huber-Lang et al. 2006).

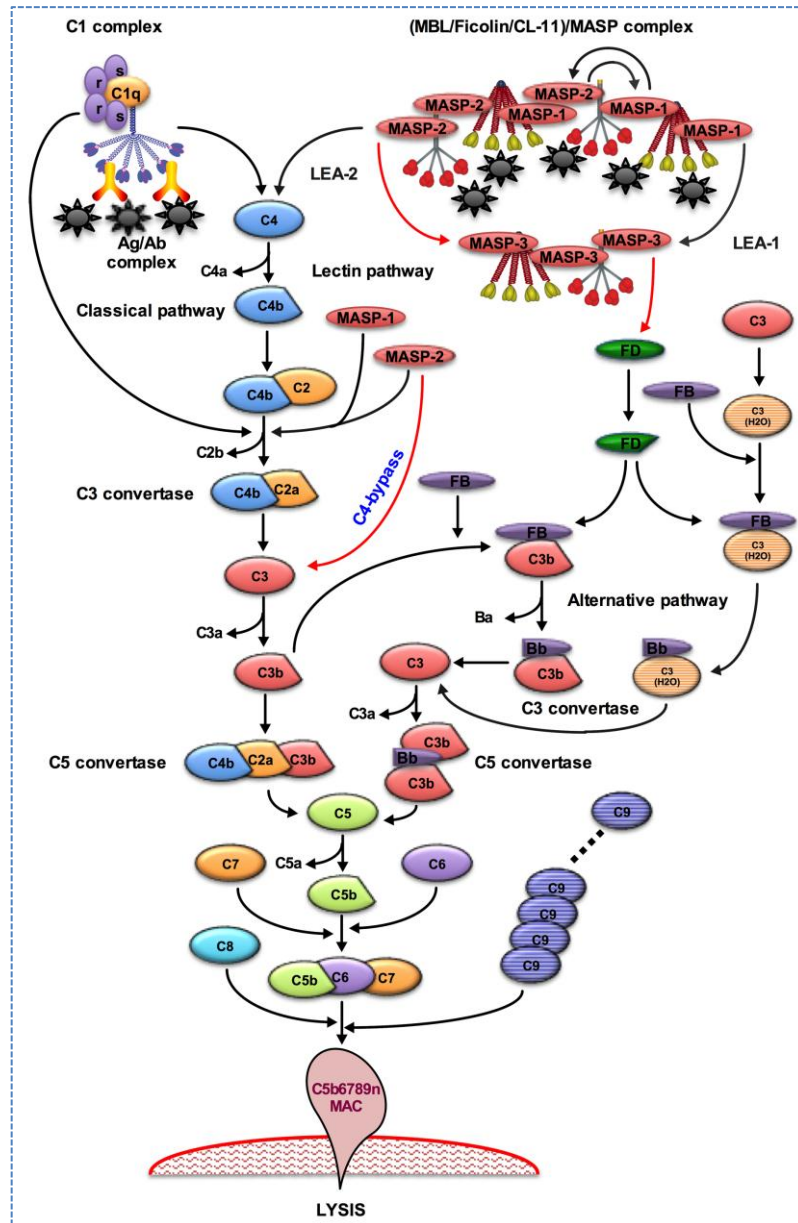


Figure 1-1: Schematic representing the activation of the complement cascade

Three distinct pathways the classical, the lectin and the alternative pathways activate the complement system. The lectin pathway has two effector arms, the Lectin pathway Effector Arm 1 (termed LEA-1), which promotes amplification of complement activation through MASP-3 dependent cleavage of pro-FD into enzymatically active FD leading to the conversion of alternative pathway convertases into their enzymatically active form and the Lectin pathway Effector Arm 2 (LEA-2), which drives complement activation through the MASP-2 dependent formation of the lectin pathway C3 and C4 convertases C4bC2a and C4bC2a (C3b)n, respectively. All three pathways converge at the stage of C3 activation. The C3 cleavage product C3b can then either initiate the generation of further alternative pathway C3 convertases (C3bBb) or become part of the C5 convertase complexes of all of the 3 pathways and initiate the terminal activation pathway leading to the formation of the Membrane Attack Complex (MAC). The diagram has been modified to include the C4-bypass mechanism and the MASP-3 dependent cleavage of the alternative pathway component pro-FD, as identified in this study (red arrows).

1.4: Biological effects of the complement activation

The major function of the complement system is to opsonize dead cells, invading microbial organisms and fragments thereof to facilitate their uptake and elimination by phagocytic cells. Upon infection, the complement system is activated locally to remove microbial invaders, a process that could affect bystander cells at the site of infection. The three pathways of the complement activation converge at the stage of C3 activation on the surface of parasites, bacteria or viruses. During the activation, pro-inflammatory signals are constantly released including C3a and C5a, which trigger phagocytosis. Anaphylatoxin C3a and C5a are strong chemoattractants that attract blood monocytes, tissue macrophages and neutrophils to the site of infection to uptake the opsonized targets (Ricklin et al. 2010). The activation of C3, on the other hand, leads to the generation of Membrane Attack Complexes (MACs), which can mediate the lysis of microorganism.

Decades after the discovery of the complement system it has become clear that its function far exceeds the elimination of the infectious agents. The complement system efficiently contributes in clearance of immune complexes and apoptotic cells (Ricklin et al. 2010, Bohlsón et al. 2014). The apoptotic cells undergo structural changes on the surface due to loss of some of the surface-bound complement regulators, such as membrane cofactor protein and protectin allowing the pattern recognition components of complement to opsonize and facilitate its uptake by phagocytes. The complement system, on the other hand, plays a pivotal role in clearance of immune complexes and cellular debris from the body to prevent development of inflammatory diseases such as Systemic Lupus Erythematosus (SLE) (Ricklin et al. 2010).

Certain proteases of the complement system, including MASP-1 and MASP-2, were found to activate the coagulation system via converting prothrombin to thrombin which enhances the clot formation consequently preventing pathogens dissemination during the infection (Hajela et al. 2002, Krarup et al. 2007, Krarup et al. 2008).

The complement system also can contribute to pathophysiology and development of several autoimmune diseases especially in individuals with deficiency or dysfunction of the regulatory complement components. Excessive complement activation leads to the development of inflammatory diseases such as Age-related Macular Degeneration (AMD), Paroxysmal Nocturnal Hemoglobinuria (PNH), atypical Haemolytic Uremic Syndrome (aHUS) and membranoproliferative glomerulonephritis (Ricklin et al. 2010). On the other hand, acute-phase disorders such as ischaemia-reperfusion injuries are complement activation-mediated tissue damage pathologies and that enhance tissue loss in stroke, myocardial infarction and following vascular surgeries. Reperfusion of ischaemic tissue induces inflammatory responses mediated by complement activation (Ricklin et al. 2010, Schwaeble et al. 2011).

1.5: Complement pattern recognition components

1.5.1: The classical pathway recognition molecule, C1q

C1q is formed of a hexamer of heterotrimers (each composed of a C1q A-chain, C1q B-chain and C1q C-chain) and forms a multimolecular complex comprised of 18 polypeptide chains with a total molecular weight of approximately 460 kDa. Each of the 3 different C1q chains consists of an N-terminal region, a collagen-like domain and C-terminal globular region, which mediates recognition of immune complexes (figure 1-2). The three different chains that form C1q (i.e. C1qA, C1qB and C1qC) are each encoded by highly homologous genes called *C1qA*, *C1qB* and *C1qC* on human chromosome 1 or mouse chromosome 4. Two chains associate together to generate a heterodimer A-B and which in turn is associated with homodimer C-C to form heterotrimeric structure ABC-CBA. Three of these structural units bind via its collagen-like region to form C1q (Wallis et al. 2010, Jlaajla et al. 2014)

C1q is the unique recognition component of the classical pathway that binds to a wide range of self and non-self patterns. C1q binds to its ligands by recognizing patterns which include bound antibody or pattern-bound C-reactive protein as well as surface features of cells and cell debris. C1q binds directly to its acceptors by its globular region and activates the complement system via the classical pathway (Nayak et al. 2012, Bohlson et al. 2014).

The binding capacity of the C1q to IgG is influenced by the nature of the IgG aggregations. Numerous molecules of IgG bind to an antigen will provide close proximity between Fc regions of IgG molecules allowing a strong binding of C1q. In addition, the interaction between IgG and C1q is ion-mediated and mainly C1qB chain is responsible for the binding of C1q to IgG or IgM (Nayak et al. 2012).

C1q binds to apoptotic cells and initiates clearance. In the late stage of apoptosis, C1q binds to apoptotic cell-bound IgM and opsonize it via activation of the classical pathway (Nayak et al. 2012). On the other hand, the acute phase

protein, C-reactive protein, can bind to lysophosphatidylcholine on the surface of dead cells such as cancer cells and expose a binding site to C1q (Nayak et al. 2012).

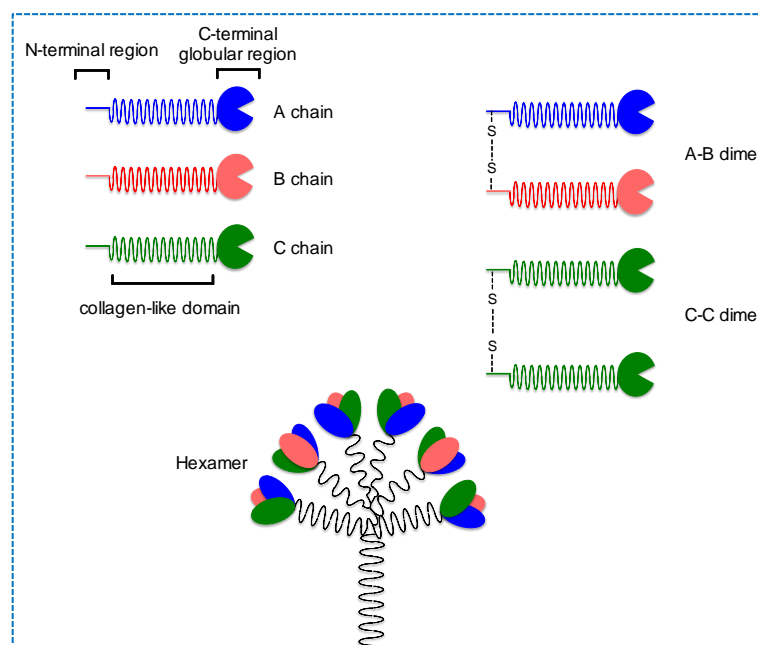


Figure 1-2: Schematic representing the structural subunit and assembly of the C1q component

Heterodimer form A-B associates with homodimer form C-C to generate heterotrimeric structure and three such a heterotrimeric structure linked to form C1q.

1.5.2: The lectin pathway recognition components

The pattern recognition components of the lectin pathway are effective initiators of the innate immune system through their ability to recognize the Pathogen Associated Molecular Patterns (PAMPs) and altered self-structures and opsonizes them via lectin pathway mediated complement activation. In man, the lectin pathway recognition components described so far are CL-11, ficolins and MBL.

1.5.2.1: Mannose binding lectin (MBL)

MBL, also named Mannan-Binding Protein (MBP), is a member of the collectin family which binds to sugar structures on the surface of broad range of microorganisms and mediates the complement activation by the lectin pathway (Garred et al. 2006).

MBL is encoded by *MBL2* gene on chromosome 10. The gene consists of four exons and three introns (Taylor et al. 1989). MBL is mainly synthesized in liver but MBL mRNA was also detected in kidney, lung, small intestine, thymus and testis but at small amount (Uemura et al. 2002). However, MBL concentration in the blood varies from undetectable concentration to up to 10 µg/mL (Takahashi 2011).

Structurally, MBL is made up of oligomers of a single type of polypeptide chain each composed of, from the C-terminus, C-type Carbohydrate Recognition Domain (CRD), coiled coil neck region, a collagen-like domain and N-terminal cysteine-rich domain (figure 1-3). The N-terminal region forms inter and intra subunit disulphide bridges, which are needed for oligomerization that is initiated by coiled/coil neck region (Garred et al. 2006).

Under reducing conditions, an individual polypeptide subunit runs at about 32 kDa on an SDS polyacrylamide gel. In the plasma homotrimers of these single chains assemble to form higher oligomers i.e. dimers, trimer or hexamer which - dependent on their state of oligomerization, run on SDS-PAGE under non-

reducing conditions as complexes of about 50-250 kDa (Wallis 2002, Garred et al. 2006).

MBL is found in plasma in complexes with MASPs as well as with the smaller spliced products of *MASP1* and *MASP2* genes, MAp44 and MAp19 respectively. MBL associates with MASPs via the interaction of its collagen domain with CUB₁ and EGF domains of MASP (Wallis et al. 2004). This association is not equally distributed as the high oligomeric forms of MBL appear to be associated mainly with MASP-2 and MASP-3 whereas the low oligomeric forms appear to be associated with MAp19 (Thiel and Gadjeva 2009).

MBL can discriminate between self and non-self surfaces and the interaction of MBL with the microbial surfaces occurs at the CRD in a Ca⁺²-dependent manner. The CRD recognizes the molecular patterns on the surface of variety of pathogens including bacteria, viruses, fungi, parasites and altered self-cells these molecular patterns include mannose, *N*-acetylglucoseamine (GlcNAc) or fucose but not sialic acids or galactose (Eisen and Minchinton 2003, Thiel and Gadjeva 2009, Takahashi 2011). However, which microorganism offers a good binding to MBL and which microorganism can be removed better by MBL-mediated mechanism is influenced by many factors including the composition of the MBL oligomers and the patterns on the antigen surface. The presentation and density of PAMPs on the antigen surface influences the affinity of MBL binding and of the MBL-mediated activation of the lectin pathway. However, all MBL oligomeric forms bind to well-presented patterns whereas only the higher oligomeric forms can bind to poorly (sparsely) presented patterns on the microbial surface (Gadjeva et al. 2004). MBL has no binding activity on self-bystander cells as the GlcNAc, fucose and mannose on the self-cell membrane are mainly hidden by sialic acid. In altered cells, such as apoptotic cells, these structures become naked and exposed to MBL (Gadjeva et al. 2004).

The main function of MBL is to recognize the pathogen and then initiate the lectin pathway complement activation via MBL-associated MASPs by bringing

MASPs together. MBL was found to bind to a broad range of Gram positive and Gram negative bacteria including *E. coli*, *Neisseria meningitidis*, *N. gonorrhea*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus* (Thiel and Gadjeva 2009). Viruses have developed many strategies to avoid recognition by recognition molecules of the immune system. However, many viruses are clearly recognized by MBL such as Human Immunodeficiency Virus (HIV), Herpes Simplex Virus (HSV) and Influenza A Virus (IAV) (Thiel and Gadjeva 2009).

MBL easily recognizes the mannan component on the surface of fungi including *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus fumigatus* (Celik et al. 2001, Thiel and Gadjeva 2009).

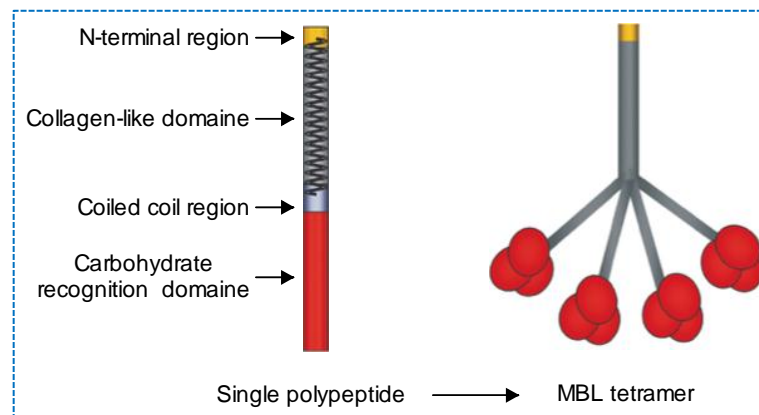


Figure 1-3: Structure of the MBL component

The MBL polypeptide subunit consists of four domains, N-terminal region, a collagenous domain, and a neck region and carbohydrate recognition domain. The polypeptide subunits assemble together to generate trimer that can be further oligomerized to form a high oligomeric form (figure modified after Garred et al. (2006).

1.5.2.2: *Collectin 11*

Collectin 11 is a serum protein (approximate serum concentration 0.3 µg/mL) associated with human MASPs as well as MAp44 (Hansen et al. 2010, Ma et al. 2013). The protein is comprised of polypeptides with a domain structure like that of MBL (Selman and Hansen 2012). Three human collectins have been described in addition to the originally identified human collectins MBL, SP-A and SP-D: Collectin-Liver 1 (CL-L1), Collectin-Placenta 1 (CL-P1) and the most recently identified collectin kidney 1 (CL-K1 or CL-11) (Ohtani et al. 1999, Ohtani et al. 2001, Keshi et al. 2006). Like MBL, CL-11 is composed of homotrimeric subunits of identical chains which themselves comprise of an N-terminal segment, a collagenous region, a neck region and a carbohydrate recognition domain (see figure 1-4) (Selman and Hansen 2012). Under reducing conditions, a single polypeptide chain of CL-11 has a molecular weight of about 35 kDa as seen on SDS-PAGE (Hansen et al. 2010, Keshi et al. 2006), while under non-reducing conditions, multimolecular human serum CL-11 complexes run, depending on their degree of polymerization at approximately 200 kDa - 300 kDa in size (Selman et al. 2012).

CL-11 is encoded by a single structural gene (*COLEC11*) located on the short arm of chromosome 2 (Selman and Hansen 2012). CL-11 is highly expressed in adrenal glands, liver and kidney as well as in ovaries, testis and in moderate levels also in the retina (Hansen et al. 2010).

CL-11 recognizes a variety of carbohydrate structures such as L-fucose, D-mannose and N-acetylglucosamine and it was shown to bind to nucleic acids and some microorganisms including *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Ohtani et al. 1999, Hansen et al. 2010, Selman and Hansen 2012, Henriksen et al. 2013).

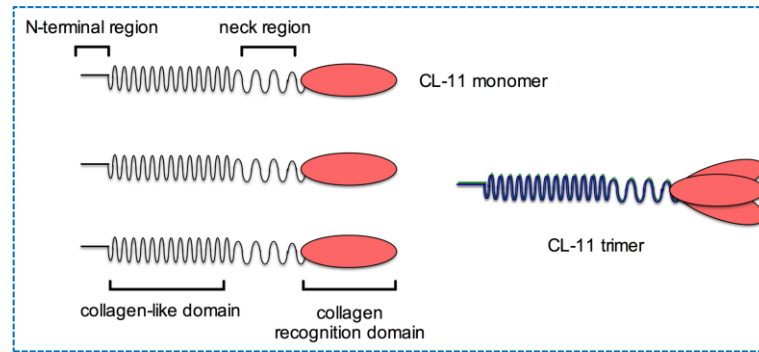


Figure 1-4: Schematic representing the structural subunit and assembly of collectin-11 component

CL-11 monomer composed of four domains, N-terminal region, a collagen-like domain, a neck region and a collagen recognition domain at the C-terminus. Three monomeric subunits assemble to form CL-11.

1.5.2.3: *Ficolins*

Ficolins are oligomeric proteins comprised of an N-terminal region, a collagen-like domain and a C-terminal fibrinogen-like domain (Endo et al. 2011). Analogous to the carbohydrate recognition domain of MBL, ficolins bind to their respective ligands via the fibrinogen-like domain which for example binds with high affinity to *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) on the surface of many pathogens (Endo et al. 2011, Matsushita et al. 2013).

Similar to MBL, ficolin complexes are composed of subunits of homotrimeric polypeptide chains. Each chain consists of a short cysteine rich N-terminal sequence, a collagen-like domain and the C-terminal fibrinogen-like domain (figure 1-5). Three monomeric subunits associate through the collagen-like domain to form trimeric structure which in turn oligomerises to form high molecular weight ficolin complexes (Carroll and Sim 2011, Matsushita et al. 2013).

To date, three ficolins have been identified in humans, L-ficolin (also known as ficolin-2, P35, EBP or hucolin), M-ficolin (also called ficolin-1) and H-ficolin (also called ficolin-3, or Hakata antigen) (Yae et al. 1991, Lu et al. 1996,

Matsushita et al. 1996). The *FCN2* gene is located on the short arm of chromosome 9 (Chromosome 9q34.3) encoding ficolin-2 or L-ficolin, which is primarily expressed in the liver and presented in serum as an oligomer at an average serum concentration of 3.9 µg/mL. H-ficolin or ficolin-3 is encoded by *FCN3* gene, which is located on human chromosome 1 (Chromosome 1p36.11) and the protein is mainly produced in the liver and the lung. In the liver, H-ficolin is expressed by hepatocytes and in the bile duct epithelium and is presented in the blood at median concentration of 18.2 µg/mL. M-ficolin is encoded by *FCN1* gene on the short arm of human chromosome 9 (Chromosome 9q34). M-ficolin is primary expressed by cells of the monocyte/macrophage lineage and abundantly expressed by the spleen and the lung. Amongst the three ficolins, M-ficolin has the lowest serum concentration with an average serum concentration of 1.2 µg/mL serum (Akaiwa et al. 1999, Endo et al. 2011, Sallenbach et al. 2011).

Ficolins form complexes with all human MASPs as well as with MAP44 and MAP19. The globular fibrinogen-like domain mediates the binding of ficolins to the pathogen surface which in turn catalyzes the activation of ficolin associated MASPs that bind to the MASPs binding site located within the collagenous region of ficolins (Matsushita et al. 2002, Liu et al. 2005, Matsushita et al. 2000a). L-ficolin binds to lipoteichoic acid (LTA), lipopolysaccharides (LPS) and capsular polysaccharides of numerous pathogens including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *E. coli* and *Salmonella typhimurium* (Krarup et al. 2004, Lynch et al. 2004, Matsushita et al. 2013). M-ficolin recognizes acetylated groups including sialic acid, GalNAc and GlcNAc and binds to *Salmonella typhimurium* and *Staphylococcus aureus* (Liu et al. 2005, Matsushita et al. 2013) H-ficolin was shown to bind to polysaccharides of *Aerococcus viridans* (Tsujimura et al. 2002) as well as to D-fucose, GalNAc, GlcNAc and the LPS from *Salmonella typhimurium* and to *Trypanosoma cruzi* parasites (Akaiwa et al. 1999, Cestari Idos et al. 2009).

In mouse, two ficolins have been identified, ficolin-A (Fujimori et al. 1998), an orthologue of human L-ficolin, and ficolin-B (Ohashi and Erickson 1998), a

orthologue of human M-ficolin (Endo et al. 2011). Mouse ficolin-A is mainly expressed in the liver and the spleen and the protein is presented in the blood. Ficolin-A recognizes acetylated compounds including GlcNAc and GalNAc and binds to several bacteria including *Listeria monocytogenes*, *S. aureus*, *E. coli* and *Pseudomonas aeruginosa*. Mouse ficolin-B is expressed in the spleen and bone marrow and recognizes GlcNAc and GalNAc and similar to M-ficolin, mouse ficolin-B recognizes acetylated sialic acid (Endo et al. 2011, Hummelshoj et al. 2012, Matsushita et al. 2013).

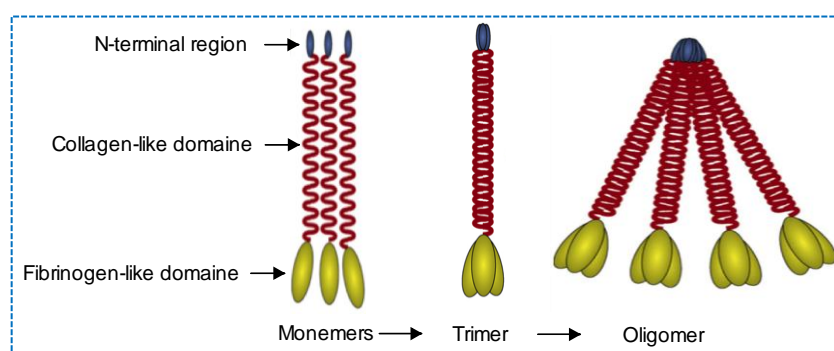


Figure 1-5: Ficolin structure

The monomeric subunit of ficolin composed of N-terminal region, a collagen-like domain and C-terminal fibrinogen-like domain. Three monomeric subunits assemble via the collagen-like domain to generate trimeric structure and these structures assemble together to form an oligomer. Figure is modified after Matsushita et al. (2013).

1.6: Complement proteases

1.6.1: Complement components C1r and C1s

The human complement components C1r and C1s are serine proteases responsible for the initiation of the classical pathway. C1r initiates C1 complex activation and C1s executes the catalytic activity of C1 complex.

C1r and C1s are modular proteins (homologous to the MASPs of the lectin pathway) each of which consists of six domains including, from the N-terminal, CUB₁ domain (CUB is named from C1r/C1s, embryonic sea Urchin protein (Uefg)-Bone morphogenetic protein 1 (Bmp1)), followed by an EGF-like domain (named from Epidermal Growth Factor (EGF) like domain), a CUB₂ domain, followed by two CCP domains, i.e. CCP₁ and CCP₂ (named from Complement Control Protein 1 (CCP₁) domain) and a C-terminal serine protease domain (figure 1-6) (Schwaeble et al. 2002, Sim and Tsiftoglou 2004, Wallis et al. 2004). The CUB domains and the EGF domain are mediating dimerization of MASPs to form homo- or heterodimers in anti parallel orientation. The binding of the MASP homo or hetero-dimers to the MASPs binding site on the recognition subcomponents is mainly mediated through the CUB domains and their binding affinity is calcium dependent. Within MASPs and C1r or C1s, the CCP domains support the binding of the substrates whereas the serine protease domain provides the catalytically active unit (Rossi et al. 2005, Degn et al. 2011).

The two C1r molecules dimerize in antiparallel orientation through binding interactions between their CCP₁ and serine protease domains to form the core unit of the C1s:C1r:C1r:C1s heterotetramer located within the C1 complex, while the heavy chain of C1r dimerizes with the heavy chain of C1s in antiparallel orientation through binding interactions between their CUB₁/EGF domains in a similar fashion to that observed for the formation of MASPs dimers. The heterotetramer C1r₂C1s₂ (340 kDa) binds to C1q in presence of Ca²⁺

via its collagenous stalk to form C1 complex (~770kDa), C1q(C1r)₂(C1s)₂ (Sim and Laich 2000, Wallis et al. 2010).

The main function of the C1r (85 kDa) is to activate zymogen C1s and conversion of C1r zymogen. C1r zymogen conversion is initiated by the catalyzation of auto-activation following binding of the C1 complex to immune complexes (Gal et al. 2009, Wallis et al. 2010). Most recent work indicated that C1r can also cleave mutant MASP-3 in which the lysine residue at the position 448 is replaced by glutamine (K448Q) (Wijeyewickrema et al. 2013).

C1s is a single polypeptide glycoprotein of 79.8 kDa present in serum in the C1 complex. Upon activation, C1s is cleaved by C1r in the region between the heavy chain and the light chain, specifically at the bond between Arg⁴³⁷-|-Ile⁴³⁸. A disulphide bond holds the two chains together. Activated C1s has two complement substrates C4 and C2 as well as it found to cleave Insulin-Like Growth Factor Binding Protein-5 (IGFBP-5) (Gal et al. 2002).

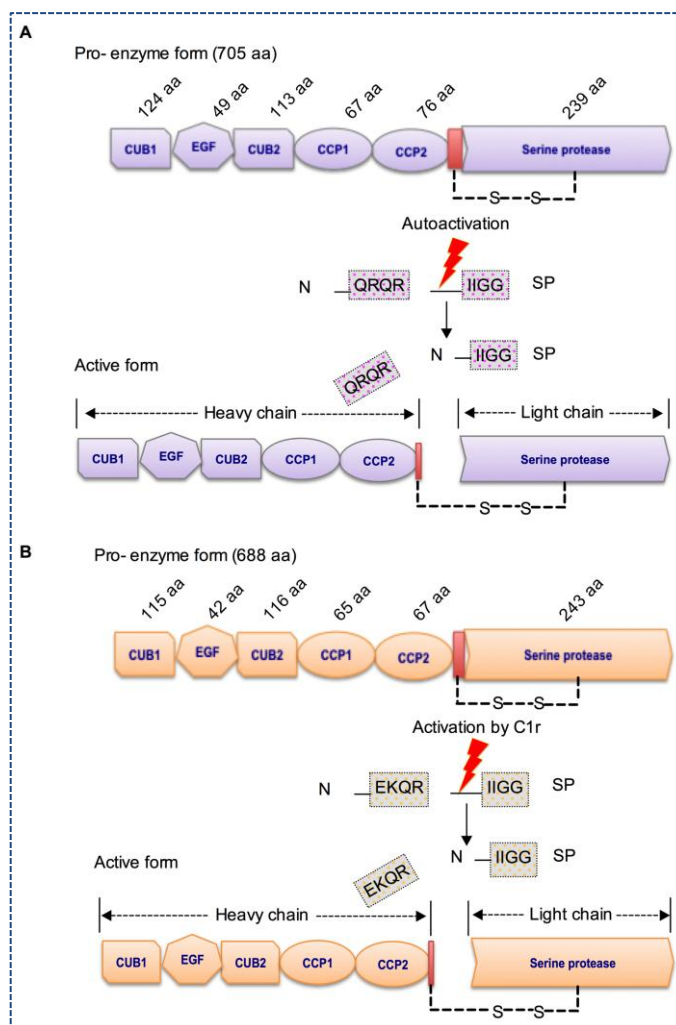


Figure 1-6: Domain structure and activation of human C1r and C1s

C1r (A) (705aa) and C1s (B) (688aa) comprised of 6 domains (CUB₁, EGF, CUB₂, CCP₁, CCP₂, SP). The residual numbering of each domain is indicated. C1r is auto-activation serine protease and its proteolytic cleavage site is Arg⁴⁶³-|-Ile⁴⁶⁴ residues. Active C1r cleaves the bonded in precursor C1s between Arg⁴³⁷-|-Ile⁴³⁸ residues (Based on: C1r UniProtKB/Swiss-Prot entry number P00736 and C1s UniProtKB/Swiss-Prot entry number P09871).

1.6.2: Complement component C2

The complement component C2 is a single polypeptide glycoprotein of 90 kDa found in serum responsible for the classical and the lectin pathways C3 convertase proteolytic activity. C2 is derived from a single gene located on the short arm of chromosome 6 (C2 gene) (Cheng and Volanakis 1994, Wetsel et al. 1996).

C2 is a modular protein which consists of, from N-terminus, three CCP domains, a von Willebrand Factor (vWF) and a serine protease domain at the C-terminus (figure 1-7). Only C4b-bound C2 is cleaved into its active form by the proteases, C1s, MASP-1 or MASP-2 (Matsushita et al. 2000b, Ambrus et al. 2003). Upon activation, C2 splits into two fragments the larger is generated from the C-terminal region, C2a (60 kDa), which associates to C4b, and the smaller fragment generated from N-terminal region, C2b (30 kDa) removed to the fluid phase (Reid and Porter 1981, Sim et al. 1993). The binding of C2a to the surface-bound C4b is mediated by Mg^{2+} ions. C2a in C3 convertase, C4bC2a can cleave both C3 and C5 components and it has no natural inhibitor but it can be regulated by decay accelerating activity of some complement regulators such as Decay Accelerating Factor (DAF), Membrane Cofactor Protein (MCP) and Complement Receptor 1 (CR1) by dissociation of C2a from C4bC2a complex (Sim and Tsiftoglou 2004).

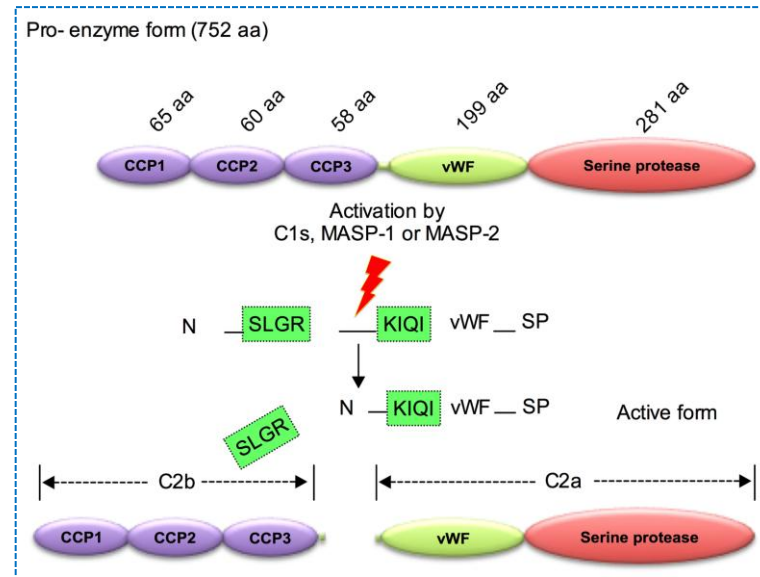


Figure 1-7: Domain organization and activation of human C2

C2 (752aa) consisted of 5 domains, three CCP domains, a von Willebrand factor and a serine protease domain. Precursor C2 is activated by C1s, MASP-1 or MASP-2 by a cleavage in the region between the third CCP domain and vWF domain (Arg²⁴³-|-Lys²⁴⁴ residues) (Based on UniProtKB/Swiss-Prot entry number P06681).

1.6.3: Factor I

Factor I (FI) is a serum glycoprotein of 88 kDa encoded by the *CFI* gene on the long arm of chromosome 4. The single chain protein consisting of five domains including, from the N-terminal region, a single FI Membrane attack complex (FIM) domain, a scavenger receptor cysteine-rich (SRCR) domain (also known as CD5-like domain), two low-density lipoprotein receptor (LDL) domains and a serine protease domain (figure 1-8) (Nilsson et al. 2011). FI is an acute phase protein mainly expressed by hepatocytes and has a median serum concentration of 35 µg/mL (Nilsson et al. 2011).

FI has two natural substrates that are formed by complement activation i.e. C3b and C4b within the convertase complex. C3b and C4b are inactivated by FI and co-factors by cleaving the alpha chain to generate iC3b and iC4b, respectively. FI acts on C3b and C4b only when bound non-covalently to a complement regulator such as CR1, FH, C4 Binding Protein (C4BP), MCP (Sim et al. 1993, Nilsson et al. 2011).

The function of the cofactors in this process is still a subject of debate. Two proposed functions for cofactors have been suggested. The first proposal adopted the idea that these cofactors induce conformational changes in the complement C3 convertase and C5 convertase complexes facilitating the access of FI to its substrate within these complexes. The other hypothesis suggests that these cofactors act on FI and redirect it towards C4b and C3b within the convertase complex (Sim et al. 1993).

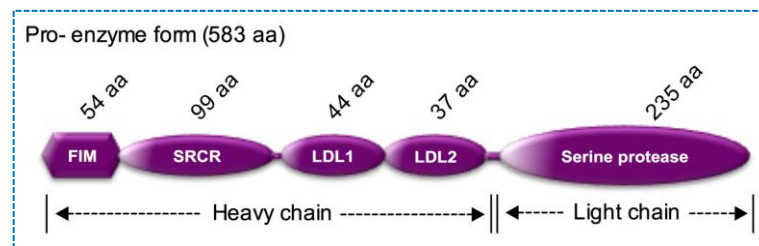


Figure 1-8: Structure of human complement FI

FI (583aa) comprises of 5 modules, a single FIM domain, a SRCR domain, two LDL domains and a serine protease domain. Its active site is composed of H³⁸⁰, D⁴²⁹ and S⁵²⁵ at the serine protease domain (Based on UniProtKB/Swiss-Prot entry number P05156).

1.6.4: MASP-1

MASP-1 is the first ever described serine protease of the lectin pathway associates with MBL or ficolin (Matsushita and Fujita 1992). The *MASP1* gene is located on the long arm of chromosome 3 in human and on mouse chromosome 16 (Takada et al. 1995). This gene is transcribed to produce three alternatively spliced mRNA species coding for three different proteins i.e. two distinct serine proteases MASP-1, MASP-3 and a non-enzymatic truncated gene product called MAp44 (see figure 1-9).

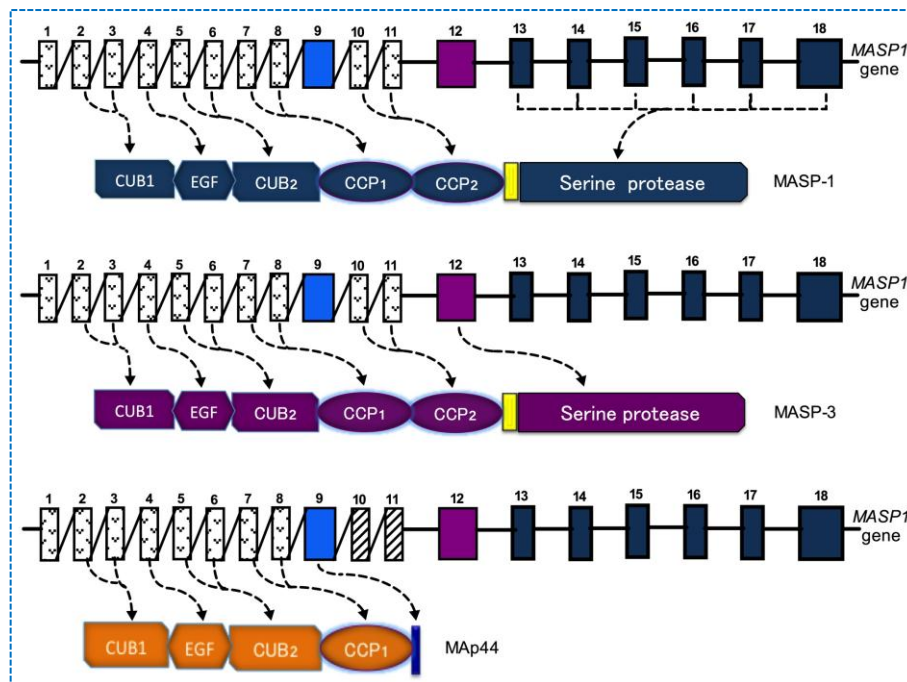


Figure 1-9: Genomic organization of *MASP1* gene

MASP1 gene comprises of 18 exons (rectangles) encoding three proteins, MASP-1 (699aa), MASP-3 (728aa) and MAp44 (361aa). Exons 2-9 encoding CUB₁, EGF, CUB₂ and CCP₁ domains in the three proteins (MASP-1, MASP-3 and MAp44). MASP-1 has a unique 17 amino acid residues at the C-terminus encoded by exon 9, which contains stop codon. Splicing out exon 9 from exons 10 and 11 generates the CCP₂ domain in MASP-1 and MASP-3. The serine protease of the MASP-1 is encoded by 6 exons (exon 13 to 18) and its stop codon located in exon 18. Whereas, MASP-3 serine protease domain is encoded by a unique exon (exon 12).

MASP-1 and MASP-3 share the same heavy chain comprising the domains CUB₁:EGF:CUB₂:CCP₁ and CCP₂, encoded by exons 2-11. The serine protease domain of MASP-3 is encoded by a single exon, i.e. exon 12, whereas the serine protease of MASP-1 is encoded by exons 13-18 (Schwaeble et al. 2002, Degn et

al. 2009b, Skjoedt et al. 2010a). MASP-1 mRNA is highly expressed in liver and also in extra hepatic sites such as small intestine, kidney, placenta, lung, colon and heart (Skjoedt et al. 2010a, Seyfarth et al. 2006). MASP-1 is a serum protein of 81 kDa present in a median serum concentration of approximately 11 µg/mL (Kuraya et al. 2003, Thiel et al. 2012). Similar to MASP-2 and MASP-3, MASP-1 is composed of six domains i.e. two CUB domains, an EGF domain, two CCP domains and a serine protease domain (see figure 1-10). MASP-1 is secreted as a single chain zymogen that associates with MBL, CL-11 or ficolins (L-ficolin, H-ficolin and M-ficolin). On an activator surface, MASP-1 autoactivates through cleavage within the linker region between the second CCP domain and the serine protease domain at the position Arg⁴⁴⁸-Ile⁴⁴⁹ while the active light chain (serine protease domain) remains attached to the heavy chain via a single disulphide bond.

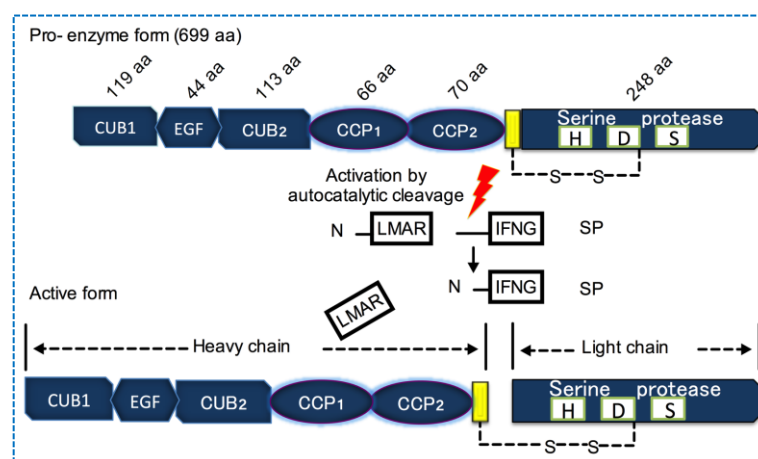


Figure 1-10: Domain organization and activation of MASP-1

MAASP-1 comprises of heavy chain and light chain linked by a link region. The serine protease domain exhibits the active site that is formed by His⁴⁹⁰ (H), Asp⁵⁵² (D) and Ser⁶⁴⁶ (S) residues (Based on UniProtKB/Swiss-Prot entry number P48740).

MAASP-1 was first considered to activate the complement components C4, C2 and C3 (Matsushita and Fujita 1992, Matsushita and Fujita 1995) but the subsequent research showed that MASP-1 does not cleave C4 (Thiel et al. 1997). However, MASP-1 is able to cleave the complement component C2 with sufficient efficiency. While MASP-1 on its own cannot drive lectin pathway

activation (due to its inability to cleave C4 to generate the lectin pathway C3 convertase C4bC2a), but it can support and augment lectin pathway activation via cleavage of C2, MASP-2 and MASP-3 (Takahashi et al. 2008, Heja et al. 2012, Moller-Kristensen et al. 2007, Megyeri et al. 2014). Recently, Teizo Fujita's team proposed another route through which MASP-1 initiates and augments the alternative pathway in a lectin pathway dependent and MASP-2 independent fashion. Fujita's team observed that the MASP-1/-3 double deficient mouse line that they have established is totally deficient in alternative pathway functional activity and demonstrated that this deficiency is caused by the absence of enzymatically active FD. MASP-1/-3 deficient mice predominantly have the enzymatically inactive zymogen precursor pro-factor D (pro-FD) in their serum. They were able to show that recombinant MASP-1 can cleave pro-FD and convert pro-FD into its enzymatically active form (Takahashi et al. 2010).

The work presented in this thesis provides clear and novel answers to the many questions resulting from this excellent work of Professor Fujita's team in Fukushima, Japan.

Another route of investigation has been opened by recent discoveries showing that the lectin pathway specific serine proteases MASP-1 and MASP-2 form a link between the complement system and the coagulation system. It was demonstrated that truncated recombinant MASP-1 (composed of CCP₁, CCP₂, SP) is able to directly cleavage and activate components of the coagulation system such as fibrinogen and factor XIII (Hajela et al. 2002, Krarup et al. 2008).

1.6.5: MASP-2

MASP-2 was first described in 1997 as the second lectin pathway specific serine protease (Thiel et al. 1997). *MASP2* gene is located on the short arm of chromosome 1 in human and on mouse chromosome 4 (Stover et al. 1999a). *MASP2* gene is composed of 12 exons encoding for two proteins MASP-2 (76 kDa) and MAP19 (19 kDa) (also called a small MBL-associated protein, sMAP). They are encoded by two distinct mRNA species generated by alternative splicing (figure 1-11) (Stover et al. 1999b, Stover et al. 2001). MASP-2 mRNA is exclusively expressed in hepatocytes (Unterberger et al. 2007).

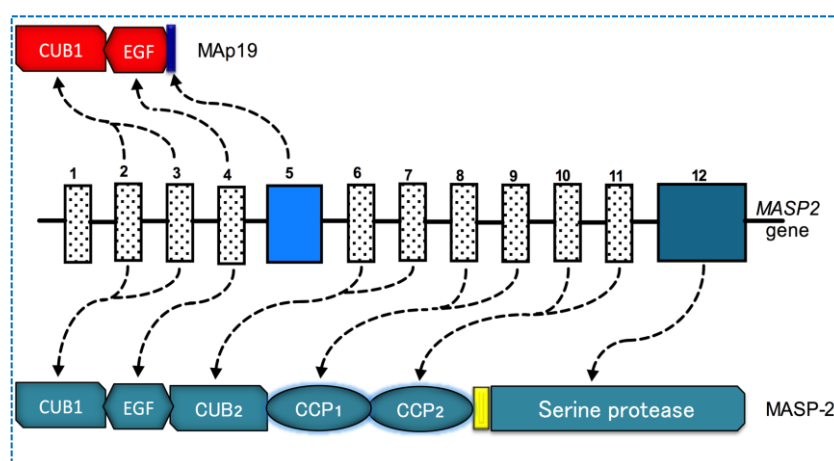


Figure 1-11: Genomic organization of *MASP2* gene

MASP2 gene contains 12 exons (rectangles) encoding MASP-2 (686aa) and MAP19 (171aa). Exons 2-4 are encoding CUB₁ and EGF domains in both MAP19 and MASP-2 proteins. MAP19 has additional 4 amino acids at the C-terminus encoded by exon 5. The second CUB domain and CCP domains are encoded by exons 6-11 whereas the serine protease domain is encoded by exon 12.

Similar to C1r, C1s and MASP-1, MASP-2 is a modular enzyme composed of six domains including (CUB₁, EGF, CUB₂, CCP₁, CCP₂, SP) (figure 1-12) (Stover et al., 2001). Over 95% of MASP-2 was found in serum circulating free and unbound to MBL (Thiel et al. 2000). The binding of MASP-2 to MBL is Ca²⁺ dependent and mediate by the first CUB and EGF domains (Chen and Wallis 2004).

The activation of MASP-2 can occur either by an autocatalytic cleavage or by direct cleavage through either activated MASP-1 or MASP-2 (Moller-Kristensen et al. 2007, Takahashi et al. 2008, Heja et al. 2012, Megyeri et al. 2014) in a defined cleavage site located between the heavy chain and the light chain at position Arg⁴⁴⁴-|-Ile⁴⁴⁵. Dissimilar to MASP-1, MASP-2 is the only serine protease of the lectin pathway that can activate the complement system on its own through its ability to cleave both complement components C4 and C4b-bound C2 forming the lectin pathway C3 convertase, C4bC2a (Vorup-Jensen et al. 1998, Matsushita et al. 2000a, Schwaebler et al. 2011). Recently it has been shown that MASP-2 mediates ischaemia-reperfusion injury through activation of the lectin pathway in C4 deficient mice via a novel C4-bypass activation of C3 (Asgari et al. 2014).

Several non-complement substrates have been reported for MASP-2 such as prothrombin, which can be converted to active thrombin that is able to cleave fibrinogen (Krarup et al. 2007).

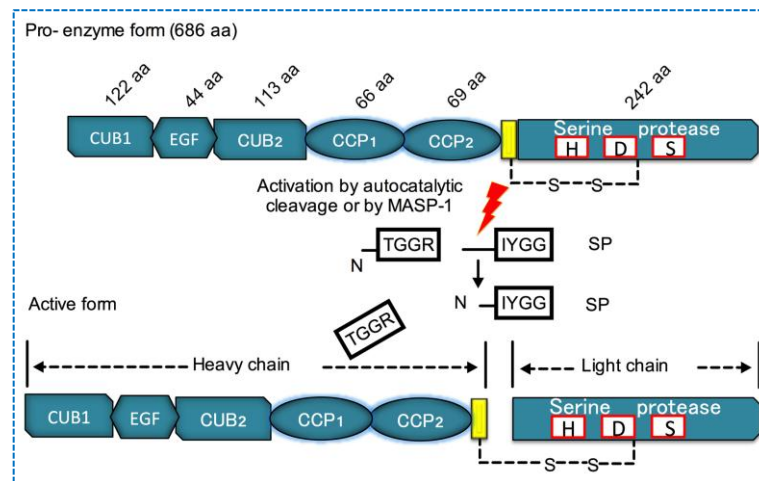


Figure 1-12: MASP-2 structure and activation

The heavy chain of MASP-2 composed of four domains (two CUB domains, an EGF domain and two CCP domains) linked to the light chain (a serine protease domain) that exhibits the active site of MASP-2, which is composed of His⁴⁸³, Asp⁵³² and Ser⁶³³ residues. MASP-2 is autoactivation enzyme and it can be also activated by MASP-1 through a cleavage of the bond between Arg⁴⁴⁴-|-Ile⁴⁴⁵ residues (Based on UniProtKB/Swiss-Prot entry number O00187).

1.6.6: MASP-3

MASP-3 is the most recently discovered MBL associated serine protease (Dahl et al. 2001) and is encoded by the *MASP1* gene through an alternative RNA splicing process of the primary gene transcript. Both MASP-1 and MASP-3 share an identical heavy chain, with an exception of the last 15 C-terminal residues of the heavy chain (linker region). The calculated molecular weight of MASP-3 is 81 kDa. In contrast to MASP-1 and MASP-2, MASP-3 cannot autoactivate and MASP-3 was recently shown to be activated by MASP-1 (Megyeri et al. 2014) or as a mutant form (K448Q) by C1r (Wijeyewickrema et al. 2013). Similar to active MASP-1, activated MASP-3 is cleaved into the heavy and the light chain held together by a disulphide-bridge (see figure 1-13).

MASP-3 is present in the serum with a median serum concentration of 6.4 µg/mL (Skjoedt et al. 2010b). Hepatocytes are considered to be the main cell type where MASPs are synthesized. However, MASP-3 was also shown to be synthesized in wide range of extrahepatic tissues including skeletal muscles, heart, small and large intestines, lung, ovary, placenta, prostate, brain and the spleen (Seyfarth et al. 2006).

Very little is known about the biological characteristics of MASP-3 and no enzymatic activity was found for MASP-3 towards the complement components C2, C4 or C3 (Dahl et al. 2001). This is the reason why MASP-3 was first thought to act as a negative regulator of the lectin pathway competing with MASP-2 and MASP-1 for the binding to MBL (Dahl et al., 2001). In 2011, Teizo Fujita's group hypothesized that MASP-3 may help to initiate the alternative pathway by cleaving FB within C3bB complex to produce C3bBb, the C3 convertase of the alternative pathway (Iwaki et al. 2011). This paper suggested that MASP-3 could be essential for activating the alternative pathway of complement on cell surfaces.

At the beginning of my PhD project, my supervisor's team had shown that in MASP-3 deficient serum, complement mediated lysis of *Neisseria meningitidis*

was totally defective and haemolytic activity against rabbit erythrocytes significantly impaired (Professor Wilhelm Schwaeble, unpublished data).

At that time, the only enzymatic activity that has been identified for MASP-3 was that towards Insulin-Like Growth Factor-Binding Protein-5 (IGFBP-5) (Cortesio and Jiang 2006).

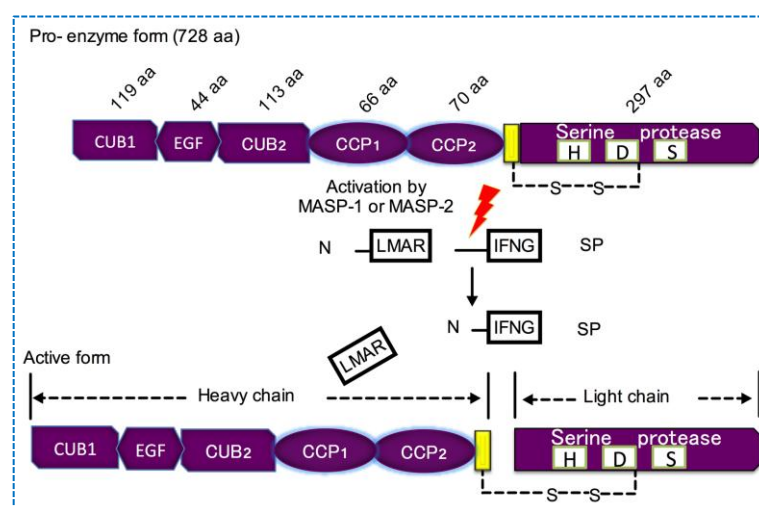


Figure 1-13: Domain organization and activation of MASP-3

The modular heavy chain of MASP-3 (two CUB domains, an EGF domain and two CCP domains) is associated with the light chain (a serine protease domain) via a disulfide bridge. The activation process initiates by MASP-1 or MASP-2 (a finding of this study) by cleaving the bond between Arg⁴⁴⁸-Ile⁴⁴⁹ residues (Based on GenBank entry number AAK84071.1).

1.6.7: Factor B

FB glycoprotein has a calculated molecular weight of 80 kDa (Mole et al. 1984) and in the serum 90 kDa (Williams et al. 1999) due to glycosylation. FB is a modular protein composed of three Complement Control Protein (CCP) domains linked together by a short stretches of amino acids, a single von Willebrand Factor (vWF) domain and a serine protease domain at the C-terminal region (Mole et al. 1984, Williams et al. 1999) (see figure 1-14).

FB activation occurs when factor B binds to covalently bound C3b on an activator surface or to hydrolyzed C3, C3(H₂O) or C3b in the fluid phase to generate C3bB or C3(H₂O)B complexes. FB binding to pathogen-bound C3b is a Mg²⁺-dependent process in which an ion binding site is formed at the apex of vWF domain (Hourcade and Mitchell 2011). After which, FB becomes susceptible to FD proteolytic activity. Enzymatically active FD cleaves FB in between amino acid positions Arg²³³ and Lys²³⁴ generating two unequal fragments (Xu et al. 2001, Milder et al. 2007, Forneris et al. 2010). The smaller fragment, Ba (residues 1-234) (approximate molecular weight of 30 kDa) representing the three CCP modules (Mole et al. 1984, Williams et al. 1999) is released into the microenvironment. It has been reported that Ba fragment has a chemotactic activity on neutrophils (Hamuro et al. 1978) as well as it has an inhibition activity on B-lymphocyte proliferation (Ambrus et al. 1990). The larger fragment Bb (comprising of residues 235-739) (approximate molecular weight of 60 kDa) consists of the vWF module and the serine protease domain (Mole et al. 1984, Williams et al. 1999). Bb fragment binds to surface-bound C3b to generate the C3 convertase, C3bBb. C3bBb is a transient complex with a half-life of approximately 5 seconds (Xu et al., 2001, Milder et al., 2007, Hourcade and Mitchell, 2011). The serine protease module exhibits the proteolytic activity of Bb fragment but it needs a cofactor of non-catalytic protein i.e. C3b. Once C3bBb has formed, it activates native C3 by cleaving the bond between Arg⁷⁷-|-Ser⁷⁸ in C3 alpha chain to generate biologically active C3a and C3b (Nagasawa et al. 1985). The another enzymatic activity that is mediated by the Bb fragment

as part of the alternative pathway C5 convertase complex C3bBb(C3b)_n is the cleavage of the C5 alpha chain between amino acid positions Arg⁷⁵¹-|-Xaa to cleave C5 into its activation fragments C5a and C5b (Hamuro et al. 1978, Xu et al. 2001, Krisinger et al. 2012).

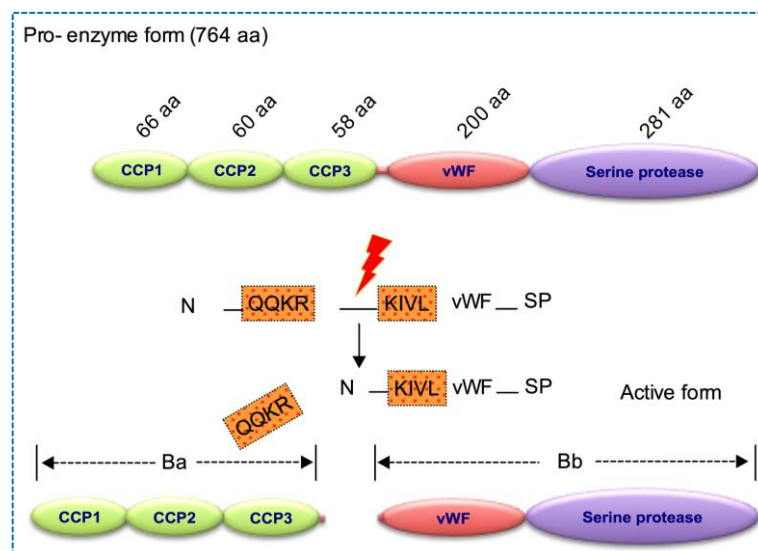


Figure 1-14: The structural domain organization and activation of FB

The precursor FB is consisted of five domains and its heavy chain comprises of three CCP domains, and its light chain that exhibits the proteolytic activity is consisted of a vWF domain and a serine protease domain (Based on UniProtKB/Swiss-Prot entry number P00751).

1.6.8: Factor D

FD is the smallest serine protease of the complement system with a molecular weight of approximately 24 kDa. FD is a unique complement serine protease. Structurally, FD consists of a single serine protease domain and is released from adipocytes as a precursor (Pro-Factor D) which needs to be cleaved to be able to cleave FB (Xu et al., 2001) (see figure 1-15). The necessity for precursor FD to be cleaved by an unknown serine protease to be converted into enzymatically active factor D was first described by Douglas Fearon in 1974 (Fearon et al. 1974).

Fearon hypothesized at the time that the conversion of pro-FD into its active form may be a physiologically meaningful process where the serine protease involved in FD activation may control activation of the alternative pathway.

This hypothesis was forgotten over decades, mainly because of the statement in Volanakis et al. (1977) saying that in serum, FD was presented in its active form. It was hypothesized that once FD is secreted, the 6-7 amino acid on the N-terminus of pro-FD are cleaved off by unknown mechanism (Yamauchi et al. 1994, Xu et al. 2001) and that consequently, FD circulates in the blood in its enzymatically active form (Kam et al. 1987). The proteolytic activity of FD was thought to be induced spontaneously by C3bB formation (Kam et al. 1987, Kim et al. 1994).

The main restriction of active FD to cleave its substrate FB is that for that to happen, FB has to be bound to C3b because only when FB is bound to C3b, FB undergoes a conformational change and this conformational change of FB is essential to make FB susceptible to FD-mediated proteolytic activity (Kam et al. 1987). FD cleaves FB in the region between the third CCP and vWF, specifically in the bond between Arg²³³ and Lys²³⁴ (Torreira et al. 2009). It is essential for the FB substrate to be cleaved that the substrate FB is attached to C3b, since active FD is devoid of any proteolytic activity towards unbound FB (Kam et al. 1987). FD is mainly synthesized by adipocytes and its zymogen form exhibits 6-7 residues at the N-terminus that need to be cleaved off by a so far not identified mechanism to convert pro-FB into its enzymatically active form. Recombinant FD expressed in a baculovirus expression system generated FD in its zymogen form and was devoid of enzymatic activity. As described in Douglas Fearon's paper in 1974, trypsin treatment achieved a partial conversion of FD into its enzymatically active form (Yamauchi et al. 1994). The presence of enzymatically active FD is crucial for alternative pathway functional activity. FD deficient mice are totally deficient of alternative pathway functional activity and despite the fact that for example plasmin can cleave FB, plasmin cannot replace FD to initiate alternative pathway functional by cleaving FB (Taylor et al. 1999, Xu et al. 2001).

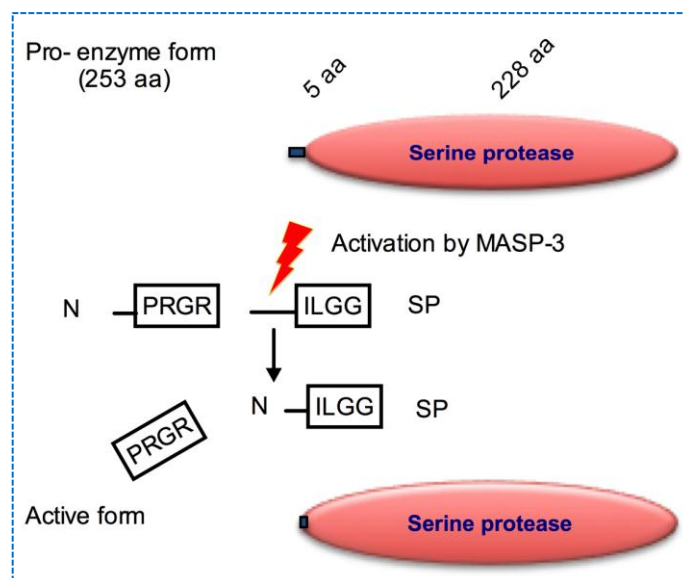


Figure 1-15: Structure and activation of human FD

Pro-FD consisted of C-terminus a serine protease domain and the N-terminus consists of 6-7 amino acid residues (Based on UniProtKB/Swiss-Prot entry number P00746).

1.7: Complement regulatory and inhibitory proteins

The complement system is an effective player in innate immune system. During the infection, the complement system can be activated locally by any of the three complement activation pathways to either lyse or opsonize invading microbes and/or altered self-cells, such as apoptotic cells. Inappropriate activation of the complement system could have detrimental effects on autologous cells and tissues and at the site of infection complement activation may destroy bystander cells. In order to prevent complement activation and cytolytic activity towards host cells, host cells are protected from autologous complement attack by fluid-phase and cell surface-bound regulators, which allow to differentiate between self and non-self antigens and limit the occurrence of immunopathology and autoimmune disease.

Several regulatory and inhibitory proteins, which control complement activation, are distributed into two classes: fluid-phase regulators and membrane-bound regulators.

1.7.1: Fluid-phase regulators

The soluble regulators of complement are found in the plasma and body fluids i.e. synovial and vitreous fluids.

C1-INH is a serpin (serine protease inhibitor) and is a single chain glycoprotein of 105 kDa. The primary sites of C1-INH synthesis are the liver, skin fibroblast and monocytes (Meri and Jarva 2001). *C1INH* gene is located on chromosome 11. In the complement system, C1-INH inhibits the classical pathway serine proteases C1r and C1s as well as the lectin pathway serine proteases MASP-1 and MASP-2 (Meri and Jarva 2001). The inhibitory activity of C1-INH is not limited to serine proteases of the complement system. C1-INH can also inhibit and regulate the activity of the blood clotting components factor XII and kallikrein of the contact system and factor XI, thrombin, plasmin, tissue

plasminogen activator in the coagulation system (Cicardi and Zingale 2007, Cugno et al. 2009).

The lectin pathway specific regulators, MAp19 and MAp44 (figure 1-16) are two alternative spliced products of *MASP2* and *MASP1* genes respectively which were found to compete with MASPs for binding to pattern recognition MBL and ficolins (Stover et al. 1999b, Skjoedt et al. 2010b, Iwaki et al. 2006).

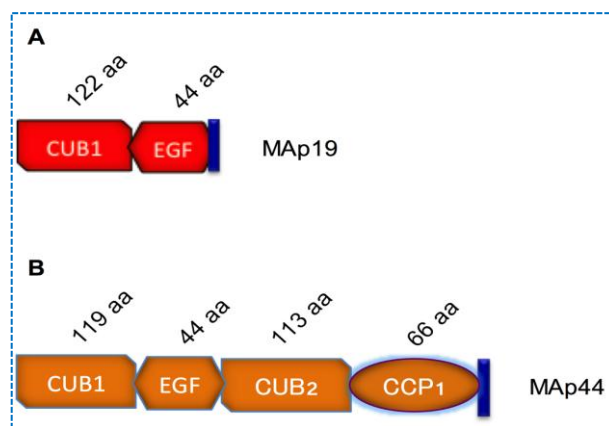


Figure 1-16: Domain structure of human MAp19 and MAp44

MAp19 (185aa) (A) consists of two domains CUB and EGF domains and its C-terminus comprised of 4 residues (Based on GenBank entry number CAB50734.1). MAp44 (361aa) (B) shares four domains structure with MASP-1 and MASP-3 including, two CUB domains, an EGF domain and a CCP domain and has an exceptional C-terminus comprised of 17aa (Based on protein data bank (PDB) entry number 4AQB_A).

Factor H (FH) is the main inhibitor of the alternative pathway C3 convertase, C3bBb. Complement FH is a glycoprotein (155 kDa) composed of 20 CCP domains and it has a serum concentration ranging between 220-540 µg/mL (Ferluga et al. 2014). FH regulates the activity of enzymatic complexes containing the complement activation product C3b by binding to complex bound C3b, (for examples in the alternative pathway C3 convertase C3bBb or alternative pathway C5 convertase C3bBb(C3b)_n or the classical pathway C5 convertase C4bC2a(C3b)_n and accelerate the decay of these convertase complexes. In addition, FH serves as a cofactor in the factor-I-mediated cleavage of C3b to haemolytically inactive iC3b (Whaley and Ruddy 1976, Pangburn et al. 1977, Meri and Jarva 2001). The cofactor activity of factor H is

located within the first 5 N-terminal domains of factor H (Misasi et al. 1989). On the other hand, FH binds to self-specific patterns such as glycosaminoglycans and sialic acid so that FH regulates the complement activation through preventing self-attack (Ricklin et al. 2010).

The recently identified FH-like protein 1 (FHL-1) is a 43 kDa protein produced by the alternative splicing of *fH* gene (Schwaeble et al. 1987).

FHL-1 has antigenicity similar to FH (Meri and Jarva 2001) and it consists of seven short consensus repeat, which are homologues of the N-terminal region of FH. The main function of FHL-1 is to regulate the alternative pathway functional activity as it has a decay accelerating activity on C3 convertase and C5 convertase as well as FHL-1 acts as a co-factor to FI to inactivate bound C3b (Misasi et al. 1989, Zipfel and Skerka 1999).

C4b-Binding Protein (C4BP) is the main inhibitory protein of the classical and lectin pathway activation. The glycoprotein has an approximate molecular weight of 570 kDa and is composed of 7 identical alpha chains and a short single beta chain held together by disulphide bonds. The alpha chain consists of 8 CCP domains while the beta chain is composed of three CCP domains. C4BP displaces C2a from C3 convertase, C4bC2a as well as C4BP acts as a co-factor for degradation of C4b by FI resulting in inhibition the formation of C3 convertase and C5 convertase (Blom et al. 2001, Meri and Jarva 2001). Similar to FH regulation activity, C4BP binds to self-surfaces and prevents autologous complement attack (Ricklin et al. 2010).

Vitronectin (protein S) and clusterin (apo-J) are found to inhibit the terminal pathway of complement. Clusterin is a plasma protein of 70-80 kDa, comprised of alpha and beta chains linked together by a disulphide bridge. The main function of clusterin is to bind to the components of the terminal pathway and inhibit the insertion of membrane attack complex into the membrane of the targeted cell (Meri and Jarva 2001).

Vitronectin is a single chain glycoprotein of approximate molecular weight of 65-75 kDa. The liver is the primary site of vitronectin biosynthesis, but it is also produced by macrophages and megakaryocytes. Similar to clusterin, vitronectin binds to the terminal pathway complexes and prevents insertion of the MAC into targeted cell (Meri and Jarva 2001).

1.7.2: The membrane bound complement regulators

The surface-bound inhibitors are expressed on most human cells and prevent convertase formations as well as participating in inhibition of the terminal pathway of complement. The complement C3 convertase and C5 convertase membrane-attached inhibitors include: Complement Receptor type 1 (CR1, CD35), Decay Accelerating Factor (DAF, CD55) and Membrane-Cofactor Protein inhibitor (MCP, CD46). CD59 or protectin is, similar to vitronectin, an inhibitor of the terminal pathway and is attached to the surface of cellular membranes through a phosphatidylinositol anchor (Meri and Jarva 2001). Protectin is a glycoprotein of 18-25 kDa presented on erythrocytes and on the epithelial and endothelial cells. Protectin inhibits complement lytic activity via binding to C5b-8 complexes and thus prevents C9 binding and MAC formation (Meri and Jarva 2001).

CR1 is a membrane-bound glycoprotein of 190-220 kDa and acts as a binding protein for C3b and C4b on erythrocytes and white blood cells. The protein is composed of 30 CCP domains and exhibits three binding sites for C3b and C4b. Similar to the fluid phase regulator factor H, CR1 acts as a decay accelerating factor on C3 convertase and C5 convertase complexes and as a cofactor for FI to convert C3b and C4b into their haemolytically inactive cleavage products iC3b, C3dg and C3c or iC4b and C4d and C4c, respectively (Meri and Jarva 2001).

With the exception of erythrocytes, all blood cells express a glycoprotein of 51-68 kDa, called membrane cofactor protein, MCP or CD46. Similar to CR1, MCP has a cofactor activity in the factor I-mediated conversion of C3b to haemolytically inactive iC3b (Meri and Jarva 2001).

The Decay-Accelerating Factor DAF or CD55 is a membrane glycoprotein of 70 kDa, expressed on the surface of most cells, including erythrocytes and vascular endothelial cells. Similar to CD59/protectin, CD55/DAF is anchored to the cellular surface by a phosphatidylinositol anchor. DAF binds to C3b and C4b contained in C3 and C5 convertase complexes and accelerates their dissociation as a negative surface regulator of complement activation (Meri and Jarva 2001).

1.8: Complement deficiency

Complement deficiencies are divided into acquired and genetic deficiencies. The acquired complement deficiency is either caused by consumption of complement proteins due to uncontrolled complement activation or by the occurrence of autoantibodies against complement factors that either deplete specific complement components or induce overshooting complement activation such as the C3 nephritic factor (Scott et al. 1978).

Genetic deficiencies are caused by mutations in complement genes that can either lead to the total absence of complement factors or loss of their functional activity or partial loss of their activity or higher activity (i.e. loss of function polymorphisms or gain of function polymorphisms).

Complement deficiencies usually lead to susceptibility of individuals to infectious diseases and/or may predispose to autoimmune diseases.

1.8.1: Deficiencies of the classical pathway components

An insufficient classical pathway leads to defective immune complex removal which in turn may result in the development of autoimmune diseases.

The second complement component, C2 is encoded by 18 exons located on the short arm of chromosome 6 (Wetsel et al. 1996). With an occurrence of 1/20000, C2 deficiency is a relatively frequently occurring inherited complement deficiency. Two types of complement C2 deficiencies have been identified, type I - in which there is neither any C2 protein detectable in the blood nor C2 mRNA presents in the peripheral monocytes. The type II deficiency is caused by a selective block in C2 translation (Knutzen Steuer et al. 1989, Johnson et al. 1993). In Caucasians, the occurrence of a homozygous deficiency is in the region of about 0.01% (Johnson et al. 1993, Wetsel et al. 1996). In contrast with that, hereditary complement C4 deficiency is very rare occurrence, mainly because for a total deficiency of complement C4, both human C4 genes have to be defective and the deficiencies have to be homozygous. So far, only about 20

cases of C4 deficiencies have been identified (Lhotta et al. 1996, Lhotta et al. 2004).

A deficiency in early components of the classical complement pathway is primarily correlated with development of autoimmune disease. The lack in C4 leads to defect in the processing of apoptotic cells and other immune complexes resulted in developing autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Henoch-Schoenlein Purpura (Lhotta et al. 1996, Lhotta et al. 2004). On the other hand, a deficiency in any of the classical pathway activating proteins increases the susceptibility to the infection with *N. meningitides*, *S pneumoniae* and *Haemophilus influenzae* (Figuerola and Densen 1991).

In general, about 75% of C1q deficient individuals or C4 deficient individuals develop SLE while C2 deficiencies are not generally predisposing to SLE (Botto et al. 2009). C2 deficiency, however, is associated with a higher susceptibility to atherosclerosis and rheumatic diseases (Jonsson et al. 2005).

1.8.2: Deficiencies of the lectin pathway components

The lectin activation pathway of complement is initiated by the binding of the lectin pathway specific recognition components to activator surfaces leading to activation of the lectin pathway specific serine proteases MASPs. MBL deficiency is the most common known complement deficiency and occurs (depending on the racial distribution) in 5-30% of the human population and are mainly caused by Single Nucleotide Polymorphism (SNPs) within the *MBL2* gene (the *MBL1* gene in humans is a pseudo gene) (Garred et al. 2003). The most common MBL deficiencies are caused by three different SNPs in exon 1 which give rise to amino acid exchanges in the coding sequence for the collagenous region of MBL. Each of these amino acid exchanges disrupts the homotrimeric structure of MBL, leading to a disrupted and dysfunctional mutant form of MBL. Because one mutant chain alone can disrupt the conformation of the

homotrimer, heterozygosity of a mutant allele results in a deficient phenotype, since the majority of the MBL homotrimeric subunits formed will contain an improperly folded form of MBL (Wallis et al. 2005).

Low plasma levels of MBL can also be caused by SNPs within the MBL promoter regions which lead to low level expression of MBL. The most common promoter SNPs are located in nucleotide positions -550 (termed H/L), -221 (termed Y/X) and -66 (termed P/Q) (Takahashi 2011).

The main function of the lectin pathway recognition component MBL is to recognize molecular patterns on the infectious pathogens, apoptotic cells and injured tissues. MBL directed lectin pathway activation then opsonizes these cells or cellular debris for elimination through phagocytosis (Takahashi 2011, Heitzeneder et al. 2012). Because of the redundancy achieved by having at least five different lectin pathway recognition molecules in plasma, individuals with MBL deficiencies are generally healthy and their increased susceptibility to some infectious diseases is only displayed when other components of immune response fail. MBL deficiency has an impact on the susceptibility on some of the infectious diseases; children with MBL deficiency have a higher risk of meningococcal or pneumococcal infections (Eisen et al. 2008, Heitzeneder et al. 2012). In addition, MBL null mice developed liver injury following the infection with *Staphylococcus aureus* (Takahashi 2011). Interestingly, low MBL concentration in the blood could benefit individuals with MBL deficiency by increasing the resistance against *Mycobacterium tuberculosis* (Soborg et al. 2003).

A very rare polymorphism of the *MASP2* gene has recently been reported, in which aspartic acid at the amino acid position 105 is replaced by glycine (D105G) (Stengaard-Pedersen et al. 2003, Thiel et al. 2009) this mutation results in the loss of an important Ca^{2+} binding site that is critical for the binding of MASP-2 to the lectin pathway recognition complexes. Although this mutant MASP-2 is present in serum and still enzymatically active, it is not bound to the lectin pathway activation complexes; the mutant MASP-2 cannot cleave C4 and C4b-bound C2 on the pathogen surface.

MASP-1/-3 deficiencies are extremely rare. It is caused by mutations in *MASP1* gene. Mutation that leads to either a MASP-3, MASP-1/-3 and CL-11 deficiency lead a developmental syndrome called Carnevale, Mingarelli, Malpuech and Michels syndrome shortly known as 3MC syndrome (Michels et al. 1978, Malpuech et al. 1983, Carnevale et al. 1989, Mingarelli et al. 1996, Rooryck et al. 2011). The 3MC syndrome is characterized by a facial cleft (lip or palate), abnormal skull growth and difficulty in learning. Four different SNPs within the *MASP1* gene lead to the 3MC syndrome: one mutation inserting a stop codon into the coding sequence for the signal peptide shared by MASP-1, MASP-3 and Map44 and three SNPs within the exon 12 of the *MASP1* gene that encodes the serine protease of MASP-3 leading to the expression of functionally inactive MASP-3 while the MASP-1 expression is unaffected (Professor Wilhelm Schwaeble personal communication).

A very recently reported CL-11 deficiency due to a substitution mutation in which the glycine at the position 204 replaced by serine and that led to instability of CL-11 in serum (Rooryck et al. 2011, Selman and Hansen 2012).

1.8.3: Deficiencies of the alternative pathway components

Properdin deficiency is the most common deficiency of components of the alternative pathway. Properdin deficiencies can be classified into three types. Type I refers to a complete deficient of properdin in the serum while type II refers to a very low properdin concentration in the serum, less than 10% of the normal level. Type III indicates that the protein has a normal blood concentrating but is dysfunctional (Skattum et al. 2011).

Properdin deficiency is X-linked so that most deficient individuals are males. Properdin deficiencies predispose to meningococcal infections because *Neisseria* are most efficiently killed by complement serum lytic activity which highly

depends on an active alternative pathway and this pathway is heavily compromised in properdin deficient individuals (Figueroa and Densen 1991).

A complete deficiency of FB has never been reported. There are a few cases of FD deficiencies that manifested themselves clinically through high susceptibility to meningococcal infections (Sprong et al. 2006, Skattum et al. 2011). C3 deficiencies are extremely rare, but it reflects a serious complement deficiency. Although, C3 deficiency is less associated with SLE (Botto et al. 2009) but the patients are susceptible to *N. meningitides*, *S. pneumoniae* and *Haemophilus influenzae* infections (Figueroa and Densen 1991).

1.8.4: Deficiencies of the terminal pathway components

A deficiency in any component of the terminal pathway components (C5-C9) leads to a defect in complement dependent serum lytic activity. The occurrence of C9 deficiency is very rare within the Caucasian populations. However, C9 deficiency is more common in the Japanese population with an allelic frequency of approximately 0.1% (Figueroa and Densen 1991). Individuals with a defect in the terminal pathway of complement have an increased risk for meningococcal infections (Fernie et al. 1996).

1.8.5: Deficiencies in complement regulatory components

Complement regulator proteins maintain a balanced state of complement activation. As described above, C1-INH, controls the classical and the lectin activation pathways by controlling C1s, and C1r esterase activities as well as controlling the proteolytic activity of MASP-1 and MASP-2 (Cicardi and Zingale 2007, Csuka et al. 2013). C1-INH deficiency causes a clinical syndrome called hereditary angioedema. There are two types of hereditary angioedema due to C1-INH deficiency caused either by a mutation within the *SERPINS1* gene, i.e. type I, in which the mutant protein is misfolded or truncated and cannot be

secreted. In type II C1-INH deficiency, the protein is secreted and present in normal levels, but dysfunctional (Cugno et al. 2009, Csuka et al. 2013).

FH is the central regulatory component of the alternative pathway. FH deficiency usually leads to develop membranoproliferative glomerulonephritis (MPGN) a severe and often fatal renal pathology. FH deficiency is also associated with atypical Haemolytic Uremic Syndrome (aHUS) a devastating inflammatory disease caused by perpetual uncontrolled complement activation with often fatal outcome through end stage renal failure (Rougier et al. 1998, Dragon-Durey et al. 2004).

1.9: *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive bacterium, a lancet shaped coccus (0.5-1.25 μm in diameter). These cocci usually form pairs or chains of single bacteria. *S. pneumoniae* is an alpha haemolytic bacterium which grows in 5-10% CO_2 at 25-42 $^{\circ}\text{C}$ in a medium containing a source of catalase (often blood or serum) to neutralize the hydrogen peroxidase produced by these bacteria (Weiser et al. 1994).

The differences in the chemical structure of the capsular polysaccharides have been used to classify *S. pneumoniae* into 91 serotypes, based on strain-specific antibodies raised in rabbits (Henrichsen 1995). These serotypes are subdivided into 46 serogroups depending on the degree of cross reactivity of these antibodies with other serotypes.

Currently, there are two types of *S. pneumoniae* serotypes classification systems, the Danish system and the American system. In the Danish system, the cross reactivity of type specific antibodies is used to allocate serotypes within a common serogroup. In the American system, the order of *S. pneumoniae* strain discovery is used as a base of its classification system (Henrichsen 1995). Recently, other techniques have been developed to identify *S. pneumoniae* strains including DNA sequencing for the gene encoding pneumolysin (Virolainen et al. 1994) and monoclonal antibody against specific serotypes (Yu et al. 2008).

S. pneumoniae infection is one of the most common causes of mortality and morbidity in adults and children throughout the world (Varon et al. 2010). It has been estimated that there are more than 1.6 million people dying of Invasive Pneumococcal Disease (IPD) annually, most of them are children less than 5 years of age (Lynch and Zhanel 2010). IPD is defined by the presence of *S. pneumoniae* in normally sterile sites such as blood, cerebrospinal fluid and pleural fluid causing invasive disease including pneumonia, septicemia and meningitis (Weiser 2010). Pneumococcal infections are the most common causes

of bacterial meningitis in developed countries. It has been estimated that there are more than 1.1 incidences per 100,000 people within the population that suffer meningitis infections caused by *S. pneumoniae* in the USA (Schuchat et al. 1997).

All age groups are under threat of pneumococcal infections, but children less than 2 years of age, adults of more than 65 years of age and immunocompromised patients are the high risk groups (Lynch and Zhanel 2010). Globally, in adults the most common serotypes associated with invasive pneumococcal disease are 14, 4, 1, 6A, 6B, 3, 8, 7F, 23F, 18C, 19F and 9V while serotypes 6, 14, 18, 19, and 23F are the most dominant serotypes responsible for invasive pneumococcal disease in children (Lynch and Zhanel 2010). However, serotypes that are associated with invasive diseases differ among geographical areas. Serotypes, in descending order, 6, 14, 8, 5, 1, 19, 9, 23, 18, 15 and 7 are the most frequent serotypes causing invasive disease in children in developed countries, while the order in developing countries is 14, 6, 19, 18, 9, 23, 7, 4, 1 and 15 (Sniadack et al. 1995).

Otitis media is the most common non-invasive disease caused by *S. pneumoniae*. In the UK, it has been estimated that there are 180 000 – 350 000 annual cases of otitis media in children aged less than 5 years. These cases can be distributed into different age groups including 14% of all cases seen in infants up to 1 year old, 18% in children of an age between 1 and 2 years, 12% of cases in children of an age between 2 and 3 years and 56% in children between an age of 3 to 5 years (Harrison et al. 1985).

S. pneumoniae colonizes the nasopharynx by the adhesion of the capsular polysaccharide to the epithelium. The capsular polysaccharide prevents complement deposition. Neuraminidases cleave host sialic acids as well as glycoproteins exposing the host cell receptors for further adhesion and attachment of streptococci (King et al. 2006). After that, the bacteria start invading the basement membranes of the host cells and that leads to the

progress the disease from colonization stages with no symptoms to the invasive stage.

The bacteria might move down to lungs and the airflow and pneumolysin play a role in that. The pneumolysin interrupts the ciliary beating consequently, facilitating a good access for the bacteria into the lungs (Hirst et al. 2004). *S. pneumoniae* have been shown to bind to the type II alveolar cells which lead to the movement of the bacteria from the lungs to the circulation resulted in sepsis (Cundell and Tuomanen 1994). In the blood stream, the pneumococci replicates and infiltrate in all organs and this filtration creates inflammatory toxic environment.

1.10: Complement interaction with *S. pneumoniae*

Experiments using animal models and sera with defects in specific complement components have shown the pivotal role of complement in the innate immune response to *S. pneumoniae* (Yuste et al. 2008, Ali et al. 2012, Ali et al. 2014). Sera from C2 deficient subjects showed impaired C3 deposition on the bacterial surface resulting in impaired phagocytosis (Yuste et al. 2008).

The targeted deficiency in complement protein in mouse lines has been extensively used to assess the importance of each pathway of complement activation in protection against *S. pneumoniae*. C1q deficient mice have shown more susceptibility to intranasal infection with *S. pneumoniae* compared to their wild-type littermates suggesting an important role for the classical pathway in protection against *S. pneumoniae* (Brown et al. 2002). Moreover, μ deficient mice (mice which lack IgM) were found to have higher mortality and high bacterial burden in blood and tissues compared to their wild-type group control (Brown et al. 2002).

The alternative pathway of complement activation was also shown to play a crucial role in the innate immune response against pneumococcal infections. FB

deficient mice were found to have significantly higher level of bacteria in blood and lung tissues compared to their wild type control group (Brown et al. 2002). In the same line, Li et al., showed a high level of activated FB in the middle ear lavage than the serum samples in the mice with pneumococcal otitis media suggesting that the alternative pathway can be activated at the site of infection as an innate defense mechanism (Li et al. 2012).

Although C1q/FB double knockout mice (mouse line with intact lectin pathway complement activity) have shown less complement C3 deposition onto *S. pneumoniae* surfaces (Brown et al. 2002), recent studies have shown a central role of the lectin pathway in complement activation against *S. pneumoniae*. Mice with a targeted deficiency of MASP-2 showed a high bacterial burden in blood and lung tissues with significantly higher mortality (Ali et al. 2012). Ficolin A and collectin-11 (the most recently described recognition molecules of the lectin pathway) were found to be the specific recognition molecules for *S. pneumoniae* mediating a rapid activation for the complement system. Mice with genetic defects of *FCNA* or *COLEC11* have a high susceptibility to *S. pneumoniae* infection with higher bacterial load in blood and lung tissues (Ali et al. 2012), Ibtehal Alkarawi and Professor Wilhelm Schwaeble, personal communication).

On the other hand, *S. pneumoniae* has several mechanisms by which it interacts and inhibits complement-mediated immunity. Pneumococcal Surface Protein C (PspC) binds to FH (the downregulator for the alternative pathway). Moreover, factor H bound *S. pneumoniae* via PspC enhances adherence of pneumococci to the epithelial and endothelial host cells (Hammerschmidt et al. 2007). Pneumococcal Surface Protein A (PspA) was found to inhibit complement deposition on the bacterial surface. PspA might inhibit C4 activation or C4b deposition on the surface of *S. pneumoniae* (Li et al. 2007).

Finally, pneumolysin was found to activate the classical pathway of complement activation by binding to Fc portion of IgG resulting in depleting the opsonic activity in the serum (Mitchell et al. 1991).

1.11:Hypotheses

The lectin pathway of complement activation is an important component of the innate immune defense against pathogens. It is activated via binding MBL and three ficolins (M, L, H) and CL-11 with their associated serine proteases (i.e. MASP-1, MASP-2, MASP-3) to a complement activating surface. The role of MASP-2 in activating the lectin pathway of complement is well established (Schwaeble et al. 2011). The functional activity of the MASP-1 is less well documented. Some proposed the necessity of the MASP-1 in triggering the initial activation step of the lectin pathway by activating the lectin pathway effector component MASP-2 (Moller-Kristensen et al. 2007, Takahashi et al. 2008, Heja et al. 2012, Megyeri et al. 2014). Others suggested that MASP-1 is essential to activate MASP-3 and complement FD (Takahashi et al. 2010, Iwaki et al. 2011). It is, however, generally accepted that MASP-1 cleaves complement factor C2 and thereby augments the lectin pathway activity.

MASP-2 can autoactivate and on its own initiate the lectin pathway complement of complement activation because it exhibits proteolytic activity on both complement components C4 and C2 required to form the lectin pathway C3 and C5 convertase complexes. In absence of MASP-2, the lectin pathway C3 convertase C4bC2a and C5 convertase C4bC2a(C3b)_n cannot form leading to a total deficiency of this route of activation. In contrast, MASP-1/-3 deficient mouse serum, which has lectin pathway serum complexes exclusively loaded with MASP-2 shows residual lectin pathway C3 cleavage activity. The reduced lectin pathway functional activity in this serum is due to the very low serum concentration of MASP-2 (0.4 microgram compared to 11 microgram of MASP-1 in serum). However, it appears that the low residual lectin pathway functional activity in MASP-1/-3 deficient mice is sufficient to mediate MASP-2 dependent ischaemia-reperfusion injury in a physiologically meaningful context (Schwaeble et al. 2011).

As noted in the introduction above, C4 deficient (Schwaeble et al. 2011, Farrar et al. 2012, Asgari et al. 2014) and MASP-1/-3 deficient mice (Gorsuch et al. 2012)

were not protected from ischaemia-reperfusion injury. The other complement components/activation products involved in the pathophysiology of ischaemia-reperfusion injury are the cleavage/activation product of complement C3, i.e. C3a and C3b (Asgari et al. 2014).

Based on these facts, the hypotheses on which my PhD project was based are as follows:

Hypothesis I

"MASP-2 can activate C3 without involving C4"

This would explain how MASP-2 mediates ischemic injury in absence of C4. In order to test this hypothesis, the ability of MASP-2 to cleave native C3 was assessed using an enzymatically active truncated form of recombinant MASP-2 composed of the three C-terminal domains (i.e. CCP₁-CCP₂-SP) and the endogenous mouse serum MASP-2.

The physiological role of the lectin pathway complement component MASP-3 is still unclear. First, it was reported that MASP-3 acts as a negative regulator for the lectin pathway functional activity, since it competes with MASP-1 and MASP-2 for the binding sites on lectin pathway activation complexes (Dahl *et al.*, 2001). More recently, however, it has been postulated that mouse MASP-3 may have an enzymatic activity towards complement FB (Iwaki et al., 2011). While MASP-1 is critical for the conversion of the alternative pathway zymogen pro-factor D into its enzymatically active form (Takahashi et al. 2010). Ongoing work in Professor Schwaebler's laboratory demonstrated that MASP-3 has an essential role in triggering the alternative pathway dependent lysis of red blood cells; as in absence of MASP-3, the haemolytic activity in plasma and serum is severely compromised.

Based on these observations, the second hypothesis on which my thesis is based is:

Hypothesis II

“MASP-3 cleaves and activates factor B and/or factor D to control the alternative pathway functional activity”

In order to test this hypothesis, human and mouse MASP-3 was expressed in CHO-K1 cells. Subsequently these recombinant materials were used to test its ability to cleave zymogen FB or pro-FD by incubating recombinant human and mouse MASP-3 with purified human FB or with recombinant human pro-FD in serum free conditions. Likewise, recombinant human and mouse MASP-3 will be used to cleave human and mouse pro-factor D in serum.

Since complement activation and C3 deposition on formalin-fixed D39 *Streptococcus pneumoniae* was compromised in MASP-1/-3 deficient sera and since the MASP-1/-3 deficient mouse line was highly sensitive to *S. pneumoniae* infection, the third hypothesis on which my thesis is based is:

Hypothesis III

“Defective alternative pathway functional activity of a MASP-1/-3 deficient mouse strain can be restored with recombinant MASP-3”

To test this hypothesis, MASP-1/-3 deficient mouse sera were reconstituted with recombinant human or mouse MASP-3 then the complement activation assay (C3 deposition) were performed on formalin-fixed D39 *S. pneumoniae*. In *in vivo* experiments, MASP-1/-3 deficient mouse line was injected with recombinant mouse MASP-3 then the sera were collected to measure the alternative pathway functional activity.

1.12: Aims

My PhD study aimed

- i) To identify the molecular composition of the lectin pathway and MASP-2 dependent C4-bypass activation of complement C3;
- ii) To assess the role of MASP-3 in the lectin pathway mediated initiation of the alternative pathway of complement;
- iii) To identify the natural substrates and activators of MASP-3.

This work intended to increase our understanding of the interactions and the molecular cross-talk between the lectin and the alternative activation pathways. This work revealed that the lectin pathway activates complement via two independent effector arms, the MASP-2 dependent Lectin pathway Effector Arm 2 (LEA-2) and the MASP-3 dependent Lectin pathway Effector Arm 1 (LEA-1) through which the lectin pathway modulates in a MASP-3 dependent fashion alternative pathway functional activity. The modulatory activity of MASP-3 on alternative pathway functional activity opens new therapeutic opportunities for the treatment of diseases caused by overshooting alternative pathway activation.

Chapter Two: Materials and methods

2.1: Materials

2.1.1: Chemical materials

<u>Material</u>	<u>Supplier</u>
1 kb plus DNA ladder	Invitrogen
2-mercaptoethanol	Sigma-Aldrich
2-Propanol	Sigma-Aldrich
3-(N-morpholino) propanesulfonic acid (MOPS)	Sigma-Aldrich
36.5% formaldehyde solution	Sigma-Aldrich
5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside (Xgal)	Sigma-Aldrich
6X DNA loading dye	Thermo scientific
Acetic acid glacial	Fisher scientific
Acrylamide bis	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Ammonium persulphate	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
<i>Bam</i> H I restriction enzyme	New England Biolabs
Barbital	Sigma-Aldrich
Blood agar base	Oxoid
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Brilliant Blue R-250	Fisher scientific
Bromophenol Blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Deoxyadinosine triphosphate (dATP)	Thermo scientific
Deoxyribo nucleotide PCR grade (dNTPs)	Promega
Dialysed fetal bovine serum	Invitrogen
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Divinyl sulfone	Fluka
EDTA- disodium salt	Sigma-Aldrich
Ethanol	Fisher Scientific

<u>Material</u>	<u>Supplier</u>
Ethidium bromide	Sigma-Aldrich
Ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Gelatin	BDH
Glycerol	Fisher scientific
Glycin	Fisher scientific
Heparin	Sigma-Aldrich
HEPES	Sigma-Aldrich
Horse blood	Oxoid
<i>Kpn</i> I restriction enzyme	New England Biolabs
Mannose	Sigma-Aldrich
<i>Not</i> I restriction enzyme	New England Biolabs
RNase A solution	Promega
Sigma-Aldrich Sepharose-6B	Sigma-Aldrich
Sigmafast <i>p</i> -Nitrophenyl phosphate tablets	Sigma-Aldrich
Skim milk	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium carbonate anhydrous	Fisher scientific
Sodium chloride	Fisher scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium hydrogen carbonate	Fisher scientific
T4 DNA ligase	New England Biolabs
T4 DNA ligase 10X buffer	New England Biolabs
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Thermoprime plus DNA polymerase	Thermo scientific
Thrombin	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100	BDH laboratory

<u>Material</u>	<u>Supplier</u>
Trizma base	Sigma-Aldrich
Trypsin from bovine pancreas	Sigma-Aldrich
Trypsin-EDTA	Sigma-Aldrich
Tryptone	BD
Tween-20	Sigma-Aldrich
VPR-AMC	R&D Systems
<i>Xho</i> I restriction enzyme	New England Biolabs
Yeast extract	BD
Zymosan	Sigma-Aldrich

2.1.2: Complement proteins

Complement protein	Supplier
Human C1-INH	Complement technology
Human C3	Complement technology
Human C3b	Complement technology
Human C4	Provided by Dr. Nicholas Lynch Department of Infection, Immunity and Inflammation, University of Leicester, UK
Human C5	Complement technology
Human FB	Complement technology
Human FH	Complement technology
Human MASP-1 (CCP ₁ -CCP ₂ -SP)	Provided by Dr. Peter Gal, Institute of Enzymology, Research Centre for Natural Sciences, Budapest, Hungary
Human MASP-2 (CCP ₁ -CCP ₂ -SP)	Provided by Dr Peter Gal, Institute of Enzymology, Research Centre for Natural Sciences, Budapest, Hungary
Rat MASP-2K	Provided by Professor Russell Wallis Department of Infection, Immunity and Inflammation, University of Leicester, UK
Human MASP-3 (CCP ₁ -CCP ₂ -SP)	Omeros Corporation Seattle, USA
Human pro-FD	Omeros Corporation Seattle, USA
OM721 HG4	Omeros Corporation Seattle, USA

2.1.3: Antibodies

Antibody	Supplier
Anti-human C4	Santa cruz
Anti-human FB	Santa cruz
Goat anti-rabbit AP	Sigma-Aldrich
Monoclonal Rat anti-human MASP-3	Hycult
Mouse anti-human MBL	Antibodyshop
Mouse anti-polyhistidin antibody	Sigma-Aldrich
Polyclonal rabbit anti-human MASP-1/-3	Santa Cruz
Rabbit anti-Rat HRP antibody	Dako
Rabbit anti-human C3c	Dako
Rabbit anti-mouse HRP	Dako
Rabbit anti-mouse HRP antibody	Dako

2.1.4: Kits

Kit	Supplier
pGEM-T easy vector	Promega
QIAquick gel extraction kit	Qiagen
Wizard genomic DNA purification kit	Promega
Wizard plus SV minipreps DNA purification kit	Promega

2.1.5: Media

Medium	Supplier
Agar	Oxoid
Brain Heart Infusion (BHI)	Oxoid
CHO-S-SFM II (-hypoxanthine, -thymidine)	Gibco
CHO-S-SFM II	Gibco
F-12 Nutrient Mix	Gibco
Minimum Essential Medium (MEM) alpha (-ribonucleosides, -deoxyribonucleosides)	Sigma-Aldrich
Opti-MEM® I Reduced Serum	Invitrogen
RPMI-1640	Sigma-Aldrich
Tryptone	BD
Yeast extract	BD

2.2: Methods

2.2.1: Molecular biology techniques

2.2.1.1: Screening of complement components deficiency in mice

2.2.1.1.1: Isolation of genomic DNA from mouse ear snips

Genomic DNA from the ear snips of MASP-1/-3 KO, MASP-2 KO, C1q KO, and C4 KO mice, were isolated using Wizard gDNA Purification Kit (Promega). 60 μ L of 0.5 M EDTA and 250 μ L of nuclei lysis solution (Promega) were mixed and 300 μ L of this mixture were added to the fresh mouse ear snip in an eppendorf tube and incubated overnight at 55°C after adding 10 μ L of 20 mg/mL of proteinase K (Qiagen). After that, 1.5 μ L of RNase A solution was added and incubated for 15 minutes at 37°C. 100 μ L of protein precipitation solution (Promega) were added to the sample and sample was mixed well and chilled on ice for 5 minutes. The mixture was then centrifuged for 4 minutes at 13000 rpm and the supernatant containing DNA was moved to 1.5 mL eppendorf tube containing 300 μ L of 2-propanol and centrifuged for 5 minutes. Precipitated DNA was washed using 70% ethanol and centrifuged for 1 minute. After discarding ethanol, the purified DNA was re-hydrated by adding 100 μ L of nuclease free water and stored at 2-8°C.

2.2.1.1.2: Polymerase chain reaction (PCR) for mouse gDNA

Isolated gDNA from mouse ear snips was PCR amplified using a standard PCR reaction mix (table 2-1) and the gDNA was amplified using specific PCR genotyping programmes (tables 2-2 and 2-3) and different sets of primers (table 2-4).

Table 2-1: Standard PCR reaction mix used for genotyping

Material	Final concentration
10 X buffer	1 X
25 mM MgCl ₂	1.5 mM
10 mM dNTP Mix	200 µM
5 mM Primer 1	0.2 µM
5 mM Primer 2	0.2 µM
5 mM Primer 3	0.2 µM
Taq DNA Polymerase	1.2 Unit
Template DNA	100 ng
dH ₂ O	Up to 50 µL

Table 2-2: Programme used for PCR genotyping of MASP-1/-3 KO mice

Step	Temperature (°C)	Time (s)	Cycles
Initiation	98	90	
Denaturation	98	30	
Annealing	60	30	
Elongation	72	30	29
Final elongation	72	300	
Hold	4.00	∞	

Table 2-3: Programme used for PCR genotyping of MASP-2 KO, C4 KO and C1q KO mouse

Step	Temperature (°C)	Time (s)	Cycles
Initiation	95	90	
Denaturation	95	30	
Annealing	62	30	
Elongation	72	30	35
Final elongation	72	300	
Hold	4.00	∞	

Table 2-4: Oligonucleotides used for mouse PCR genotyping

Gene	Primer name	Sequence 5' --> 3'
<i>MASP1</i>	NeoU	CAT CGC CTT CTA TCG CCT TCT TGA
	M1U	CTC CCT GCC TCA GAC TGT TTG ATA
	Mil	GCT GAT GCT GAT GTT AGG ATG GTA TTC
<i>MASP2</i>	M2 screen_F1	CAT CTA TCC AAG TTC CTC AGA
	Neo5_R1	CTG ATC AGC CTC GAC TGT GC
	M2wto_R1 (WT)	AGC TGT AGT TGT CAT TTG CTT GA
<i>C4B</i>	mC4wto_F2 (WT)	GCA TTT CTC TCC CTT CTA GAA CA
	mC4_R1	TGT AGC CCG TGG GTC TTA AG
	Neo3_F4	CCT TCT TGA CGA GTT CTT CTG A
<i>C1q</i>	mC1qA/5+	GGG GCC TGT GAT CCA GAC AG
	mC1qN/2 (WT)	TAA CCA TTG CCT CCA GGA TGG
	Neo3	GGG GAT CGG CAA TAA AAA GAC

The PCR products were run in 1% agarose gel for 45 minutes at 120V.

Table 2-5: Protocol used to make agarose gel electrophoresis

Material	Composition
Agarose gel	1% agarose in TAE buffer then ethidium bromide was added 0.5 µg/mL final concentration
Tris- Acetic acid-EDTA (TAE) buffer, pH 8.5	2.0 M Tris-HCl, 50 mM EDTA-Na ₂ , 1.0 mM acetic acid glacial

2.2.1.2: PCR amplification of human and mouse MASP3

Human cDNA, corresponding to human MASP-3 mRNA, was used as a template to amplify *MASP3* with a high-fidelity platinum *pfu* DNA polymerase (Thermo Scientific). The following synthetic oligonucleotide primers sense primer (*Kpn* I site underlined) 5' GGT ACC CAC ACC GTG GAG CTA AAC AAT ATG TTT G 3' and antisense primer (*Not* I site and underlined) 5' GCG GCC GCA CAC CGT TCC ACC TG GGG CT 3' were used to amplify the full-length coding sequence of human *MASP3* and sense primer (*Kpn* I site and underlined) 5' GGT ACC ATT GTA GAC TGT AGA GCC CCA GGA 3' was used as a forward primer to amplify the coding sequence of human MASP-3 serine protease domain [based on nucleotide sequence of GenBank Accession Number: AF284421].

Mouse *MASP3* was amplified from mouse cDNA using phusion high-fidelity DNA polymerase (Thermo Scientific) and the following primers sense primer (*Bam*H I site and underlined) GGA TCC ATG AGG TTC CTG TCT TTC TGG CG 3' and antisense primer (*Xho* I site and underlined) 5' CTC GAG CGC ACC GTT CTA CCT GGA GG 3' [based on nucleotide sequence of GenBank Accession Number: AB049755].

The hexahistidine tag was added at 3' end of the *MASP3* sequence in place of the stop codon. The PCRs were carried out with following cycling programmes:

Table 2-6: Programme used for PCR amplifying of human MASP-3

Step	Temperature (°C)	Time (s)	Cycles
Initiation	98	90	
Denaturation	98	15	
Annealing	70	30	
Elongation	68	40	15
Denaturation	98	15	
Annealing	58	30	
Elongation	68	40	25
Final elongation	68	300	
Hold	4.00	∞	

Table 2-7: Programme used for PCR amplifying of mouse MASP-3

Step	Temperature (°C)	Time (s)	Cycles
Initiation	98	90	
Denaturation	98	15	
Annealing	75	30	
Elongation	72	40	15
Denaturation	98	15	
Annealing	65	30	
Elongation	72	40	25
Final elongation	72	300	
Hold	4.00	∞	

The PCR products were run in 1% agarose gel for 45 minutes at 120V.

2.2.1.3: *Purification of DNA from agarose gel*

DNA bands were purified using QIAquick gel extraction kit (Promega). The agarose gel contains desired fragments was cut using clean and sharp scalpel. QG buffer was added and incubated at 50°C for 10 minutes with shaking. After

the gel completely dissolved, the dissolved DNA was moved to QIAquick spin column and centrifuged for 1 minute. After that, 500 μL of QG buffer were added and centrifuged for 1 minute. The column was washed using 750 μL of washing buffer (PE) and centrifuged for 1 minute. After discarding the flow-through, the column was centrifuged for another 1 minute and the DNA was then eluted by adding 50 μL of nuclease free water.

2.2.1.4: A tailing of PCR products

A 3' terminal A overhang was added to the purified PCR products according to the following protocol:

Table 2-8: Protocol used for A-tailing of the purified PCR products

Material	Amount
Gel extracted DNA	3 μg
100 mM dATP	0.1 μM
10X taq PCR buffer	1 X
Taq DNA polymerase	1.2 Unit

The reaction was run at 70°C for 25 minutes.

2.2.1.5: Cloning of PCR products into pGEM-T easy vector

The DNA construct was ligated into pGEM-T easy victor (Promega) using T4 DNA ligase (Promega) according to the following protocol:

Table 2-9: Protocol used for cloning PCR product into pGEM-T easy vector

Material	Final concentration
2X rapid ligation buffer	1 X
pGEM-T easy vector (50 ng)	50 ng
PCR product	10 ng
T4 DNA ligase	3 Units
dH ₂ O to a final volume of	10 μL

The reaction was incubated at room temperature for 2 hours or at 4°C for overnight.

2.2.1.6: Preparation of chemically competent cells

Top 10 *E. coli* from the frozen stock were cultured onto Luria-Bertani (LB) agar plate (table 2-10) and incubated for 16 hours at 37°C. After that, one colony was picked up and transferred into 5 mL of LB broth medium (table 2-10) containing 20 mM MgSO₄ and incubated overnight at 37°C. Next day, 1 mL of the overnight culture was transferred into 100 mL of LB broth medium and incubated at 37°C for 2.5 to 3 hours until OD_{550nm} reaches 0.7. After centrifugation the culture for 10 minutes at 3000 rpm, the cells were re-suspended in 30 mL of Tfb I (table 2-11). Another centrifugation was done for 10 minutes at 3000 rpm at 4°C and the pellet was re-suspended into 4 mL of Tfb II (table 2-11). Single use aliquots were made and stored at -80.

Table 2-10: Media used for bacterial culture

Medium	Composition
Luria-Bertani (LB) broth	1% trypton (w/v), 0.5% yeast extract (w/v) , 0.5% NaCl (w/v).
Luria-Bertani (LB) agar	1% trypton (w/v), 0.5% yeast extract (w/v) , 0.5% NaCl (w/v), 1.5% agar (w/v).

Table 2-11: Buffers used for competent cells preparation

Buffer	Composition
Transformation buffer (Tfb I, pH 7.4	3 mM CH ₃ CO ₂ K, 50 mM MnCl ₂ , 100 mM KCl, 15% glycerol (v/v)
Transformation buffer (Tfb II, pH 7.4	10 mM Na-MOPS, 10 mM KCl, 75 mM CaCl ₂ , 15% glycerol (v/v)

2.2.1.7: *Transfecting chemically competent cells*

LB agar was prepared according to composition given in protocol (table 2-10) after autoclaving, the medium was left to cool down to 60°C and ampicillin (Sigma-Aldrich) was then added 100 µg/mL final concentration. After pouring the plates, plates were left to dry in a laminar flow hood for an hour. 40 µL of 0.1 M IPTG (table 2-12) and 40 µL of 20 mg/mL Xgal (table 2-12) were added to the plate surface.

Heat shock method was used to transfer the plasmid DNA into Top 10 *E. coli*. The competent cells were placed on ice until thawed (for about 5 minutes) and 2 µL of the ligation reaction (pGEM-T easy/MASP3 construct) were added to a sterile eppendorf tube on ice then 50 µL of the competent cell stock were added to the tube and incubated in ice bath for 20 minutes followed by heat shock for 4 minutes in a water bath at 37°C. The tube was then returned immediately to the ice bath for 2 minutes. After that, the cells were transferred into 450 µL of LB broth and incubated for 90 minutes at 37°C with gentle shaking. Two different volumes, 50 µL and 100 µL, of this culture were plated on LB agar plates and incubated for overnight at 37°C.

Table 2-12: Protocol used to prepare IPTG and Xgal stock solutions

Buffer	Composition
IPTG	0.1 M of isopropyl thiogalactoside, (Isopropyl beta-D-thiogalactopyranoside) in distilled water
Xgal	20 mg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside dissolved in DMSO

2.2.1.8: *Isolation and purification of plasmid DNA*

Single colony was picked from the overnight culture and cultivated in LB broth medium containing 100 µg/mL ampicillin final concentration and incubated overnight at 37°C with shaking. Plasmid DNA was prepared using wizard plus SV minipreps DNA purified system (Promega). Briefly, the overnight bacterial

culture was centrifuged for 10 minutes at 3400 rpm. 250 μ L of cell re-suspension solution were added and mixed by vortexing. Then, 250 μ L of cell lysis solution were added and mixed by inverting the tube four times and tubes were incubated for 2 minutes at room temperature. After that, 10 μ L of alkaline protease solution were added and kept at room temperature for 4 minutes. The bacterial lysate was centrifuged at 13000 rpm for 10 minutes after adding 350 μ L of neutralization solution. For plasmid DNA isolation and purification, the supernatant of bacterial lysate was moved to a spin column and centrifuged for 1 minute. The centrifugation was repeated two times for 1 and 2 minutes after adding 750 μ L and 250 μ L of column wash solution, respectively. The purified plasmid DNA was eluted by adding 50 μ L of nuclease free water.

2.2.1.9: Restriction digestion

Isolated plasmid DNA was digested using restriction enzymes as the following protocol:

Table 2-13: Protocol used for DNA digestion from pGEM-T easy vector

Material	Final concentration
DNA sample	0.1 μ g
<i>Kpn</i> I Restriction enzyme*	5 Units
<i>Not</i> I Restriction enzyme*	5 Units
10X Restriction buffer 2	1 X
10X BSA	1 X
Deionized water to a final volume of	10 μ L

* *Bam*H I and *Xho* I restriction enzymes were used to digest mouse *MASP3* construct.

The mixture was incubated at 37°C for 2 hour at room temperature or at 4°C for overnight. Then, the desired DNA was detected by 1% agarose gel.

2.2.1.10: Sub-cloning of purified DNA into expression vector pSecTag 2/ Hygro B

DNA construct was sub-cloned into pSecTag 2/ Hygro B by firstly, digestion the desired DNA and the expression vector by the same restriction enzymes. Then, ligation of the purified DNA into pSecTag 2/ Hygro B according to the following protocol:

Table 2-14: Protocol used for sub-cloning of DNA product into pSecTag 2/ Hygro B vector

Material	Final concentration
pSecTag 2/ Hygro B	50 ng
10X ligase Buffer	1 X
Purified DNA	10 ng
T4 DNA ligase	3 Units
Deionized water to a final volume of	10 μ L

After overnight incubation at 4°C, the mixture was transformed into Top-10 *E. coli*.

2.2.2: Cell culture techniques

2.2.2.1: Cultivation of Chinese Hamster Ovary (CHO)-K1 Cells

CHO-K1 cells were cultivated in Ham's F-12 Nutrient Mix, GlutaMax (Gibco) culture medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) in presence of 5 mL of 100U penicillin/0.1 mg/mL streptomycin (Sigma-Aldrich) (table 2-15) and incubated at 37°C into CO₂ incubator.

Table 2-15: Preparation of serum CHO-K1 cell line medium

Medium	Composition
Serum CHO-K1 medium	500 mL Ham's F-12 Nutrient Mix, GlutaMax 10% FBS (v/v), 5 mL 100U penicillin / 0.1 mg/mL streptomycin

2.2.2.2: Cultivation of DUX B11- MBL cell line

DUX B11-MBL cells were cultivated in a minimum essential medium (MEM) (Sigma-Aldrich) with 10% dialyzed FBS (Invitrogen) in presence of 5 mL of 100U penicillin/0.1 mg/mL streptomycin (Sigma-Aldrich) (table 2-16) and 0.5 µM of methotrexate and cells were incubated into CO₂ incubator at 37°C. When the cells were approximately 80% confluent, cells were sub-cultured into triple layers flasks and incubated at 37°C until become confluent.

Table 2-16: Preparation of serum DUX B11 cell line medium

Medium	Composition
Serum DUX B11 medium	500 mL MEM, 10% dialysed FBS (v/v), 5 mL 100U penicillin / 0.1 mg/mL streptomycin

2.2.2.3: Transfecting CHO-K1 cells with MASP3 construct

Once the CHO-K1 culture was approximately 80% confluent, cells were sub-cultured into 6 well plate and incubated at 37°C until become 50-80% confluent.

GeneJuice transfection reagent (Novagen) was used to transfect CHO-K1 cells with human MASP-3 plasmid DNA. 3 μL of the GeneJuice were added into 100 μL of RPMI-1640 medium (Sigma-Aldrich) and mixed thoroughly by vortexing and incubated for 5 minutes. After that, 2 μL of plasmid DNA, standing for 502.8 ng/ μL , were added to the mixture and mixed by pipetting and another incubation was made for 10 minutes. The mixture was added over entire surface of the well then the plate placed into CO₂ incubator at 37°C.

Lipofectamine™ LTX reagent (Invitrogen) was used to transfect CHO-K1 cells with mouse MASP-3 plasmid DNA. 3 μL of plasmid DNA (500 ng/ μL) were diluted in 100 μL of Opti-MEM I reduced serum medium (Invitrogen). And then 3 μL of Lipofectamine™ LTX were added to the diluted DNA solution. Reaction was mixed gently and incubated for 25 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. The growth medium was removed from cells and replaced with 1.5 mL of complete growth medium (table 2-15). Then, 100 μL of the DNA-Lipofectamine™ LTX mixture were added directly to each well containing cells and mixed by rocking the plate back and forth. Cells were incubated at 37°C in a CO₂ incubator for overnight.

Next day, the transfection mixture was removed and replaced with Ham's F-12 Nutrient Mix serum medium (table 2-15) containing 300 $\mu\text{g}/\text{mL}$ hygromycin B (Invitrogen). After 48 hour of transfection, cells were washed three times with the phosphate buffered saline (PBS) (table 2-17) then 100 μL of trypsin-EDTA (Sigma-Aldrich) were added to each well and the plate placed in incubator at 37°C for 1 minute. After that, cells were re-suspended immediately with Ham's F-12 Nutrient Mix serum medium (table 2-15) containing 300 $\mu\text{g}/\text{mL}$ hygromycin B and distributed into 24 well plate.

When the cells were 80-90% confluent, serum medium was substituted with serum free medium (CHO-S-SFM II) (Gibco) containing 100 U penicillin/ 0.1

mg/mL streptomycin and 300 µg/mL hygromycin B (table 2-18). After 48 hours of incubation at 37°C, supernatant was collected and protein was detected by dot blot and confirmed by Western blotting techniques.

Table 2-17: Preparation of phosphateed buffer saline

Buffer	Composition
PBS, pH 7.4	18 mM KH ₂ PO ₄ , 101 mM NaH ₂ PO ₄ , 1.369 M NaCl, 27 mM KCl

Table 2-18: Preparation of serum free CHO-K1 cell line medium

Medium	Composition
Serum free CHO-K1 medium	500 mL CHO-S-SFM II, 5 mL 100U penicillin / 0.1 mg/mL streptomycin, 1.5 mL of 100 mg/mL hygromycin B

2.2.3: Protein methods

2.2.3.1: Dot blot

Dot blot is a simple technique used for detecting positive clones expressing recombinant protein. 30 μ L of the culture supernatant were spotted onto nitrocellulose membrane (Bio-Rad) and allowed to dry. Then, the membrane blocked with 5% skim milk (Sigma-Aldrich) in PBS for 1 hour at room temperature with shaking. Monoclonal mouse anti-polyhistidine HRP conjugated antibodies (Sigma-Aldrich) diluted 1:10000 in blocking buffer were added and incubated at room temperature for 1 hour with shaking. After washing three times with PBS containing 0.05% Tween-20, the luminata crescendo Western HRP substrate (Millipore) was added onto nitrocellulose membrane surface and the membrane was then exposed to Fuji film (Fisher Scientific).

2.2.3.2: Protein purification

2.2.3.2.1: Purification of recombinant MBL

2.2.3.2.1.1: Preparation of mannose-Sepharose column

Fifty milliliters of Sepharose-6B (Sigma-Aldrich) were washed through a large filter with 2-3 litres of nano-pure water and transferred into a 200 mL beaker containing 50 mL of 0.5 M sodium bicarbonate buffer pH 11 (table 2-19) and 10 mL of divinyl sulfone (DVS) (Fluka) (DVS; for cross-linking the Sepharose) and left to stir gently for 70 minutes. The Sepharose was washed again with 2-3 litres of nano-pure water. The mannose (Sigma Aldrich) was dissolved in 0.5 M sodium bicarbonate buffer pH 10 (20%, w/v) and 50 mL of mannose solution were then added to the Sepharose and left to stir gently for overnight.

After filtering and washing mannose-coupled Sepharose with 2-3 litres of nano-pure water, 50 mL of 0.5 M sodium bicarbonate buffer pH 8.5 containing 1 mL

of 2-mercaptoethanol (Sigma-Aldrich) were added to block the uncoupled sites on the Sepharose and gently stirred for 2 hours. The mannose-coupled Sepharose washed again with 2-3 litres of nano-pure water and stored in clean filtered water containing 0.05% sodium azide (Sigma-Aldrich) at 4°C.

Table 2-19: Preparation of sodium bicarbonate buffer

Buffer	Composition
Sodium bicarbonate buffer, pH 8.5, 8.0, 11.0	0.5 M Na ₂ HCO ₃

2.2.3.2.1.2: MBL purification on mannose-Sepharose column

DUX B11-MBL cell line was cultivated in triple layers flasks and incubated at 37°C in CO₂ incubator and when the cells were approximately 80% confluent, cells were washed trice with PBS and the serum medium was substituted with serum free medium (Gibco) (table 2-20) containing 5 mL of 1 M HEPS pH 7.4, 5 mL of 100U penicillin / 0.1 mg/mL streptomycin and 0.5 µM of methotrexate. After 48 hours of incubation at 37°C, supernatant was collected to purify the recombinant protein.

Serum free medium was collected from DUX B11-MBL cell line and centrifuged at 3000 g for 10 minutes to remove the cell debris. High salt buffer (table 2-21) was added to the supernatant in a ratio 1:1 (v/v) and the pH was adjusted to 7.4. Three milliliters of mannose-Sepharose (for 500 mL of cell culture medium) were washed with 5X column volumes of high salt loading buffer. After that, the mixture of supernatant and high salt buffer was applied over the column in the cold room. After that, the column was washed with 10 mL of high salt buffer then with 10 mL of low salt buffer (table 2-21). 10 mL of elution buffer were applied to elute the recombinant protein (table 2-21).

Table 2-20: Preparation of serum free DUX B11 cell line medium

Medium	Composition
Serum free DUX B11	500 mL CHO-S-SFM II (-hypoxanthine and -thymidine), 5 mL HEPS pH 7.4, 5 mL 100U penicillin / 0.1 mg/mL streptomycin

Table 2-21: Buffers used for purification of recombinant MBL from mannose-Sepharose column

Buffer	Composition
High Salt Buffer (HSB), pH 7.4	50 mM Tris-HCl, 1.25 M NaCl, 10 mM CaCl ₂
Low Salt Buffer (LSB), pH 7.4	50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl ₂
Elution Buffer (EB), pH 7.4	50 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA

2.2.3.2.2: Purification of recombinant human and mouse MASP-3

Positive clones in dot blot assay were grown in large triple layers flasks in serum medium (table 2-15) containing 300 µg/mL hygromycin B. When the cells were 80% confluent, the culture medium was replaced with serum free medium (table 2-18) and incubated at 37°C in CO₂ incubator. After two days, the medium was collected and centrifuged at 3400 rpm for 10 minutes to remove the cell debris. The supernatant was mixed with binding buffer 50% (v/v) (table 2-22) and the pH was adjusted to 7.4. After that, the mixture was applied into HisGravi Trap column (GE Healthcare) for overnight in a cold room. After washing the column with 10 mL of binding buffer (table 2-22). Two milliliters of binding buffer containing 100 mM, 200 mM, 300 mM and 500 mM imidazole (table 2-22) were applied to elute the recombinant protein.

Table 2-22: Buffers used for Ni²⁺ affinity chromatography

Buffer	Composition
Binding buffer, pH 7.4	500 mM NaCl, 20 mM NaH ₂ PO ₄ , 20 mM imidazole
Wash buffer, pH 7.4	500 mM NaCl, 20 mM NaH ₂ PO ₄ , 20 mM imidazole
Elution buffer, pH 7.4	500 mM NaCl, 20 mM NaH ₂ PO ₄ , 300 mM imidazole

2.2.3.3: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to study the characterisations of recombinant human and mouse MASP-3 and recombinant human MBL under reducing and non-reducing conditions. In the reducing conditions, 20 µL of 0.1 mg/mL recombinant protein were boiled at 90°C for 10 minutes with SDS protein-loading buffer containing 5% of 2-mercaptoethanol (table 2-23). The non-reduced SDS-PAGE was performed by adding the SDS protein-loading buffer without boiling and exclusive of reducing reagent. After that, the samples and PageRuler as a molecular weight marker (Thermo Scientific) were loaded into 10% SDS polyacrylamide gel (for recombinant human and mouse MASP-3) or onto 12% SDS polyacrylamide gel (for recombinant human MBL) and run in 1X SDS running buffer (table 2-23) at 150V for 1 hour. After that, the gel was fixed with fixing buffer (table 2-23) for 15 minutes and stained with Coomassie Blue (table 2-23) for 30 minutes at room temperature with gentle shaking. Then, the gel was de-stained using de-staining solution (table 2-23) until the protein bands become visible.

Table 2-23: Protocol used to prepare SDS-PAGE buffers

Buffer	Composition
5X SDS protein-loading buffer	5.8 mL Tris-HCl pH 6.8, 2.5 mL glycerol, 0.83 g SDS, 1 mg bromophenol Blue
5X SDS protein-loading buffer for reducing protein	5% 2-mercaptoethanol in 5X SDS protein-loading buffer
Ammonium per-sulphate	10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (w/v) in distilled water
Coomassie Blue dye	1.2 g Coomassie brilliant Blue, 50% methanol, 10% acetic acid glacial (v/v), 40% Milli-Q water
De-staining solution	45% methanol, 10% acetic acid glacial (v/v), 45% Milli-Q water
Fixing solution	50% methanol, 10% acetic acid glacial (v/v), 40% Milli-Q water
SDS solution	10% SDS (w/v) in distilled water
Tris solution, pH 6.8	1 M Tris-HCl
Tris solution, pH 8.8	1.5 M Tris-HCl
Tris-Buffer Saline (TBS), pH 7.4	10 mM Tris-HCl, 140 mM NaCl
Tris/glycin/SDS running buffer, pH 8.3	25 mM trizma base, 192 mM glycin, 0.1% SDS (w/v)

2.2.3.4: Western blotting

Western blotting was performed to identify the recombinant proteins and their size. The SDS-PAGE separated proteins were transferred electrophoretically at 300 mA for 90 minutes in transfer buffer (table 2-24) onto supported nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS for 1 hour with shaking. After washing three times with PBS containing 0.05% Tween-20, monoclonal rat anti-human MASP-3 antibodies (Hycult) diluted 1:2000 in blocking buffer were added and incubated for 1 hour at room temperature with shaking. The membrane was then washed three times and rabbit anti-rat IgG HRP conjugated antibodies (Dako) diluted 1:5000 in blocking buffer were added and incubated for another 1 hour at room temperature with gentle shaking. After that, the membrane was washed three times and luminata crescendo Western HRP substrate was added onto the entire nitrocellulose membrane surface and exposed to Fuji film for 5 minutes.

In another Western blotting, monoclonal mouse anti polyhistidine antibodies HRP conjugated (Sigma-Aldrich) diluted 1:5000 in blocking buffer were used to detect the recombinant human and mouse MASP-3.

Western blotting of recombinant human MBL was probed with mouse anti-human MBL antibodies (Antibodyshop) diluted 1:2000 in washing buffer and rabbit anti-mouse HRP antibodies (Dako) diluted 1:5000 in washing were used as secondary antibodies.

Table 2-24: Protocol used to prepare Western blotting transfer buffers

Buffer	Composition
Tris/glycin transfer buffer, pH 8.3	25 mM trizma base, 192 mM glycin, 20% methanol (v/v)

2.2.4: Preparation of serum for functional complement assay

2.2.4.1: Human serum

Human sera were obtained from the blood of healthy individuals and left to clot for 15 minutes at room temperature. After centrifugation at 3000 g for 10 minutes at 4°C, serum was aspirated and immediately placed in -80°C freezer until used (Lachmann 2010).

2.2.4.2: Mouse serum

Blood from mice previously genotyped for complement deficiency was collected by cardiac puncture under general anaesthetic and immediately transferred onto ice for 3 hours to prevent complement activation. The blood was then spun down at 7000 rpm for 7 minutes at 4°C using a refrigerated centrifuge. Serum was collected and centrifuged again and then aliquot and stored in -80°C freezer until used.

2.2.5: MASP-2-dependent, lectin pathway mediated C4-bypass activation of C3

2.2.5.1: C3 activation by MASPs

The proteolytic activity of the lectin pathway serine proteases i.e. MASP-1, MASP-2, and MASP-3 on the third complement component was assayed. 100 µg/mL of purified human C3 (CompTech) were mixed with 7.5 µg/mL of catalytic domains (CCP₁-CCP₂-SP) of MASP-1, MASP-2 or MASP-3 in a total volume of 20 µL in TBS-Ca²⁺-Mg²⁺ buffer (table 2-25) and incubated at 37°C for 90 minutes with gentle shaking (250 rpm).

Reactions were stopped by adding 5 µL of 5X SDS loading buffer containing 5% of 2-mercaptoethanol and boiled at 90°C for 10 minutes. After brief centrifugation, samples were separated in 8% SDS polyacrylamide gel and stained with Coomassie Blue.

In another experiment, 7.5 µg/mL of MASP-2 (CCP₁-CCP₂-SP) were incubated with 100 µg/mL of human C3 in TBS-Ca²⁺-Mg²⁺ buffer (table 2-25) at different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0).

Table 2-25: Preparation of TBS-Ca²⁺-Mg²⁺ buffer

Buffer	Composition
TBS-Ca ²⁺ -Mg ²⁺ buffer, pH 7.3, 8.0	2 mM MgCl ₂ , 5 mM CaCl ₂ , TBS up to 1 litre

2.2.5.2: C3 cleavage by mouse MASP-2K

The recombinant mouse full-length MASP-2K proteolytic activity on C3 was examined. The assay was performed by mixing 1 µg/mL of recombinant mouse MASP-2K with 1 µg/mL of recombinant human MBL in MBL binding buffer (table 2-26) for overnight at 4°C. Nunc MaxiSorp 96 well plate was coated with 10 µg/mL of mannan in coating buffer (table 2-26) for overnight at 4°C. Protein binding sites were blocked with 1% bovine serum albumin (BSA) in TBS for 2 hours at room temperature. After washing the plate three times with washing buffer (table 2-26), 100 µL of MBL(MASP-2K)₂ mixture (corresponding to 0.1 µg each) were then added to the plate and plate was incubated at room temperature for an hour. Plate was washed trice with washing buffer and after that, 100 µL of purified human C3 diluted in barbital buffer saline (BBS) starting at maximum concentration of 0.01 mg/mL were added to the plate and incubated for an hour at 37°C.

Following washing three times, rabbit anti-human C3c antibodies (Dako) diluted 1:5000 in washing buffer were added and incubated at 37°C for 90 minutes. After washing trice, 100 µL of alkaline phosphatase goat anti-rabbit antibodies (Sigma-Aldrich) diluted 1:5000 in washing buffer were added and incubated at room temperature for an hour. After that, plate was washed three

times and 100 μL /well of colorimetric substrate *p*-nitrophenyle phosphate (*p*NPP) were added and incubated at room temperature for approximately 10 minutes and then the absorbance was measured at 405 nm using BioRad microtiter plate reader.

Table 2-26: Protocol used to prepare buffers used in ELISA assays

Buffer	Composition
Barbital buffer saline (BBS), pH 7.4	4 mM Barbital (veronal), 145 mM NaCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 0.05% Tween-20
Blocking buffer	1% BSA in TBS
Coating buffer, pH 9.6	15 mM Na_2CO_3 , 35 mM NaHCO_3 ,
MBL binding buffer, pH 7.4	0.1% HSA (w/v), 10 mM CaCl_2 , 0.05% triton X-100 (v/v), up to 1 litre in TBS
Washing buffer, pH 7.4	5 mM CaCl_2 , 0.05% tween-20 (v/v), up to 1 litre in TBS

2.2.5.3: Time-course of C3 cleavage by MASP-1 and MASP-2

Recombinant human MASP-1 (CCP₁-CCP₂-SP) and recombinant human MASP-2 (CCP₁-CCP₂-SP) enzymatic activity on C3 was studied in a time-course experiment in which 100 $\mu\text{g}/\text{mL}$ of human C3 were mixed with 7.5 $\mu\text{g}/\text{mL}$ of catalytically active fragments of MASP-2 or MASP-1 in a total volume of 20 μL in TBS- Ca^{2+} - Mg^{2+} pH 8.0 (table 2-25) and incubated at 37°C with gentle shaking for different time points (120, 80, 40, 20, 10, 5, 0.00 minutes). After that samples were boiled and separated in 6% SDS polyacrylamide gel and stained with Coomassie Blue.

2.2.5.4: MASP-2 inhibition by C1-INH and OM721 HG4

The specificity of MASP-2 enzymatic activity on C3 was examined by using specific MASP-2 inhibitors. This experiment was performed by pre-incubating 15 µg/mL of catalytically active MASP-2 with varying concentrations of C1-INH (CompTech), or OMS721 HG4 inhibitory antibodies starting at a minimum molar ratio of two folds (C1-INH concentrations were 90 µg/mL, 45 µg/mL, 22.5 µg/mL and OMS721 HG4 inhibitory antibodies concentrations were 60 µg/mL, 30 µg/mL, 15 µg/mL) for 20 minutes at room temperature in a total of 10 µL in TBS-Ca²⁺-Mg²⁺ pH 7.3 (table 2-25). After that, 10 µL of 0.2 mg/mL purified human C3 were added to the reaction and incubated at 37°C with gentle shaking for an hour the samples were then denatured and separated in 6% SDS polyacrylamide gel and the gel was stained with Coomassie Blue.

2.2.5.5: C3 cleavage by endogenous human and mouse MASP-2

In order to use the full-length MASP-2 and examine its activity on C3, the endogenous MASP-2 was immobilised from mouse sera on mannan coated ELISA plate. The experiment was performed by coating Nunc MaxiSorp 96 well plate with 1 µg/well of mannan for overnight at 4°C. The remaining protein binding sites were blocked with 0.1% human serum albumin (HSA) (Sigma-Aldrich) in TBS for 2 hours. Following washing the plate trice with TBST-Ca²⁺, 100 µL of 20% MASP-1/-3 KO, MASP-2 KO, C4 KO, C1q KO, C3 KO or wild type mouse sera diluted in high salt buffer (table 2-27) were added to the plate, in another experiment, 20% of normal human serum diluted in high salt buffer was added to the plate, and plate was incubated at 37°C for an hour.

Plate was washed once with high salt buffer then washed twice with washing buffer after that 100 µL of descending concentrations of purified human C3 diluted in BBS (table 2-26) starting at a maximum concentration of 0.01 mg/mL were added to the plate and incubated for 90 minutes at 37°C. Afterwards plate

was washed and C3 deposited on the plate was probed with rabbit anti-human C3c antibodies (Dako) diluted 1:5000 in washing buffer.

In another experiment, 100 μ L of 20% MASP-1/-3 KO, MASP-2 KO, C1q KO, C4 KO or wild type mouse sera diluted in high salt buffer were added to the plate and after incubation for an hour at 37°C, 100 μ L of 10 μ g/mL purified human C3 diluted in BBS were added to the plate and reaction was stopped at different time points (0.0, 2.0, 5.0, 10, 20, 40, 60, 90 minutes) by adding 100 μ L of GVB-EDTA (table 2-27).

Table 2-27: Preparation of GVB-EDTA and high salt buffers

Buffer	Composition
GVB-EDTA buffer, pH 7.4	10 mM EDTA, 5 mM barbital, 145 mM NaCl, 0.1% gelatin
High salt buffer (dilution buffer), pH 7.4	20 mM Tris-HCl, 1 M NaCl, 10 mM CaCl ₂

2.2.6: MASP-3 dependent, lectin pathway mediated activation of the alternative pathway C3 convertase

2.2.6.1: Human recombinant MASP-3 activation assay

2.2.6.1.1: Turnover of recombinant human MASP-3 by trypsin

Trypsin from bovine pancreas (Sigma-Aldrich) was used to activate recombinant human MASP-3. The enzyme was serially diluted in TBS-Ca²⁺ buffer (table 2-28) starting at a maximum concentration of 5 µg/mL. Then, 4 µg of recombinant human MASP-3 were added to the trypsin in a total volume of 20 µL. Samples were mixed and incubated at 37°C for 1 hour with shaking. Reactions were stopped immediately by adding 5 µL of 5X SDS protein-loading buffer containing 5% of 2-mercaptoethanol and heated at 90°C for 10 minutes. Samples were separated in 10% SDS polyacrylamide gel and gel was fixed and stained with Coomassie Blue.

Table 2-28: Preparation of TBS-Ca²⁺buffer

Buffer	Composition
TBS-Ca ²⁺ buffer, pH 7.4	5 mM CaCl ₂ , TBS up to 1 litre

2.2.6.1.2: Activation of recombinant human MASP-3 by different serine proteases

Catalytic domains (CCP₁-CCP₂-SP) of recombinant human MASP-1 and recombinant human MASP-2 were used to activate the recombinant full-length human MASP-3 in serum free condition. Each enzyme was serially diluted in TBS-Ca²⁺ buffer starting at a maximum concentration of 50 µg/mL. Then 4 µg of recombinant human MASP-3 were added to the diluted enzymes at maximum ratio of 1:4, MASP-1 or MASP-2:MASP-3 (1µg:4µg, w/w) in a total volume of 20 µL. Reactions were then mixed and incubated in an eppendorf heatblock at

37°C for 1 hour. Reactions were stopped and samples were separated in 10% SDS polyacrylamide gel and the gel was stained with Coomassie Blue. In another preparation, proteins were electrophoretically transferred into nitrocellulose membrane and activated protein was detected by rabbit anti-human MASP-1/-3 antibodies (Santa Cruz H-90) diluted 1:5000 in blocking buffer.

In other experiments, plasmin and thrombin were used to activate recombinant full-length human MASP-3 starting at a maximum enzyme:substrate ratio of 1:4, thrombin or plasmin:MASP-3 (1µg:4µg, w/w) in TBS-Ca²⁺ buffer (table 2-28). Reactions were performed under the same conditions used above.

2.2.6.2: *Amidolytic assay*

Amidolytic activity of catalytic fragments of recombinant human MASP-3 (CCP₁-CCP₂-SP) on fluorogenic Boc-Val-Pro-Arg (VPR) aminomethylcoumarin (AMC) conjugated substrate was assessed by measuring of AMC released upon cleavage.

Nunc MaxiSorp 96 well plate was blocked with 0.1% HSA in TBS at room temperature for 2 hours. Plate was washed three times with TBST/Ca²⁺. Varying MASP-3 concentrations, (starting at a maximum concentration of 10.0 µg/well) diluted in TBS-Ca²⁺-Mg²⁺ buffer pH 8.0 were added to 0.1 µM VPR-AMC (R&D Systems) and 200 ng of MASP-2 (CCP₁-CCP₂-SP) in a total volume of 100 µL. Immediately plate was placed into fluroskan and emission was read at 360 nm every 30 seconds for 90 minutes. The Michaelis constant K_m and maximum velocity, V_{max} , were calculated for MASP-3 using nonlinear regression analysis (GraphPad Prism version 6.00).

2.2.6.3: *Assessment the proteolytic activity of MASP-3 on the complement components FH and C5*

The proteolytic activity of MASP-3 (CCP₁-CCP₂-SP) on the complement components FH and C5 was assessed by incubating 100 µg/mL of purified human factor H (CompTech) or purified human C5 (CompTech) with 25 µg/mL of recombinant human MASP-3 (CCP₁-CCP₂-SP) or 25 µg/mL of recombinant human MASP-1 (CCP₁-CCP₂-SP) or recombinant human MASP-2 (CCP₁-CCP₂-SP) in a total volume of 20 µL in TBS-Ca²⁺-Mg²⁺ pH 7.3 at 37°C with gentle shaking for 1 hour.

Reactions stopped by 5 µL of 5X SDS protein-loading buffer containing 5% of 2-mercaptoethanol and proteins were separated in 10% SDS polyacrylamide gel. The gel was fixed and then stained with Coomassie Blue.

2.2.6.4: *Alternative pathway assay in FB depleted serum and FD depleted serum on zymosan*

Microtiter plate was coated with 1 µg /well zymosan from *Saccharomyces cerevisiae* in coating buffer (table 2-26). After overnight incubation at 4°C, the remaining protein binding sites were blocked with 1% BSA in TBS for 2 hours at room temperature followed by washing the plate with TBST.

Purified human FB was added to complement FB depleted (FBdpl) serum (CompTech) at a final concentration of 2 mg/mL of diluted serum in presence or absence of 6 µg/mL of recombinant human MASP-3 (CCP₁-CCP₂-SP) and serum was diluted in GVB-Mg²⁺-EGTA buffer (table 2-29) starting at a maximum serum concentration of 13% and plate was incubated at 37°C for 90 minutes. Plate was washed three times with washing buffer then 100 µL/well of 1:5000 rabbit anti-human C3c antibodies were added and plate was incubated at 37°C for 90 minutes. Afterwards, 100 µL of alkaline phosphatase goat anti-rabbit antibodies diluted 1:5000 in washing buffer were added.

In FD depleted (FDdpl) serum, recombinant human pro-FD was pre-incubated with recombinant human MASP-3 (CCP₁-CCP₂-SP) in an enzyme:substrate starting at maximum ratio of 1:10, MASP-3:pro-FD (0.3µg:3µg, w/w) for 40 minutes and the mixture was then added to FDdpl at FD final concentration of 2 µg/mL of undiluted serum.

In another experiment, 10 µg of recombinant human MASP-3 (CCP₁-CCP₂-SP) or recombinant human MASP-1 (CCP₁-CCP₂-SP) or both enzymes were added to 1 mL of MASP-1/-3 KO mouse serum and serum was diluted in GVB-Mg²⁺-EGTA buffer (table 2-29) starting at a maximum serum concentration of 20%. The diluted serum was then added to zymosan coated plate and plate was incubated at 37°C for 90 minutes.

Table 2-29: Protocol used to prepare the alternative pathway buffer

Buffer	Composition
GVB-Mg ²⁺ -EGTA buffer, pH 7.3	10 mM EGTA, 5 mM MgCl ₂ , 5 mM barbital, 145 mM NaCl, 0.1% gelatin

2.2.6.5: *Proteolytic activity of human MASP-3 on the complement components*

FB and FD

The previous experiments revealed the necessity of MASP-3 in initiation the alternative pathway functional activity; so that recombinant human MASP-3 (CCP₁-CCP₂-SP) enzymatic activity was investigated on FB and FD in serum free condition. In this experiment 100 µg/mL of purified human C3b (CompTech) and 100 µg/mL of purified human FB were incubated with 12.5 µg/mL of recombinant human MASP-3 (CCP₁-CCP₂-SP) and 5 µg/mL of pro-FD in a total volume of 20 µL in GVB⁺⁺ (table 2-30) for an hour at 37°C with gentle shaking.

Reactions stopped and proteins were separated in 10% SDS polyacrylamide gel. The gel was fixed and stained with Coomassie Blue.

Table 2-30: Protocol used to prepare GVB⁺⁺

Buffer	Composition
GVB ⁺⁺ , pH 7.3	0.5 mM MgCl ₂ , 0.15 mM CaCl ₂ , 5 mM barbitol (veronal), 145 mM NaCl, 0.1% gelatin

2.2.6.6: *FD activation by MASP-3*

The proteolytic activity of the catalytically active recombinant human MASP-3 (CCP₁-CCP₂-SP) on pro-FD is examined by incubating 150 µg/mL of recombinant human pro-FD with varying concentrations of recombinant human MASP-3 (CCP₁-CCP₂-SP) starting at a maximum enzyme:substrate ratio of 1:10, MASP-3:pro-FD (0.3µg:3µg, w/w) in a total volume of 20 µL in GVB⁺⁺ at 37°C with gentle shaking for 4 hours. After that, 5 µL of 5X SDS protein-loading buffer containing 5% reducing reagent were added and proteins were boiled at 90°C for 10 minutes. After brief centrifugation, proteins were separated in a Novex 4-20% Tris-Glycin gel (Life technologies). The gel was fixed and stained with Coomassie Blue.

2.2.7: Studies the role of the alternative pathway of complement activation against *S. pneumoniae*

2.2.7.1: *In vitro* studies

2.2.7.1.1: Bacterial culture

S. pneumoniae was cultured from frozen stock on blood agar (Oxoid) plates (table 2-31) at 37°C for overnight in presence of 5% CO₂. To prepare working stocks, one colony of *S. pneumoniae* was transferred into 10 mL of brain heart infusion (BHI) (Oxoid) (table 2-31) and incubated at 37°C for overnight.

Table 2-31: Bacterial growth media

Medium	Composition
Brain heart infusion (BHI) medium	37 g of BHI dissolved in 1 litre of distilled water
Blood agar medium	4% blood agar base (w/v) in 1 litre of distilled water and sterilised by autoclaving and the media was then supplemented with 5% (v/v) horse blood

2.2.7.1.2: Optochin sensitivity test

S. pneumoniae was plated on blood agar plates in presence of optochin disks (Sigma-Aldrich) and incubated at 37°C in 5% CO₂ for 18-24 hours.

2.2.7.1.3: Preparation of formalin fixed *S. pneumoniae*

Serotype 2 *S. pneumoniae* D39, used in all the *in vitro* studies, was fixed with 0.5% formalin (Sigma-Aldrich). 10 µL from the frozen stock were inoculated in 10 mL of BHI (table 2-31) and incubated overnight at 37°C. After centrifugation for 10 minutes at 3000 rpm, the pellet was washed three times with PBS and re-

suspended in PBS containing 0.5% formalin and incubated at room temperature for 3 hours. After that, bacteria were washed two times and re-suspended in coating buffer (table 2-26) and the OD_{550nm} was adjusted to 0.6 (Lynch et al. 2004).

2.2.7.1.4: Preparation of heat-killed bacteria

S. pneumoniae, was heat-killed at 70°C for 1 hour. The overnight culture in BHI was centrifuged for 10 minutes at 3000 rpm, the pellet was washed three times with PBS and distributed into eppendorf tubes and incubated for 1 hour at 70°C. After that, the cells were washed two times with PBS and re-suspended in PBS and the OD_{550nm} was adjusted to 0.6. The killed bacteria were sub-cultured onto blood agar plates and incubated at 37°C for overnight to prove that no viable bacteria remained.

2.2.7.1.5: The alternative pathway assay on *S. pneumoniae*

The alternative pathway of complement activation is studied on the surface of *S. pneumoniae* in MASP-1/-3 KO mouse sera reconstituted with recombinant full-length mouse MASP-3. The experiment was performed by coating Nunc MaxiSorp 96 well plate with 100 µL /well of OD₅₅₀ 0.6 formalin fixed *S. pneumoniae* in coating buffer (table 2-26) for overnight at 4°C. Protein binding sites were blocked with 1% BSA in TBS at room temperature for 2 hours. After washing the plate three times, 10 µg of recombinant full-length mouse MASP-3 were added to 1 mL of MASP-1/-3 KO mouse serum and serum was diluted in GVB-Mg²⁺-EGTA buffer (table 2-29) starting at a maximum serum concentration of 13% and the diluted serum was then added to the plate and incubated at 37°C for 90 minutes. Following three times washing, 100 µL of 1:5000 rabbit anti-human C3c diluted in washing buffer were added. Subsequently 100 µL of 1:5000 alkaline phosphatase conjugated goat anti-rabbit diluted in washing buffer were added.

In additional experiment, different concentrations of recombinant full-length mouse MASP-3 i.e. 6 µg/mL, 12 µg/mL, and 24 µg/mL of undiluted serum

were used to reconstitute MASP-1/-3 KO mouse serum diluted in GVB-Mg²⁺-EGTA alternative pathway buffer.

2.2.7.1.6: C3 breakdown on the surface of heat-killed *S. pneumoniae*

This experiment was performed to investigate the fluid phase C3 breakdown products (Hyams et al. 2011) in MASP-1/-3 KO mouse sera in presence of heat-killed bacteria. 2X10⁸ heat-killed *S. pneumoniae* were washed with GVB-Mg²⁺-EGTA buffer at 14000 rpm for 15 minutes and re-suspended in 100 µL of GVB-Mg²⁺-EGTA buffer (table 2-29) containing 10 µL of wild type mouse serum, MASP-1/-3 KO serum or MASP-1/-3 KO serum reconstituted with 10 µg/mL of recombinant full-length mouse MASP-3 then incubated at 37°C for 1 hour with gentle shaking (250 rpm). After that, 25 µL of SDS protein-loading buffer containing 2-mercaptoethanol (5%) were added and samples were reduced and spun down. Five microliters of the supernatant were then separated in 10% SDS polyacrylamide gel. Proteins were electrophoretically transferred into nitrocellulose membrane and probed with polyclonal anti-human C3c antibody.

2.2.7.2: *In vivo* study

All *in vivo* procedures used were under the project licence 60/4327 in accordance with standard operating procedures following the guidelines from the Animal Scientific Procedure Act 1986 of the UK Home Office.

2.2.7.2.1: Mice

Female C57BL/6 wild type mice were obtained from Charles River Laboratories, UK. MF1 mice used for bacterial passage were purchased from Harlan Olac Ltd, (Bicester, UK). MASP-1/-3 KO mice on C57BL/6 background were breeding in house and the mice PCR genotyped for complement component deficiency. Mice 8-10 weeks aged were used in experiments.

2.2.7.2.2: MASP-3 *in vivo* reconstitution

Three MASP-1/-3 KO mice on C57BL/6 background were injected i.v. with 20 µg/mouse of recombinant full-length mouse MASP-3. Mice were bled 24, 48 and 72 hours post reconstitution. The alternative pathway assay was performed on zymosan coated microtiter plate (1 µg /well). The residual protein binding sites were blocked with 1% BSA. After washing the plate three times with TSBT, MASP-1/-3 KO serum, MASP-1/-3 KO serum *in vivo* reconstituted with recombinant full-length mouse MASP-3 and the wild type serum were diluted in GVB-Mg²⁺-EGTA buffer (table 2-29) starting at a maximum serum concentration of 13%. The diluted serum was then added to the plate and after 90 minutes of incubation at 37°C, C3b deposited on the plate was detected with rabbit anti-human C3c.

2.2.7.2.3: Animal passage of *S. pneumoniae*

To obtain virulent *S. pneumoniae* D39, the bacteria were passaged through MF1 mouse strain as described by Canvin et al. (1995) and Kadioglu et al. (2000). *S. pneumoniae* was streaked onto blood agar plate overnight at 37°C. On the following day, a streak of colonies was inoculated in BHI (table 2-31) and incubated statically for overnight at 37°C. The bacterial culture was centrifuged at 3000 rpm for 10 minutes and pellet was re-suspended in 5 mL of PBS. After that, 100 µL of this suspension were intraperitoneally injected in two MF1 mice. Next 22-28 hours of infection, the disease symptoms reached ++ starry coat and the blood was collected by cardiac puncture under general anaesthetic and the animal were culled by cervical dislocation.

Fifty microliters of the collected blood were inoculated in 10 mL of BHI and incubated statically for overnight at 37°C. Next day, the bacterial growth was collected and centrifuged at 3000 rpm for 10 minutes. Pellet was re-suspended in 1 mL of BHI containing 20% fetal calf serum (FBS). After that, 667 µL of this suspension were added to 10 mL of BHI serum broth medium and the OD₅₀₀ was adjusted to 0.7 and incubated at 37°C until OD₅₀₀ reached 1.6. The culture was aliquot and stored at -80 until use. On the following day, plating onto

blood agar plates was made to check the passaged stock viability.

Virulence testing of the passaged *S. pneumoniae* was made in MF1 mice. One tube of the frozen stock was thawed and centrifuged at 13000 rpm for 2 minutes, and pellet was re-suspended in 500 μ L of PBS and diluted in PBS to achieve the 1×10^6 CFU per 50 μ L. Five MF1 mice were anaesthetised (2.5% (v/v) of inhaled flurane and 2 litres O₂/minute) and intranasally infected with 1×10^6 CFU in 50 μ L of PBS. Mice were then checked for signs of disease and when that reached +/+ lethargic, mice were culled by cervical dislocation. If 80% or more of the infected mice succumbed to infection within 48-72 hours, dose considered being virulent.

2.2.7.2.4: Infection of mice

Female MASP-1/-3 KO mice and their wild type littermate were infected intranasally with passaged *S. pneumoniae* as following: Mice were anaesthetised with 2.5% (v/v) of inhaled flurane and 2 litres O₂/minute. Dose of 25 μ L corresponding to 3×10^5 CFU/25 μ L of *S. pneumoniae* in PBS was given into the mouse nostrils. Cages containing infected mice were placed on separated infectious rack.

Following the infection, the administered dose was confirmed by viable count on blood agar plates.

Mice were monitored for signs of disease and mice were culled by cervical dislocation when reached ++ lethargic.

2.2.7.2.5: Bacterial burdens in blood and lungs

Blood was collected by tail bleeding of infected mice at different time points. Mice were placed into a thermocage at 37°C for 20 minutes. Then 20 μ L were withdrawn from each mouse. The collected blood was serially diluted in PBS and then 60 μ L of the diluted blood were spotted onto blood agar plates. Plates were placed into a candle jar and incubated at 37°C for overnight.

To calculate bacterial loads in infected mouse lungs, lungs were collected aseptically at the time of death and weighed and then transferred into a tube containing 5 mL of PBS. Tissues were homogenised and 20 µL of this homogenate were serially diluted 10 folds in PBS. Afterwards, 60 µL were plated from each dilution onto blood agar plates. CFU/mg was calculated according to the following equation:

$$\text{CFU/mg} = [(\text{No. colonies} \times \text{dilution factor} \times 1000 \times \text{volume of homogenising solution}) / \text{volume plated in } \mu\text{L}] / \text{organ weight in mg.}$$

2.2.8: Statistical analysis

Student t test was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. Differences were considered significant at P values of <0.05.

*Chapter three: Revealing the molecular
basis of the C4-bypass mechanism of the
lectin pathway of complement activation:
MASP-2 mediated activation of C3 in
absence of complement C4*

3.1: Introduction

The lectin pathway of complement activation is part of the innate immune defense against invading microorganisms. This activation pathway is composed of five different pattern recognition molecules in man, i.e. ficolins (M, L and H) (Yae et al. 1991, Lu et al. 1996, Matsushita et al. 1996) Mannose-Binding Lectin (MBL) (Turner 1996) and the recently discovered collectin-11 (CL-11, another member of the collectin family with a high degree of similarity to MBL; (Ohtani et al. 1999, Hansen et al. 2010), as well as three MBL-associated serine proteases named MASP-1 (Matsushita and Fujita 1992), MASP-2 (Thiel et al. 1997) and MASP-3 (Dahl et al. 2001), and two enzymatically inactive proteins MAp44 or MAP-1 (Degn et al. 2009b, Skjoedt et al. 2010a) and MAp19 or sMAP (Stover et al. 1999b, Takahashi et al. 1999). In mouse, five independent lectin pathway recognition molecules have been described: MBL-A and MBL-C, which are encoded by two different MBL genes, CL-11 and two members of the ficolin family, ficolin-A and ficolin-B. The lectin pathway specific enzymes described in the mouse are MASP-1, MASP-2 and MASP-3. MASP-1 and MASP-3 are alternative splicing products of a single structural *MASP1* gene (Stover et al. 2003). A third alternative splice product of the *MASP1* gene, MAp44 or MAP-1 is enzymatically inert as it is devoid of a serine protease domain and has recently been described as a competitive regulator of the lectin pathway, since it competes with the MASPs for the binding of the MASPs binding site on the recognition subcomponents (Skjoedt et al. 2012, Degn et al. 2013). Likewise, the gene encoding MASP-2 located on mouse chromosome 4 (Stover et al. 2004) or human chromosome 1p36.2-3 (Stover et al. 2001).

Activation of the lectin pathway is initiated by the binding of the lectin pathway recognition components to their particular substrate (Schwaeble et al. 2002, Thiel et al. 2002).

Amongst the 3 different lectin pathway specific serine proteases, MASP-2 plays an essential role as the essential effector enzyme, since MASP-2 cleaves both C4 and C2 to form the lectin pathway C3 convertase, C4bC2a and C5 convertase,

C4bC2a(C3b)_n, respectively (Thiel et al. 1997, Schwaebler et al. 2011). MASP-1 can activate both C2 and MASP-2 (Ambrus et al. 2003, Takahashi et al. 2008).

MASP-1, MASP-2 and MASP-3 zymogens are found in blood as a dimer (preferentially homodimers) formed through a calcium-dependent binding interaction with their CUB₁, EGF and CUB₂ domains (binding to each other in anti-parallel orientation). These MASP dimers bind to a conserved MASPs binding domain located in the collagenous region of the lectin pathway recognition complexes (Wallis and Dodd 2000). The antiparallel orientation of MASPs in this relatively inflexible dimer formation leads to a distant positioning of each of the two individual serine protease domains, so distant that they cannot interact and activate each other. Within each lectin pathway activation complex, the serine protease domains of the MASP dimers stick out 90° from the axis of the collagenous stalks of their respective recognition subcomponent. In fact, the orientation of the serine protease domains of the MASP dimers within each complex only allows them to transactivate, i.e. to cleave MASPs in neighboring complexes. This critically discriminates the activation events of the lectin pathway from those of the classical pathway activation complex where the heterotetramer C1s:C1r:C1r:C1s situated within the hexameric recognition subcomponent C1q can be activated independently within the same C1 complex. In contrast, initiation of lectin pathway activation depends on the binding of many lectin pathway initiation complexes to their ligands in close proximity to each other on activator surfaces. This is necessary to allow transactivation to occur where a MASPs dimer in one complex cleaves the MASPs dimer in the neighbouring complex to form a chain reaction of activation events. In these chain events, MASP-1 can cleave neighboring MASP-1, MASP-2 or MASP-3 zymogens, as MASP-2 can cleave neighboring MASP-2 or MASP-1 or MASP-3 zymogens, while MASP-3 appears not to cleave MASP-1 or MASP-2 (Zundel et al. 2004, Gal et al. 2005). While activated MASP-2 cleaves both C4 and C4b-bound C2, (Rossi *et al.*, 2001), MASP-1 is devoid of ability to cleave C4 and can only cleave C4b-bound C2 or C2 on its own (Matsushita et al. 2000a, Ambrus et al. 2003) and therefore, in absence of MASP-2, the C4bC2a C3

convertase of the lectin pathway cannot be formed (Schwaeble et al. 2011). Interestingly, MASP-1 has been reported to be able to cleave dead C3, i.e. C3 where the thioester bond has been hydrolyzed resulting in the loss of most of its biological activity (Matsushita and Fujita 1995, Hajela et al. 2002). Recent work described that MASP-1 activates lectin pathway serine proteases MASP-2 and MASP-3 (Takahashi et al. 2008, Kocsis et al. 2010, Iwaki et al. 2011).

Since MASP-1 can cleave MASP-2 and vice versa and since MASP-1 is significantly more abundant than MASP-2 (11 microgram per mL compared to 0.5 microgram/mL for MASP-2) it seems only plausible that MASP-1 can directly accelerate MASP-2 activation and that the cooperation between MASP-1 and MASP-2 is essential to obtain optimal rates of MASP-2 dependent lectin pathway activation. While in absence of MASP-2 the lectin pathway C3 convertase C4bC2a and C5 convertase C4bC2a(C3b)_n are not formed and no activation of C3 can be measured under lectin pathway specific assay conditions (i.e. mannan or zymosan coated activator surfaces, high serum dilutions, presence of calcium etc...), the absence of both MASP-1 and MASP-3 in serum of MASP-1/-3 deficient mouse serum allows a residual, but physiologically meaningful degree of C3 activation and C5 activation to occur (Schwaeble et al. 2011).

It has become clear that primary immunodeficiency disorders, due to the lack of complement components, present with a diminished ability to form membrane attack complexes (Walport 2001). The deficiency of the complement component C2 was amongst the first complement deficiencies reported more than half a century ago and surprisingly no clinical symptoms were apparent (Silverstein 1960). Since then, many genetic studies have identified a whole array of different complement deficiencies (Skattum et al. 2011). The occurrence of C2 deficiency estimated to be 1:20,000 in the Western populations (Walport 2001). The deficiency in the fourth complement component is very rare with fewer than 30 cases have been reported so far in total, since humans have two highly homologous genes for C4, a total C4 deficiency requires a combined deficiency

of both genes (Lhotta et al. 2004). Deficiencies of the complement components C2 and or C4 are strongly associated with the predisposition for Systemic Lupus Erythematosus (SLE) and an increase susceptibility to pneumococcal infections (Roberts et al. 1978, Yuste et al. 2010, Boteva et al. 2012). The first description of a functional bypass mechanism that allows complement activation to take place in C4 deficient serum was proposed more than four decades ago by Joseph May and Michael Frank showing that C4 deficient guinea pig serum was able to lyse sheep red blood cells (May and Frank 1973). Both suggested the presence of a C1-like serum component being involved, a prediction long before anyone knew of the existence of a lectin pathway route of complement activation and the role of lectin pathway specific serine proteases in haemolysis.

More recently, evidence for the existence of a MASP-2 dependent and C4 independent C4-bypass route of complement activation became apparent from several independent studies carried out as part of the research programme coordinated by my PhD supervisor Professor Wilhelm Schwaeble in Leicester and in other closely collaborating laboratories. These studies all pointed to the same conclusion: Ischaemia-reperfusion injury is lectin pathway dependent, leading to the activation of complement C3. However, C4 deficient animals are not protected from MASP-2 dependent ischaemia-reperfusion injury (Schwaeble et al. 2011, Farrar et al. 2012, Asgari et al. 2014).

My work described in this chapter has provided a strong evidence for the molecular events that lead to the C4-independent activation of complement C3 that critically contributes to MASP-2 dependent post-ischaemic tissue injury and tissue loss known as ischaemia-reperfusion injury.

Here for the first time, the mechanism of lectin pathway activation in the absence of C4 is explained.

3.2: Results

3.2.1: Screening complement deficiency in mice

Mouse lines with gene-targeted deficiencies in one or more complement components were genotyped by PCR to confirm the homozygous presence of the gene disruption in the targeted allele. The *MASP2* gene in mouse is located on chromosome 4. The gene consisted of 12 exons encode for two proteins, MASP-2 and MAp19 (Stover et al. 1999b, Iwaki and Fujita 2005). The MASP-2 heavy chain is encoded by exons 1-11 and the light chain is encoded by a single exon 12 (Stover et al. 1999a). In the *MASP2* gene targeted mouse, the exons 11 and 12 of *MASP2* gene (exon 11 encodes the C-terminal half of CCP₂ and exon 12 encodes the serine protease domain) were replaced with a neomycin-resistance cassette (*neomycin* gene) (Schwaeble et al. 2011). The chosen multiplex primers will amplify an 800bp PCR product of the wild type allele, and a 500bp PCR product of the gene targeted disrupted allele of *MASP2* (figure 3-1A).

The classical pathway recognition subcomponent C1q is composed of three chains A, B, and C which are encoded by three closely linked genes, i.e. *C1qA*, *C1qB* and *C1qC* located on mouse chromosome 14. These three different C1q chains associate to heterotrimeric subunits and six of these heterotrimeric subunits for the tulip-like shape of the approximately 420 kDa C1q macromolecule (Petry et al. 1996). The *C1q* A-chain gene in C1q deficient mice was disrupted by the insertion of a *neomycin* gene disruption cassette within the first exon of the targeted *C1qA* gene (Botto et al. 1998). The size of the multiplex PCR product amplified from the wild type allele is about 360bp and the PCR product amplified from the disrupted allele is about 160bp in size (figure 3-1B).

The mouse *C4B* gene is located on mouse chromosome 17 and encodes the fourth complement component. The C4 precursor protein consists of three chains i.e. α , β , and γ . In the C4 targeted mouse line used in my work the *C4B* gene is disrupted by a *neomycin* cassette replacing part of exon 23 and the exons 24 -29 (Fischer et al. 1996). The size of the PCR product amplified in a multiplex

PCR from the wild type allele is about 458bp and the PCR product amplified from the disrupted allele is about 600bp (figure 3-1C).

MASP1 gene is located on chromosome 16 (B2-B3) in mouse (Takada et al. 1995). Three different proteins MASP-1, MASP-3 and a truncated gene product of 44 kDa MAp44 are encoded by this gene. The gene consists of 18 exons. Exons 1-11 encode for the heavy chain that both MASP-1 and MASP-3 have in common. The light chain or the serine protease domain of MASP-3 is encoded by a single exon (i.e. exon 12) while the light chain of MASP-1 is encoded by six exons (i.e. exons 13-18).

The targeted exon in *MASP1* gene is exon 2 so that, MASP-1 and MASP-3 as well as Map44 are absent since the disruption of exon 2 terminates the translation of all gene products (Takahashi et al. 2008). The size of the PCR product amplified in a multiplex PCR from the wild type allele is about 539bp and that amplified from the gene targeted allele about 639bp (see figure 3-1D).

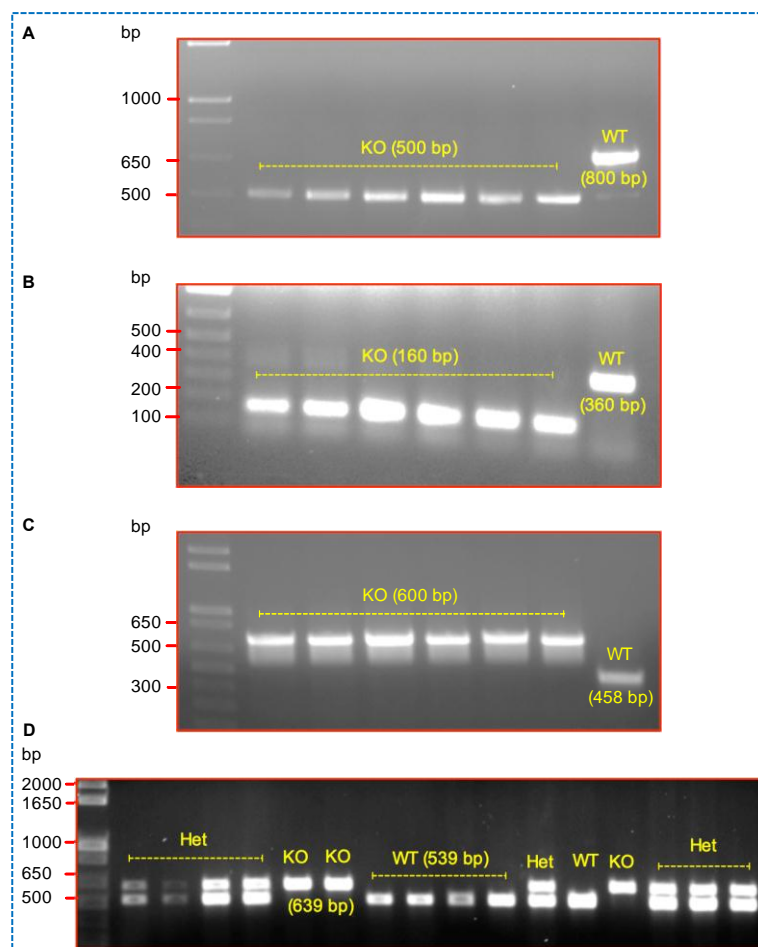


Figure 3-1: Screening for complement component genes targeted in mice

(A) Screening for *MASP2* gene. *MASP-2* KO mice show a band of approximately 500bp and its wild type of approximately 800bp. (B) Screening for *C1q* gene. The wild type mice show a band of approximately 539bp while the *C1q* KO mice show a band of approximately 639bp. (C) Screening for *C4B* gene. The *C4* KO mice show a band of approximately 600bp and its wild type of approximately 458bp. (D) Screening for *MASP1* gene. The *MASP-1/-3* KO mice show a band of approximately 639bp and the wild type band is 539bp.

3.2.2: Purification of recombinant MBL on mannose-Sepharose column

The first preparation of MBL was made from rabbit serum (Kawasaki et al. 1978) and later isolated from human and rodent sera (Townsend and Stahl 1981, Kawasaki et al. 1983).

Mannose-Sepharose affinity chromatography was used to purify the recombinant protein from the supernatant of the DUX B11- MBL cell line (figure 3-2). MBL binding to mannose-Sepharose complexes was enhanced by adding

Ca^{2+} and the bound protein was then eluted in a buffer containing EDTA. MBL is composed of structural subunits each of which made of three identical (homotrimeric) polypeptide chains (each subunit of an approximate molecular weight of 32 kDa). The different size of the MBL is due to the nature of the oligomerization of its structural subunits. The tetrameric form of the human MBL is the most common form in which four subunits bind together in their N-terminal region by disulphide bonds (Garred et al. 2006).

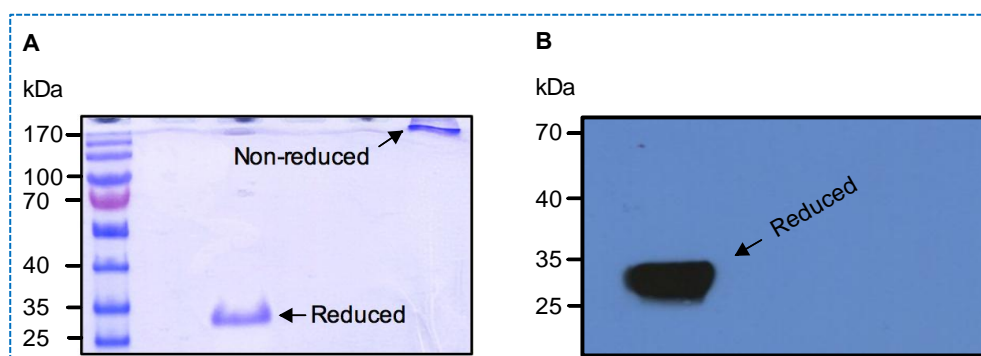


Figure 3-2: SDS-PAGE and Western blotting analysis for recombinant human MBL

(A) Purified recombinant human MBL shows a band of approximately 32 kDa under reducing condition and a band of approximately 170 kDa under non-reducing conditions. (B) Western blotting analysis for the recombinant human MBL probed with a monoclonal mouse anti-human MBL antibody.

3.2.3: Turnover of C3 by MASPs

Precursor complement C3 protein consists of two polypeptide chains α - chain (110 kDa) and β -chain (75 kDa) linked together by a disulphide bond. The C3 convertase cleaves C3 α -chain into C3 α' (101 kDa) and C3a (9 kDa) (an anaphylatoxic peptide that is released into microenvironment) (Sim et al. 1981). The larger activation product C3b (C3 α -chain and C3 β -chain) can easily be seen in the SDS-PAGE gel shown in figure (3-3A). The purified human precursor C3 was blotted into nitrocellulose membrane and probed with anti-human C4 or anti-human FB antibodies to confirm the absence of C4 and FB in this preparation (see figure 3-4).

In this experiment C3 was mixed with catalytic fragments (CCP₁- CCP₂-SP) of recombinant human MASPs, i.e. MASP-1, MASP-2, and MASP-3 in an enzyme:substrate ratio of 1:10, MASP:C3 (w/w). All reactions containing MASP-1 or MASP-2 showed C3 cleavage (figure 3-3A, lanes 2-5). MASP-3 (lane 1) showed no proteolytic activity on C3 while native C3 (lane 6) showed no activation when incubated alone under the same condition indicating that the C3 preparation was not contaminated.

C3 activation in presence of MASP-2 is greater than in presence of MASP-1 (figure 3-3B) and the addition of MASP-3 showed no synergistic role in enhancing C3 activation (figure 3-3A, lanes 2 and 3 and figure 3-3B).

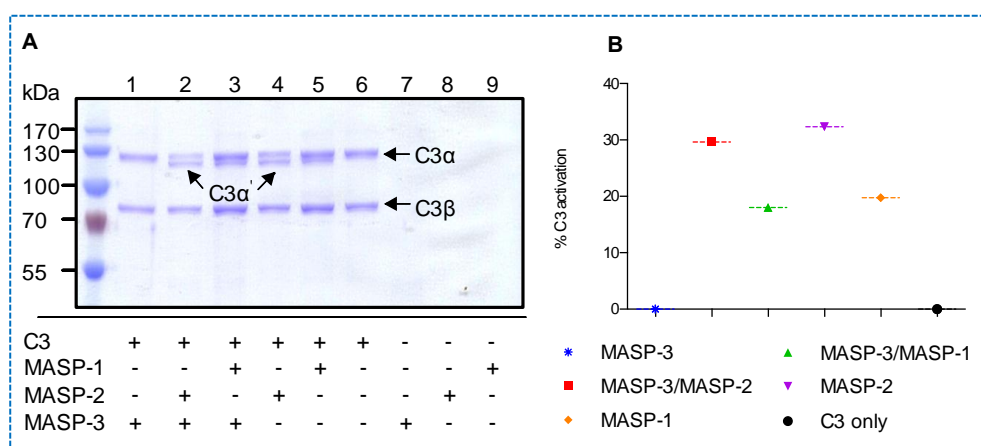


Figure 3-3: Turnover of C3 by MASP-1 and MASP-2

Purified human C3 was mixed with recombinant human MASP-1 (CCP₁- CCP₂-SP), MASP-2 (CCP₁- CCP₂-SP) and MASP-3 (CCP₁- CCP₂-SP) in an enzyme:substrate ratio of 1:10, MASP:C3, (0.2μg:2μg, w/w) and incubated for 90 minutes. (A) The proteins were separated in SDS polyacrylamide gel and gel was stained with Coomassie Blue. Activation of C3 in presence of recombinant human MASP-1 or recombinant human MASP-2 results in the cleavage of the C3α chain into C3α' and C3a (77 residues; not visible on the gel). (B) The intensities of the C3α and C3α' bands were determined by densitometry and the percentage activation calculated. The figure shows typical experiment of three independent experiments carried out.

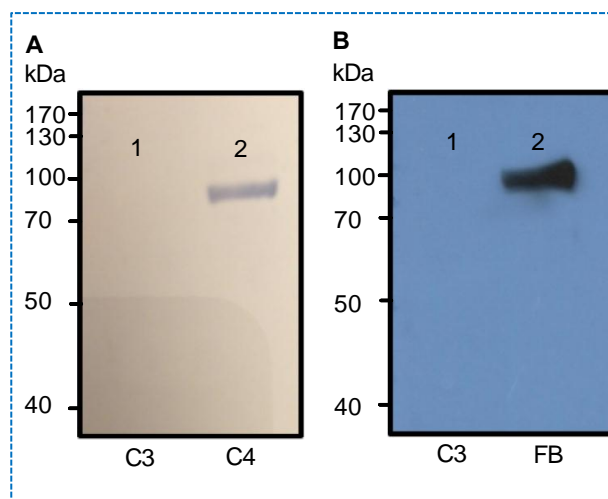


Figure 3-4: Western blotting analysis to test the quality of human C3 preparation

Purified human C3 (2 μ g) was separated in 10% SDS polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane and tested for possible contamination with C4 and FB. (A) Blotted C3 preparation was probed with anti-human C4. Lane 2 purified C4 used as a positive control. (B) The blot probed with anti-human FB antibody and purified human FB used as positive control (lane 2).

3.2.4: Optimal pH for C3 cleavage by MASP-2

The pH value affects the activity of the complement components as the pH value greater than 8.0 leads to de-association of the C1-INH from MASP-2 (Professor Robert B. Sim personal communication) and the same pH value leads to auto-activation of FB and resulted in C3 cleavage (Le et al. 2007). This experiment designed to investigate the optimal pH for MASP-2 activity towards C3. Purified human C3 was incubated with recombinant human MASP-2 (CCP₁- CCP₂-SP) in an enzyme:substrate ratio of 1:10, MASP-2:C3 (w/w) in TBS-Ca²⁺-Mg²⁺ at different pH values (figure 3-5). The acidic pH value showed no activity of MASP-2 towards C3 (figure 3-5A, lane 1) and very low activity in basicity and acidity pH values (figure 3-5A, lanes 2 and 6 and 3-5B). MASP-2 works best at pH values ranging from 6.0 to 8.0, optimally at pH 7.0.

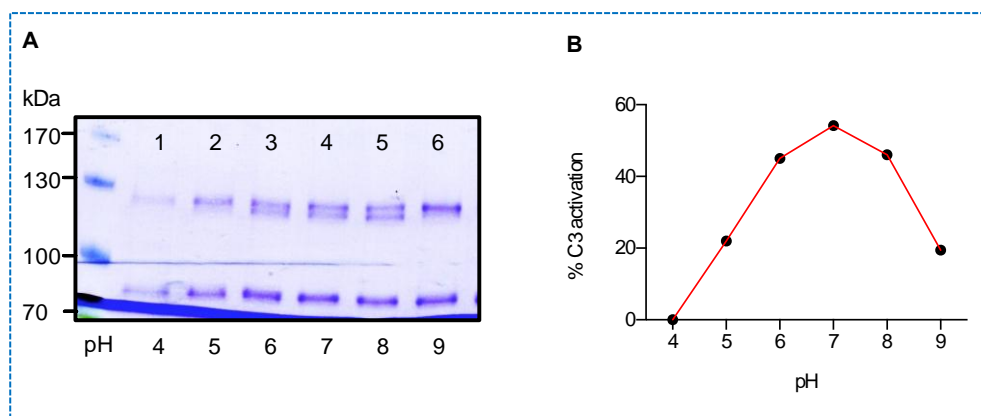


Figure 3-5: Neutral pH is optimal for the cleavage of C3 by MASP-2

C3 (0.15 μ g) and MASP-2 (2 μ g) reaction were prepared in buffers with pH values ranging from 4.0 to 9.0, and incubated for 90 minutes. (A) SDS-PAGE analysis for C3 and MASP-2 proteins stained with Coomassie Blue. (B) Percentage of C3 activation was determined by measuring the intensities of the C3 α and C3 α' bands. The figure shows typical experiment of three independent experiments carried out.

3.2.5: C3 cleavage by MASP-2K

In MASPs, the C-terminal region consists of a serine protease domain linked to N-terminal domains (CUB₁, EGF, CUB₂, CCP₁, CCP₂) via a linker region. MASP-2 is synthesized in the liver and secreted into the blood stream as a zymogen (Seyfarth et al. 2006). The MASP-2 zymogen can autocativate when it binds to a recognition molecule in presence of an activator surface or be activated by MASP-1 (Moller-Kristensen et al. 2007, Takahashi et al. 2008, Heja et al. 2012, Megyeri et al. 2014). MASP-2K, in which the arginine residue at the cleavage site changed to lysin at position 424 (Chen and Wallis 2004). To test whether the catalytically active rat MASP-2K has an enzymatic activity on C3, recombinant rat MASP-2K was incubated with recombinant human MBL to form a complex and added to a mannan coated plate. C3 deposition was then measured by ELISA assay after adding purified human C3.

MASP-2K was able to cleave C3 producing C3b deposited on the ELISA plate (figure 3-6).

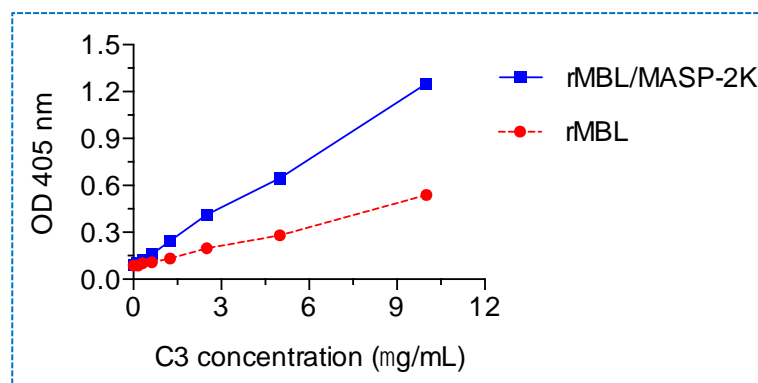


Figure 3-6: C3 cleavage by recombinant rat MASP-2K

Overnight mixture of recombinant MASP-2K and recombinant MBL was incubated in MBL binding buffer and added to a mannan coated ELISA plate. After washing the plate, the MBL(MASP-2K)₂ complexes adhered to the mannan coated plate were exposed to purified human C3 starting at maximum C3 concentration of 10 µg/mL. C3b (deposited on the plate by binding through the reactive thioester) was detected using an anti-human C3c antibody and C3b binding quantified by ELISA.

3.2.6: Time-course of C3 cleavage by MASP-1 and MASP-2

In the C3 preparation there are two kinds of C3, C3 with hydrolyzed thioester bond, (so called C3(H₂O) or dead C3) (Hajela et al. 2002) and C3 with intact thioester bond (live C3). Beside its activity towards the second complement component, MASP-1 cleaves C3(H₂O) but has no enzymatic activity towards live C3 (Hajela et al. 2002) whereas MASP-2 cleaves C4 and C4b-bound C2 (Rossi et al. 2001). To compare the enzymatic activity of the catalytic domains (CCP₁- CCP₂-SP) of recombinant human MASP-1 and recombinant human MASP-2, constant concentrations of each enzyme were incubated with purified human C3 for different time points. C3 cleavage was visualized by SDS-PAGE (figure 3-7A and 3-7B) and measured by densitometer (figure 3-7C).

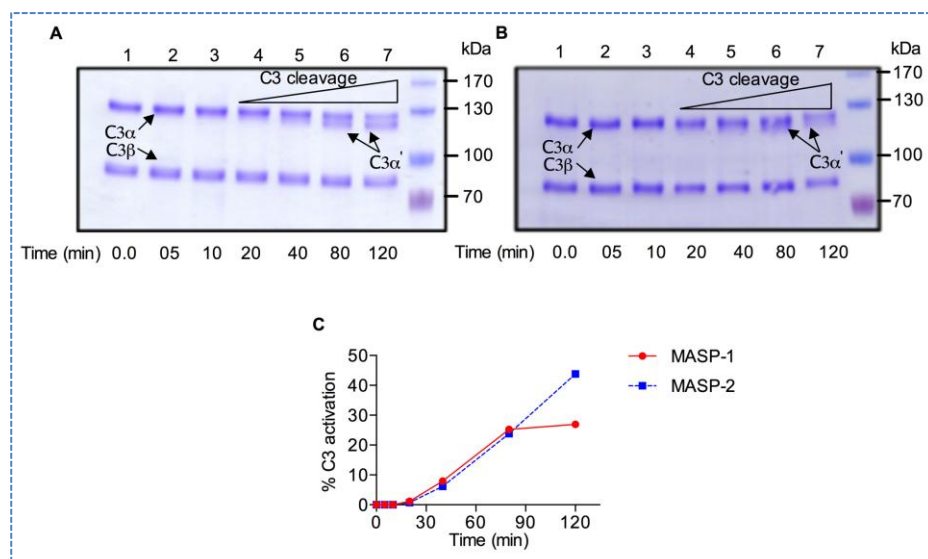


Figure 3-7: Time-course of C3 cleavage by catalytic fragments (CCP1- CCP2-SP) of MASP-1 and MASP-2

Purified human C3 was mixed with recombinant human MASP-1 (CCP₁- CCP₂-SP) (A) or MASP-2 (CCP₁- CCP₂-SP) (B) at enzyme:substrate ratio of 1:10, MASP:C3, (0.2μg:2μg, w/w) for 0.00 to 120 minutes. The proteins were separated in 6% SDS polyacrylamide gel and stained with Coomassie Blue. The C3α and C3α' band densities were measured by densitometer and plotted against time (C). The figure shows typical experiment of two independent experiments carried out.

3.2.7: C1-INH and OMS721 HG4 efficiently inhibit MASP-2

OMS721 HG4 is a MASP-2-specific inhibitory antibody that inhibits MASP-2 functional activity. C1-INH is a naturally occurring plasma protein inhibitor that amongst other serine proteases inhibits the complement serine proteases, i.e. C1s, C1r MASP-1 and MASP-2 (Matsushita et al. 2000b, Meri and Jarva 2001). Proteolytic MASP-2 activity against C3 was examined in presence of OMS721 HG4 and C1-INH. In this experiment, recombinant human MASP-2 (CCP₁- CCP₂-SP) was pre-incubated with various amounts of purified C1-INH or OMS721 HG4 for 20 minutes then human C3 was added to the mixture and further incubated for 90 minutes. Proteins were separated in 6% SDS polyacrylamide gel.

MASP-2 proteolytic activity was completely inhibited by OMS721 HG4 (figure 3-8A) and C1-INH (figure 3-8B) at the lowest concentrations tested (i.e. a two-fold molar ratio excess of inhibitor).

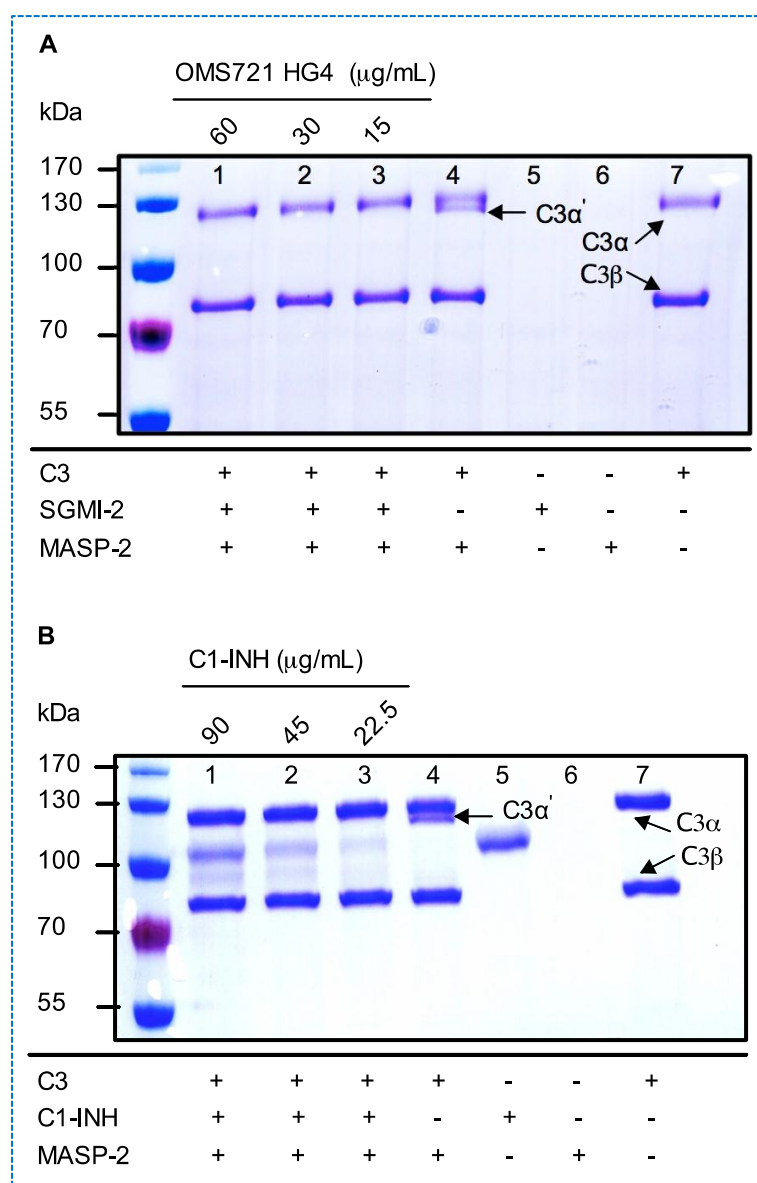


Figure 3-8: Inhibition of MASP-2 proteolytic activity by OMS721 HG4 and C1-INH

MAASP-2 was pre-incubated with various amounts of OMS721 HG4 (**A**) or with C1-INH (**B**) starting at a minimum molar ratio of two-fold then 2 μg of purified human C3 were added and further incubated for an hour at 37°C. Both inhibitors, OMS721 HG4 and C1-INH were blocked C3 proteolysis by MASP-2 at different inhibitor concentrations (**A** and **B** lanes 1-3). The figure shows typical experiment of two independent experiments carried out.

3.2.8: *Endogenous human and mouse MASP-2 cleaves C3*

The fast rate of autoactivation of MASP-2 poses a challenge for expressing and purifying full-length wild type protein. Other forms of MASP-2, rather than the wild type form, have been expressed such as the catalytic fragments (CCP₁-CCP₂-SP) of MASP-2 or by using a replacement mutation in the active site serine residue (Wallis et al. 2007). MASP-1/-3 KO mouse serum, that has intact MASP-2 (figure 3-9), C1q KO serum, that contains the lectin pathway serine proteases MASP-1, -2, -3 (figure 3-10), C4 KO mouse serum (figure 3-11), and C3 KO mouse serum (figure 3-10) were added to mannan coated wells in high salt buffer that prevents firstly C3 cleavage then the binding of the natural antibodies, consequently abolishing the classical pathway activation. In the C4 KO mouse serum, there is no classical pathway nor the normal route of the lectin pathway due to the absence of the fourth complement component, which makes the production of the classical and the lectin pathways C3 convertases (C4bC2a) impossible so that the only route of C3 activation is via the C4-bypass pathway (figure 3-11). In another experiment, sera were added to the mannan coated plate and 1 µg/well of C3 was then added. The reactions were stopped at various time points (from 00-90 minutes) using GVB containing 10 mM EDTA (figure 3-12). The normal human serum was used to immobilised human MASP-2 on mannan coated plate and exogenous C3 deposition was probed by anti-human C3c antibody (figure 3-13).

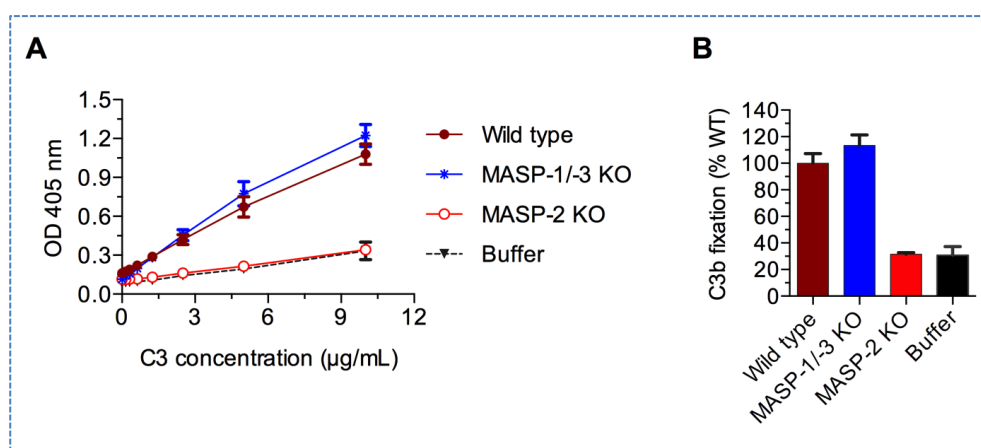


Figure 3-9: C3 cleavage by endogenous mouse MASP-2 immobilized from MASP-1/-3 KO mouse serum

Endogenous MASP-2 was immobilized to mannan coated ELISA plate from MASP-1/-3 KO serum in high salt buffer then purified human C3 was added (A). The highest C3 deposition was plotted against C3 deposition in wild type (B). C3 cleavage was statistically different in wild type vs MASP-2 KO ($P < 0.0015$) and in MASP-1/-3 KO vs MASP-2 KO ($P < 0.0004$). This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

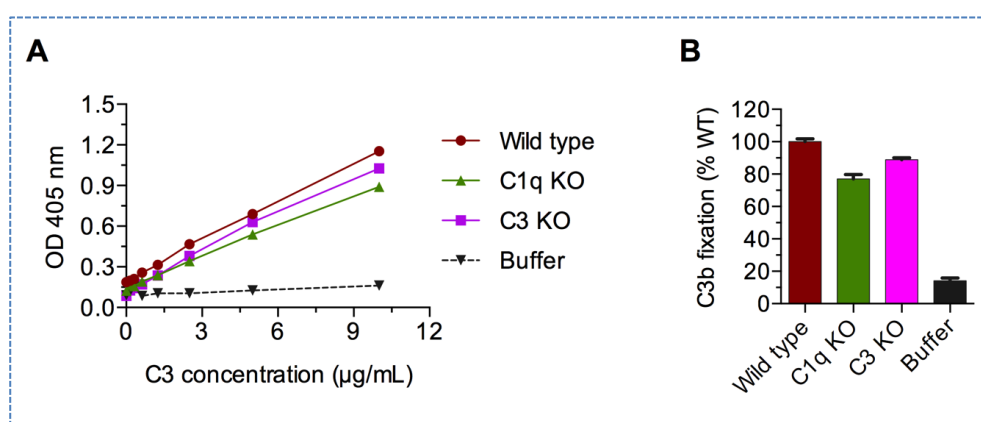


Figure 3-10: C3 cleavage by endogenous mouse MASP-2 immobilized from C1q KO and C3 KO mouse serum

C1q KO and C3 KO mouse serum was added to a mannan coated ELISA plate in high salt buffer then purified human C3 was added to the immobilized endogenous MASP-2 (A). The highest C3 deposition was plotted against C3 deposition in wild type (B). C3 cleavage was not statistically different in wild type vs C1q KO and wild type vs C3 KO ($P > 0.05$). This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

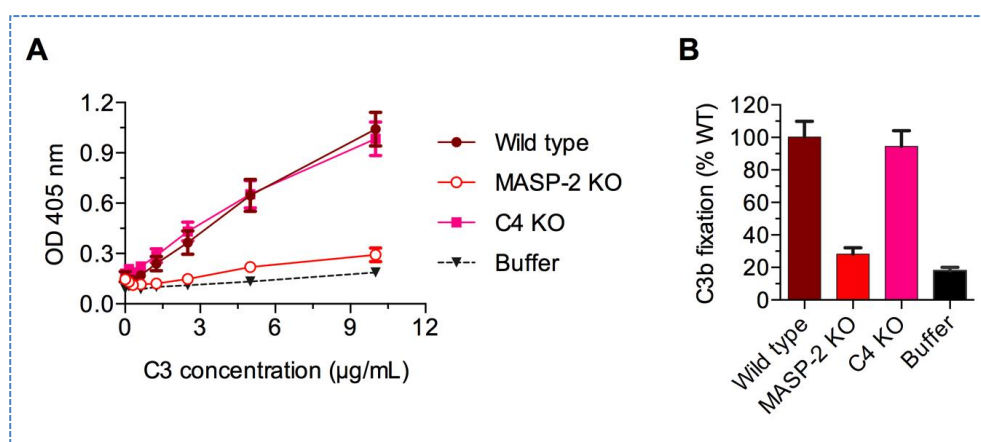


Figure 3-11: C3 cleavage by endogenous mouse MASP-2 immobilized from C4 KO mouse serum

Mannan coated ELISA plate was used to immobilize endogenous MASP-2 from C4 KO mouse serum diluted in high salt buffer then purified human C3 was added (A). The highest C3 deposition was plotted against C3 deposition in wild type (B). Statistically difference was seen between wild type vs MASP-2 KO ($P < 0.003$) and C4 KO vs MASP-2 KO ($P < 0.007$). This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

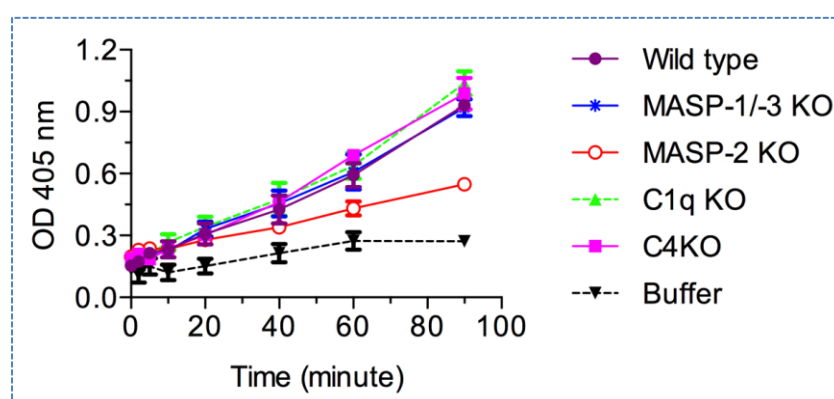


Figure 3-12: Time course of C3 cleavage by endogenous mouse MASP-2

Immobilised endogenous MASP-2 to mannan coated ELISA plate from MASP-1/-3 KO MASP-2 KO, C1q KO, C4 KO mouse sera diluted in high salt buffer then purified human C3 was added. C3 was incubated for various time points (0.00 to 90 minutes). Data are the means \pm SEM from two independent experiments carried out.

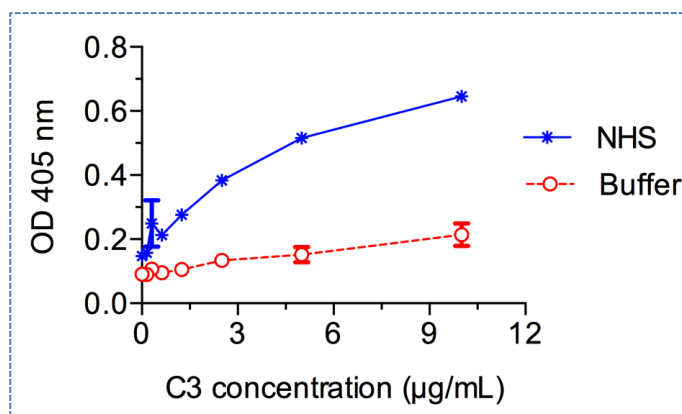


Figure 3-13: C3 cleavage by endogenous human MASP-2

Endogenous human MASP-2 was immobilized to mannan coated ELISA plate from normal human serum diluted in high salt buffer then purified human C3 was added and C3b deposition was probed by anti-human C3c antibody. This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

3.3: Discussion

The complement system is composed of more than 40 components that either circulate in the plasma or act as complement receptors or complement regulators on the cell surfaces of all of our cells. Activation of complement is initiated through a cascade of sequential enzymatic reactions which are carefully controlled by fluid-phase and cell surface bound complement regulatory components to prevent damage of autologous tissues. The system is designed to respond appropriately to challenges such as invasive infections, injuries, and to fulfill physiological maintenance tasks such as the clearance of dead cells, immune complexes and debris. In this maintenance task, complement assists the phagocytic system by targeting cells and cellular and microbial debris through a process called opsonization, where complement serves as a sensor which coats all surfaces that are not equipped with inhibitory regulators of complement activation with the most abundant complement activation product C3b or iC3b. C3b and iC3b opsonization and the release of chemoattractant complement activation products such as C3a and C5a, significantly enhance clearance of opsonized cells, microorganisms and debris through C3 receptor bearing phagocytic cells. C3 is cleaved by specific C3 convertase complexes which cleave the alpha chain of native C3 releasing a small cleavage fragment of about 9 kDa (C3a) and the major C3b cleavage fragment C3b that can bind covalently to complement-activating surfaces via its reactive internal thioester bond (Sim et al. 1993).

In addition, some microbial pathogens, including *Neisseria meningitidis*, can be lysed in serum directly through Membrane Attack Complexes (MAC) formed through activation of the terminal activation cascade C5b-C9 as described in the introduction.

The first part of my work aimed to find a molecular explanation for a paradox feature of the lectin pathway. The central role of lectin pathway dependent C3 activation in the pathophysiology of ischaemia-reperfusion injury has been documented in many recent publications from different independent research

teams (Schwaeble et al. 2011, Farrar et al. 2012, Asgari et al. 2014). While mice deficient of either C3 or lectin pathway recognition subcomponents (i.e. MBL) or the lectin pathway effector enzyme MASP-2 showed a significant degree of protection from post-ischaemic-reperfusion injury resulting in significant inflammation, tissue loss and loss of organ function, C4 deficient mice showed no degree of protection in any of the ischaemia-reperfusion injury models studied (Asgari et al. 2014). This result was highly unexpected, because according to the present text book view of the lectin pathway, the only convertase complex that mediates the cleavage of complement C3 is the lectin pathway C3 convertase, C4bC2a which forms after a MASP-2 dependent cleavage of C4 and C4b-bound C2 (Vorup-Jensen et al. 1998, Matsushita et al. 2000a, Fujita et al. 2004). It was therefore, expected that in absence of either C2 or C4, no C3 can be activated via the lectin pathway activation route. The existence of a lectin pathway specific MASP-2 dependent C4-bypass activation route was postulated based on *in vitro* evidence showing that in the total absence of C4, in human and mouse serum, a residual lectin pathway dependent activation of complement C3 can clearly be measured (Schwaeble et al. 2011).

My first task was to elucidate the molecular composition and the sequence of activation events leading to lectin pathway mediated activation of C3 in C4 deficient mouse serum. Since MASP-2 was shown to be essential for the C4-bypass activation of complement C3, I tried to revisit previously published work that reported a direct, albeit low abundant cleavage of purified complement C3 by any of the three different MASPs. The first report that indicated that C3 can be directly cleaved by MASP-1 was published more than 20 years ago using preparations of MASP-1 from serum (Matsushita and Fujita, 1993; Matsushita and Fujita, 1995). In 2001, Rossi *et al.* generated recombinant C1s, recombinant MASP-1 and recombinant MASP-2 in a baculovirus expression system and tested their substrate specificity against purified C2, C4 and C3 (Rossi et al. 2001). This work confirmed that MASP-1 can cleave C2, but not C4, while MASP-2 effectively cleaves both C2 and C4 similar to the classical

pathway effector enzyme C1s. In addition, both recombinant MASP-1 and recombinant MASP-2 were shown to cleave C3, although the rate of activation was so slow and inefficient (3 orders of magnitude lower than that of the C3 convertase, C4aC2b), that the authors concluded that cleavage of C3 by either MASP-1 or MASP-2 may not play a critical physiological role. In 2003, Ambrus *et al.* produced enzymatically active fragments of both MASP-1 and MASP-2 (composed of either the C-terminal CCP₁-CCP₂-SP domains, or the CCP₂-SP or the SP domain alone) in a bacterial expression system. Because most of the recombinant proteins accumulated as inclusion bodies, the proteins were harvested by centrifugation, refolded and purified by ion exchange chromatography. The substrate specificity assays revealed that both MASP-1 and MASP-2 cleave C2 efficiently and that the serine protease domains alone are sufficient to cleave C2 efficiently, while the CCP₂ domain of MASP-2 was required to effectively cleave C4. Both MASP-1 and MASP-2 showed a low degree of C3 cleavage activity, but interestingly, this cleavage was most effective when using hydrolyzed C3 [C3(H₂O) or C3i or as used in this paper C3(NH₃)], where the active thioester has been inactivated through spontaneous or chemically induced hydrolysis (Matsushita and Fujita 1995, Hajela et al. 2002, Ambrus et al. 2003). While C3(H₂O) can bind to FB to initiate the “tickover activation” of the alternative pathway (Lachmann and Hughes-Jones 1984).

As a start, I tested and compared the direct proteolytic activity of enzymatically active recombinant fragments of the lectin pathway serine proteases MASP-1, MASP-2 and MASP-3 against purified human C3. C3 cleavage was seen when C3 was either incubated with recombinant MASP-1 or MASP-2, while no C3 cleavage was detectable when C3 was incubated in presence of recombinant MASP-3. Since MASP-1 shares many similarities with trypsin (Ambrus et al. 2003) an enzyme that cleaves C3 effectively *in vitro*, it was somewhat surprising that recombinant MASP-2 appeared to be more effective in cleaving C3 than MASP-1 as shown in the time course experiment shown in figure (3-7).

The ability of recombinant human MASP-2 to cleave purified serum C3 revealed low rate cleavage of the C3 alpha-chain (see figures 3-3 and 3-7) as reported previously (Rossi et al. 2001, Ambrus et al. 2003).

As I was using recombinant MASP-1 and MASP-2 fragments produced in a bacterial expression system, I had to exclude the remote possibility that some of the proteolytic activity seen could have been caused by trace contaminations with a bacterial protease. To address this concern, I used specific known inhibitors of MASP-2 functional activity i.e. C1-INH which inhibits the esterase activity of C1s, C1r, MASP-1 and MASP-2 (Matsushita et al. 2000a, Meri and Jarva 2001, Rossi et al. 2001, Ambrus et al. 2003), and the MASP-2 specific inhibitory antibody OMS721 HG4 (Schwaeble et al. 2011, Ali et al. 2012) to check whether *in vitro* cleavage of purified C3 can be specifically inhibited. As shown in figure (3-8), both C1-INH and OMS721 HG4 effectively blocked C3 cleavage by MASP-2, excluding that C3 cleavage is mediated by a trace contamination with an unspecific protease.

In order to evaluate if MASP-2 is capable of cleaving native C3 and/or hydrolyzed C3, I used an ELISA based assay system that measures the amount of the C3 cleavage product C3b binding to the ELISA plate via its active thioester. This assay specifically monitors the cleavage of native C3, since the cleavage of hydrolyzed C3 would not result in C3b deposition, since the internal thioester to bind C3b to nucleophilic surface compounds is no longer reactive in C3(H₂O) or C3i or C3(NH₃).

For the first set of experiments, I used recombinant MBL in combination with recombinant full-length rat MASP-2K, both expressed in CHO cells. MASP-2K is a mutant form of recombinant MASP-2 where the cleavage/activation site has been changed by site-directed mutagenesis to replace the arginine residue at the cleavage site for zymogen activation (R⁴²⁴) with a lysine residue (K⁴²⁴) in order to slow down the degree of auto-activation of MASP-2 (Chen and Wallis, 2004). This mutant form of MASP-2 cleaved native C3 effectively to allow C3b to deposited on the ELISA plate. As shown in figure (3-6), pre-incubation of the

mannan-coated ELISA with 1 $\mu\text{g}/\text{mL}$ recombinant MBL and 1 $\mu\text{g}/\text{mL}$ of recombinant MASP-2K for overnight at 4°C yielded an MBL(MASP-2K)_2 complex that bound to the mannan-coated surface that cleaved the C3 substrate as monitored by C3b deposition on the ELISA plate. Pre-coating of the mannan-coated ELISA plate with recombinant MBL alone (i.e. without addition of recombinant rat MASP-2K) showed a residual degree of C3b deposition. This residual background activity of the recombinant MBL alone was reported previously and was previously explained by the possible binding of bovine MASPs from the fetal calf serum contained in the cell culture medium of the MBL-expressing cell line (Chen and Wallis 2004).

In order to assess the activity of lectin pathway activation complexes captured from different serum sources, I exposed mouse and human sera with and without defined complement deficiencies on mannan-coated ELISA plates. Defined mouse and human sera were diluted to a final concentration of 20% in high salt buffer and incubated at 4°C for 1 hour to allow lectin pathway activation complexes to adhere to the mannan-coated surface. The buffer allows MBL(MASP)_2 complexes to bind to mannan, but dissociates the classical pathway C1 complex (Petersen et al. 2001) and prevents activation of the lectin pathway. After incubation, the ELISA plates were washed once with high salt buffer and twice with washing buffer to remove any non-surface bound complement while MBL(MASP)_2 complexes remained attached to the mannan-coated ELISA plate. The use of wild type sera and sera with defined complement deficiencies allowed to determine the composition of the adhering lectin pathway complexes and exclude the contribution of any defined complement component to C3 convertase activity of the lectin pathway activation complexes sticking to the mannan-coated ELISA plate. The use of MASP-1/-3 deficient mouse serum and MASP-2 deficient mouse serum allowed to define whether both MASP-1 and MASP-2, or MASP-2 alone, or none of the MASPs can cleave native C3 in a lectin pathway dependent manner as a function of naturally occurring lectin pathway serum complexes. Exposing mannan-coated ELISA plates to MASP-1/-3 deficient mouse serum yielded in

coating the surface with lectin pathway activation complexes exclusively composed of MBL and MASP-2, while the use of MASP-2 deficient serum yielded in coating the ELISA plate in complexes exclusively composed of MBL and MASP-1 and MASP-3. In order to exclude a contribution of any traces of C3 convertase complexes that could have bound to the ELISA plate surface during the exposure of the plates to serum, I also coated ELISA plates with C4 deficient mouse serum, C3 deficient mouse serum and C1q deficient mouse serum. Following the washing steps, 100 microliter of purified native human serum C3 in GVB⁺⁺ buffer (0.001 microgram C3/mL) in were exposed at 37°C for 1 hour to the lectin pathway complexes coated ELISA plates, washed once with high salt buffer and twice with washing buffer. C3b binding was quantified using rabbit-anti-human C3c antibody and probed with an alkaline phosphatase labeled goat anti-rabbit antibody.

Figures (3-9 to 3-12) that summarize the results of the analysis using sera from wild type and gene-targeted mice, show a considerable degree of C3 deposition that was seen in wild type mouse serum, MASP-1/-3 deficient mouse serum, C4 deficient mouse serum, C3 deficient mouse serum, and C1q deficient mouse serum. However, no deposition of C3b was detected in any of the ELISA plates coated with MASP-2 deficient mouse serum. These results clearly indicate that: i) MASP-2 is essentially required for lectin pathway activation complexes to cleave native C3, ii) MASP-1 does not cleave native C3 within lectin pathway activation complexes and iii) neither endogenous C4, nor C3, nor C1q are required for lectin pathway complexes to cleave native C3 in a MASP-2 dependent manner.

Taken together my results clearly indicate that serum resident lectin pathway activation complexes can cleave exogenous native C3 on lectin pathway activating surfaces in a strictly MASP-2 dependent fashion. Although this direct cleavage activity of MASP-2 towards C3 may be low, compared to the C3 convertase activity measured for the classical and lectin pathway specific C3 convertase complex, C4bC2a or the alternative pathway C3 convertase, C3bBb,

this direct cleavage and activation of C3 by MASP-2 could be of physiological importance in the mediation of pathogenic inflammatory conditions such as ischaemia-reperfusion injury. Interestingly, my *in vitro* test assessing the direct cleavage of native C3 by lectin pathway activation complexes sequestered from serum through affinity binding to their carbohydrate ligands also revealed that the absence of MASP-1 and MASP-3 in MASP-1/-3 deficient does not affect the ability of MASP-2 to cleave C3, since the MBL(MASP-2)₂ complexes captured from MASP-1/-3 deficient serum cleaved native C3 as efficiently as complexes containing all three lectin pathway specific serine proteases i.e. wild type mouse serum. This phenotype is particularly surprising, since recent work by others claims that MASP-1 is essentially required to activate and drive MASP-2 dependent complement activation (Heja et al. 2012). If this was totally true, then it could be expected that MASP-1/-3 targeted mice would also be compromised in their ability to activate MASP-2 and be protected from MASP-2 mediated ischaemia-reperfusion injury. However and most surprisingly, MASP-1/-3 deficient mice do show no degree of protection from ischaemia-reperfusion injury (Gorsuch et al. 2012).

Taken together, my results strongly support the hypothesis that this remarkable phenotype of ischaemia-reperfusion injury depending on C3 activation in a lectin pathway and MASP-2 dependent fashion, but independent of the presence or absence of C4 and MASP-1 is caused by direct cleavage of native C3 by MASP-2, a lectin pathway mediated C4-bypass activation of C3.

Figure (3-14) below depicts the MASP-2 mediated activation steps of the lectin pathway, including the C4-bypass activation route identified through my work.

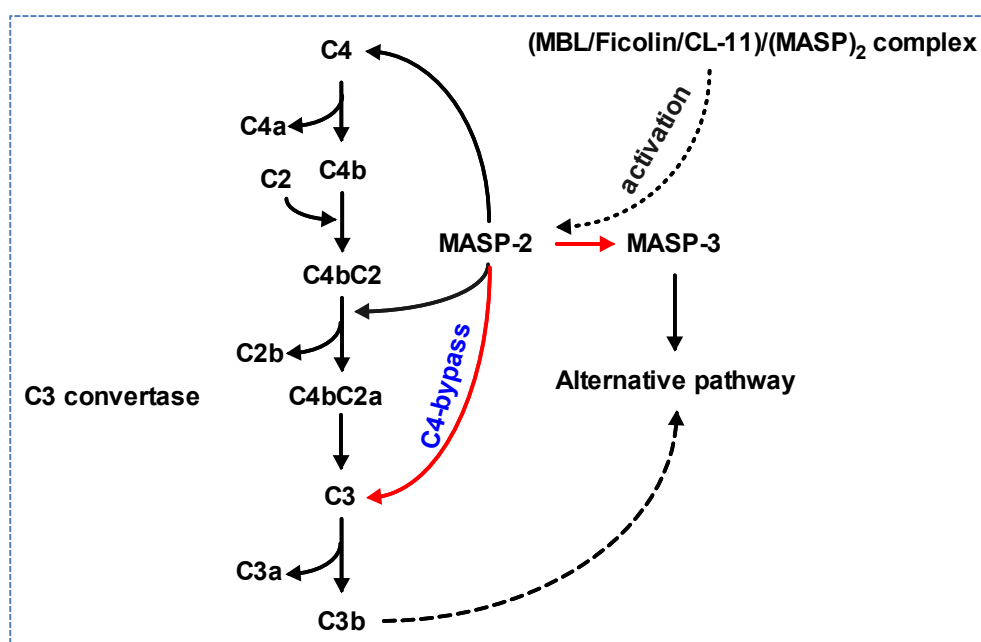


Figure 3-14: MASP-2 enzymatic activities within the activation of complement via the lectin pathway

MASP-2 cleaves complement components C4 and C4-bound C2 and this study identified two new enzymatic activities of MASP-2 (red arrows) i.e. C3 proteolysis and MASP-3 activation.

*Chapter four: MASP-3 dependent, lectin
pathway mediated activation of the
alternative pathway C3 convertase*

4.1: Introduction

The lectin pathway of complement is an antibody-independent route of complement activation and represents a key player in the innate immune defense by recognizing bacterial surface carbohydrates such as mannose, *N*-acetylglucoseamine, *N*-acetylmannoseamine, *N*-acetylgalactoseamine (Carroll and Sim 2011).

The lectin pathway is initiated when a pattern recognition molecule and its associated serine protease binds to an activator surface. In man, there are five different recognition molecules i.e. MBL, ficolins M, L, H, (ficolin 1, 2, 3, respectively) and Collectin 11 (CL-11) (Kawasaki et al. 1978, Yae et al. 1991, Lu et al. 1996, Matsushita et al. 1996, Hansen et al. 2010) while in mouse there are two MBLs, MBL-A and MBL-C, two ficolins, ficolin-A and B and CL-11 (Stover et al. 1999c, Hansen et al. 2010, Schwaeble et al. 2002). The pathway is also contained three homologous MBL-Associated Serine Proteases (MASP) -1, -2 and -3 as well as two MBL-Associated Proteins MAp19 (19 kDa) and MAp44 (44 kDa) (Matsushita and Fujita 1992, Thiel et al. 1997, Stover et al. 1999b, Takahashi et al. 1999, Dahl et al. 2001, Degn et al. 2009a, Skjoedt et al. 2010a).

MASP-1 is the first discovered serine protease of the lectin pathway associated with MBL (Matsushita and Fujita 1992). It was originally thought to cleave C3 but its activity is mostly towards hydrolyzed C3, C3(H₂O). MASP-1 was then shown to cleave the complement component C2, but not C4 (Hajela et al. 2002). So that, MASP-1 supports and augments the lectin pathway activity of MASP-2 (see below). However, there is some evidence that MASP-1 cleaves FD and converts it into its active form (Takahashi et al. 2010) nonetheless whether this cleavage activity is essential to maintain alternative pathway functional activity still unclear. Recently, some studies suggested a role of MASP-1 in driving the lectin pathway via activation of MASP-2 (Takahashi et al. 2007, Heja et al. 2012). On the other hand, recombinant catalytic fragments of MASP-1 (CCP₁-CCP₂-SP) directly cleave some factors in the coagulation system such as prothrombin and factor XIII and fibrinogen (Krarup et al. 2008, Hess et al. 2012) indicating the

role of the MASP-1 in the linkage between the complement system and the coagulation system.

MASP-2 was first described in 1997 (Thiel et al. 1997) and dissimilar to MASP-1, MASP-2 cleaves the fourth complement component and C2 to generate the lectin pathway C3 convertase C4bC2a (Vorup-Jensen et al. 1998). In addition, MASP-2 showed enzymatic activities in activating prothrombin (Krarup et al. 2007).

The most recently discovered MASP, MASP-3 (Dahl et al. 2001) is encoded through an alternative splicing process of the *MASP1* gene. Similar to MASP-1, MASP-3 is composed of six domains including CUB₁ and CUB₂ (domains found in C1r/C1s, Uegf, Bmp 1 and 2), Epidermal Growth Factor (EGF) like domain, Complement Control Protein 1 and 2 (CCP₁, and CCP₂) followed by a Serine Protease (SP) domain. The first five domains are identical in MASP-1 and MASP-3, but the SP domain is different (Virolainen et al. 1994, Takahashi et al. 2008). MASP-3 has a median serum concentration of 6.4 µg/mL (Skjoedt et al. 2010b) and MASP-3 mRNA is expressed in a wide range of tissues including liver, colon tissue, prostate, skeletal muscles, heart, small intestines, lung, ovary, placenta, brain and the spleen (Seyfarth et al. 2006). MASP-1 and MASP-2 expression, however is liver specific and have average serum concentrations of 11 µg/mL and 0.5 µg/mL, respectively (Moller-Kristensen et al. 2007, Thiel et al. 2012). Very little is known about the biological characteristics and role of the MASP-3 and its role in complement activation remained mysterious for several years until Takahashi et al. (2010) suggested that MASP-3 cleaves FD and the most recent publications described that the MASP-3 can initiate the alternative pathway by cleaving FB in the C3bB complex to produce C3bBb, the specific the alternative pathway C3 convertase (Iwaki et al. 2011).

Activation of MASP-3 has also been unexplained: it was reported that it does not auto-activate and no enzymatic activity of MASP-3 was found towards C2, C4 or C3 (Dahl et al., 2001) and the only clear proteolytic activity of MASP-3

that was identified until recently is that towards insulin-like growth factor-binding protein 5 (IGFBP-5) (Cortesio and Jiang 2006).

MASP-3 was more recently implied to be essential for activating the alternative pathway of complement on cell surfaces. In the absence of MASP-3, complement mediated lysis of *Neisseria meningitidis* and the haemolytic activity of serum is impaired (Professor Wilhelm Schwaeble, personal communication).

This part of the thesis is aimed to identify the natural substrates and activators of MASP-3 to contribute to understand the molecular basis of the alternative pathway activation by MASP-3. The results obtained clarified that the activation of the MASP-3 is catalyzed by both MASP-1 and MASP-2 and MASP-3 efficiently activates pro-FD but not zymogen FB. Additionally it was found that MASP-1 reconstitution fails to restore alternative pathway functional activity in MASP-1/-3 deficient mouse serum.

4.2: Results

4.2.1: Expression and purification of recombinant human and mouse

MASP-3

4.2.1.1: Cloning and expression of recombinant human and mouse MASP-3

MASP-3 is not an auto-activating enzyme so that there was no need to use site directed mutagenesis and the protein was cloned and expressed in its wild type form.

4.2.1.1.1: Cloning of human and mouse MASP-3

Human and mouse cDNAs encoding the full-length MASP-3 were kindly provided by Professor Wilhelm Schwaeble and used as a template for cloning of the full-length human and mouse MASP-3 and the human MASP-3 serine protease domain.

Three primers were used to amplify the coding sequence of the full-length human MASP-3 and the coding sequence of MASP-3 serine protease domain (figure 4-1A). Two primers were used to amplify the coding sequence of mouse MASP-3 (figure 4-1B). The PCR products were then cloned into pGEM-T easy vector (figure 4-1, C and D) and sub-cloned into expression vector pSecTag 2/Hygro B (figure 4-2, A and B).

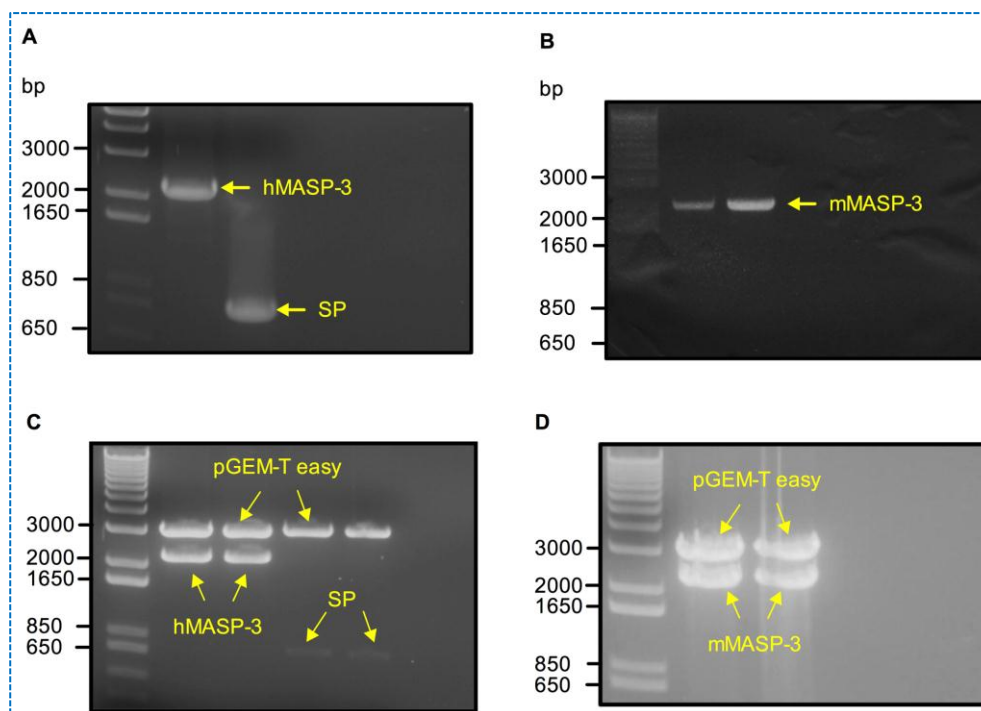


Figure 4-1: Agarose gel electrophoresis of PCR amplified human and mouse MASP-3 and cloning into pGEM-T easy vector

Generation of the full-length coding sequence and the coding sequence of serine protease of human MASP-3 using *pfx* DNA polymerase (A). The full-length coding sequence of mouse MASP-3 was PCR amplified using phusion high-fidelity DNA polymerase (B). The full-length human and mouse MASP-3 shows a band of approximately 2175bp and the coding sequence for human MASP-3 serine protease domain shows a band of approximately 850bp. The PCR products of human (C) and mouse (D) MASP-3 were cloned into pGEM-T easy vector.

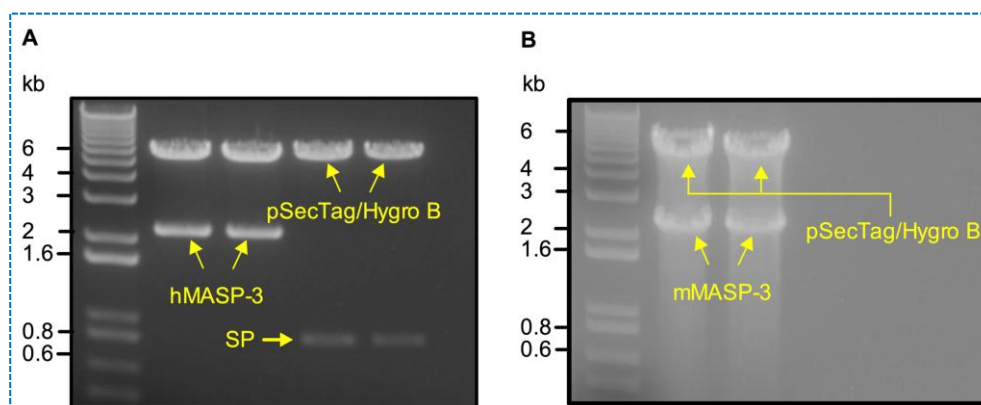


Figure 4-2: Agarose gel electrophoresis shows sub-cloning of human and mouse MASP-3 constructs into pSecTag 2/Hygro B expression vector

The full-length coding sequence of human and mouse MASP-3 and the coding sequence of the human MASP-3 serine protease domain were sub-cloned into the 5.7 kb expression vector, pSecTag 2/Hygro B. Human MASP-3 constructs were digested with *Kpn* I and *Not* I restriction enzymes (A) and mouse construct was digested with *Bam*H I and *Xho* I restriction enzymes (B).

4.2.1.1.2: Expression of human and mouse MASP-3

Following the cloning of DNA construct into pSecTag2/Hygro B, the plasmid DNA was sequenced to ensure that no mutations took place during the cloning steps.

After transfecting CHO-K1 cells with the expression vector containing the construct of human or mouse MASP-3, the cells were maintained in a selection medium. Three weeks after, clones were screened for protein expression. The human MASP-3 construct encoded for the serine protease domain was failed to give any positive clones.

4.2.1.1.3: Purification of recombinant human and mouse MASP-3

The positive clones in a dot blot test (figure 4-3) were maintained in triple layers flasks and the supernatant containing recombinant MASP-3 was applied to a His GraviTrap column. The supernatant, flow through, column wash and elution fractions were analyzed by SDS-PAGE (figure 4-4, A and B) under reducing and non-reducing conditions (figure 4-5) and the presence of the expected His-tagged recombinant proteins confirmed by Western blotting using HRP conjugated monoclonal mouse anti polyhistidine antibodies (figure 4-6A) and monoclonal rat anti-human MASP-3 antibodies that probed with HRP conjugated rabbit anti-rat antibodies (figure 4-6B).

By immunoblotting and protein sequencing technique (Appendix A), the identity of the recombinant proteins were confirmed as human and mouse MASP-3.

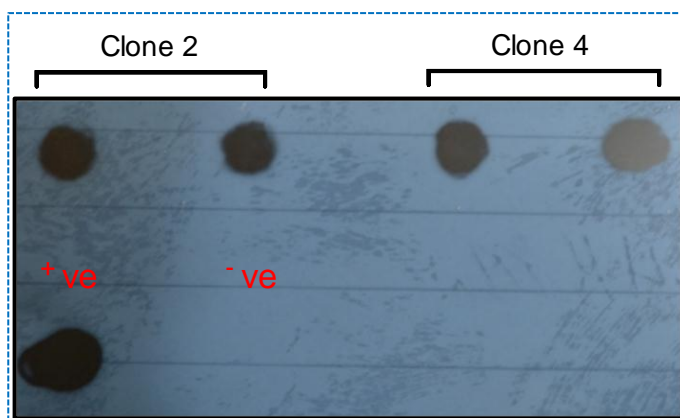


Figure 4-3: Dot blot assay of the supernatant of different clones for expression of human MASP-3

Screening of some selected clones for expression of human MASP-3. The protein expression was visualized using HRP conjugated monoclonal mouse anti-polyhistidine antibodies. Recombinant properdin with histidine linker was used as a positive control (+ve). Supernatant from non-transfected CHO-K1 cells was used as a negative control (-ve). Clones 2 and 4 were positive.

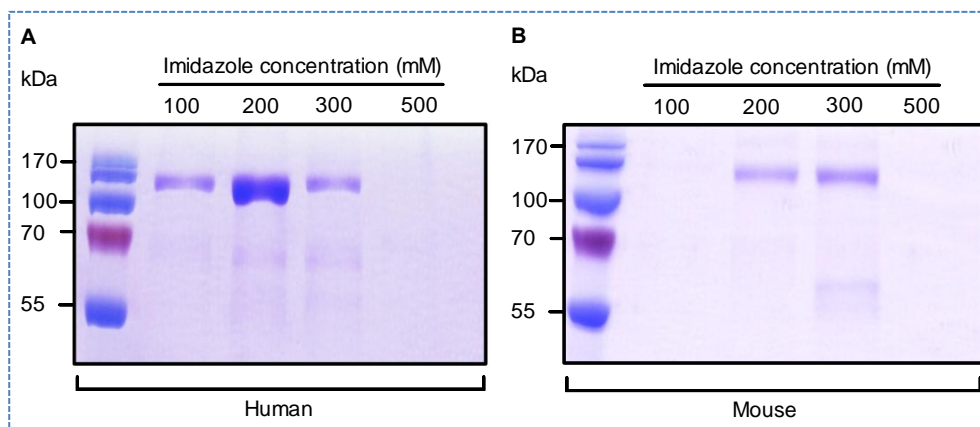


Figure 4-4: SDS-PAGE analysis of the purified human and mouse MASP-3 fractions

Purification of recombinant human (A) and mouse (B) MASP-3 was performed by using His GraviTrap column. Different concentrations of imidazole (100 mM, 200 mM, 300 mM and 500 mM) were used to elute the recombinant proteins from His GraviTrap column. Proteins were denatured and separated in 10% SDS polyacrylamide gel and stained with Coomassie Blue. The purified proteins showed a single band of approximately 115 kDa under reducing conditions. Recombinant human MASP-3 was eluted with 200 mM imidazole and mouse MASP-3 was eluted with 300 mM imidazole.

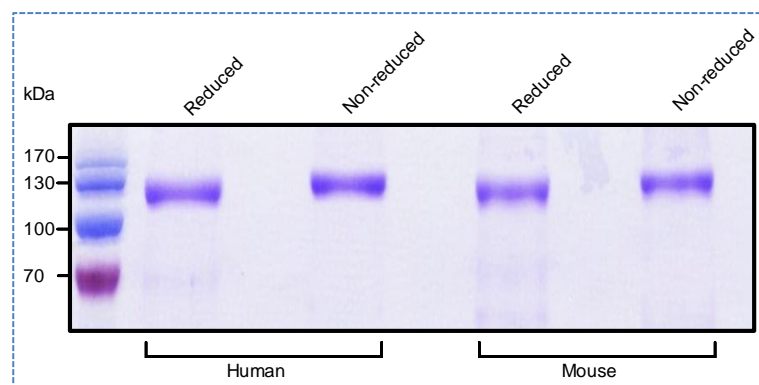


Figure 4-5: SDS-PAGE analysis of the purified recombinant human and mouse MASP-3 under reducing and non-reducing conditions

Recombinant human and mouse MASP-3 was eluted with 200 mM imidazole or 300 mM imidazole, respectively. 2 μ g of the recombinant proteins were analyzed in 10% SDS polyacrylamide gel and the gel was stained with Coomassie Blue. Recombinant MASP-3 shows a band of approximately 115 kDa under reducing conditions and 130 kDa under non-reducing conditions.

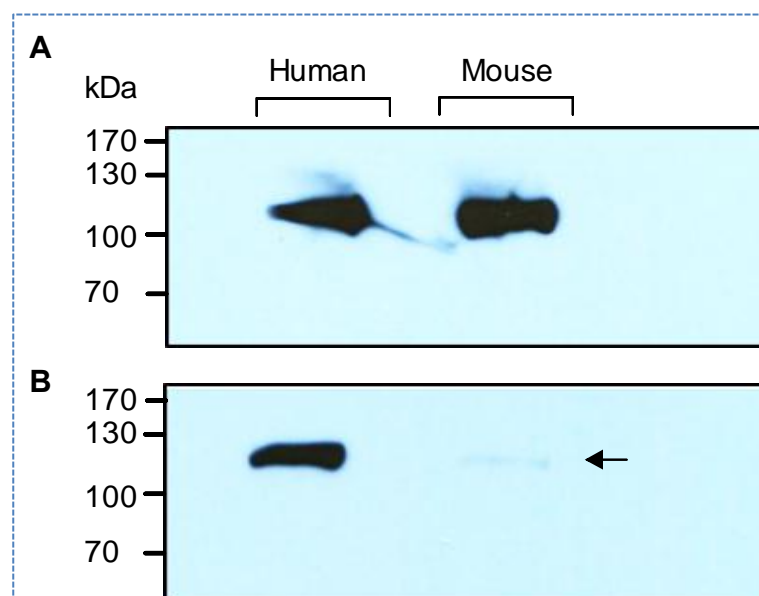


Figure 4-6: Western blotting analysis of the recombinant human and mouse MASP-3

One microgram of the recombinant human or mouse MASP-3 was separated on SDS polyacrylamide gel and proteins were electrophoretically transferred onto a nitrocellulose membrane. The recombinant proteins were visualized using HRP conjugated monoclonal mouse anti-polyhistidine antibodies (A) or using monoclonal rat anti-human MASP-3 (38:12-3) antibodies probed with HRP conjugated rabbit anti-rat antibodies (B).

4.2.2: Recombinant human MASP-3 activation assays

4.2.2.1: Recombinant human MASP-3 cleavage by trypsin

MASP-3 is a serine protease that consists of a heavy chain (also called A chain) and light chain (also called B chain). The two chains are separated upon activation in the region between the serine protease domain and link region but both attached together through a disulphide bridge (Dahl et al. 2001). Recombinant full-length human MASP-3 was incubated with various concentrations of trypsin from bovine pancreas starting at a maximum ratio a ratio of 1:80, trypsin:MASP-3 (w/w) (figure 4-7). Trypsin hydrolysis the peptide bonds following arginine (Arg-|-Xaa) or lysine (Lys-|-Xaa). The number of arginine and lysine residues in the amino acid sequence of human MASP-3 is 30 residues each. Arginine residues are distributed as 17 residues in the MASP-3 heavy chain and 13 residues in the light chain and for lysine 22 residues are in the MASP-3 heavy chain and 8 residues in the light chain. So that, there are about 60 potential cleavage sites for trypsin in the MASP-3 sequence residues (Based on GenBank entry number AAK84071.1). The reason behind the limitation of trypsin proteolytic activity is that the recombinant MASP-3 is a highly folded protein as it has been expressed in mammalian cell line so that it is relatively resistant to proteolysis at most of the Arg, Lys sites.

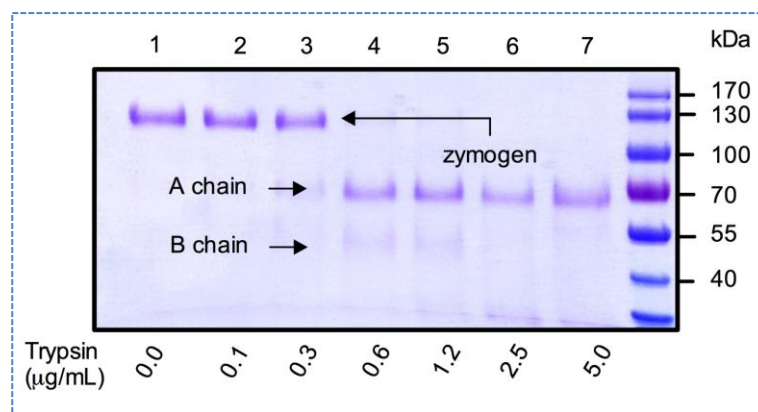


Figure 4-7: SDS-PAGE analysis of MASP-3 cleavage by trypsin

Recombinant full-length human MASP-3 was incubated with various concentrations of trypsin from bovine pancreas starting at a maximum ratio of 1:80, trypsin:MASP-3, (0.05 μg:4 μg, w/w) in TBS-Ca²⁺ buffer for an hour at 37°C and separated in 10% SDS polyacrylamide gel. The full-length recombinant MASP-3 zymogen (115 kDa) is cleaved by trypsin into A chain (60 kDa) and B chain (55 kDa) (lanes 1-4). 0.6 μg/mL trypsin (1:10) (lane 4) gives midpoint of MASP-3 cleavage. The figure shows typical experiment of two independent experiments carried out.

4.2.2.2: *Activation of recombinant human MASP-3 by different serine proteases*

The hydrolysis activity of catalytic fragments (CCP₁-CCP₂-SP) of recombinant human MASP-1 and MASP-2 and also plasmin and thrombin on recombinant full-length human MASP-3 was assayed at various enzyme:substrate ratios, the highest being 1:4 (w/w). MASP-3 conversion from zymogen to active enzyme was apparently achieved by incubating recombinant full-length human MASP-3 with catalytic fragments (CCP₁-CCP₂-SP) of recombinant human MASP-1 or MASP-2 (figures 4-8 and 4-9). The activation site of MASP-3 is located between lysine 448 and arginine 449 (Based on GenBank entry number AAK84071.1) resulted in two chains: the heavy chain consists of 429aa and the light chain is about 280aa linked together by a disulphide bond which can be reduced in denaturation in presence of reducing reagent. Cleavage at exactly the correct site was not confirmed (e.g. by N-terminal sequencing) but activation was later assessed by enzymatic tests.

Fifty % zymogen MASP-3 conversion was achieved by MASP-1 concentration of 50 μg/mL (1:4) (figure 4-8, lane 1 and figure 4-10) (lane 1) and the same conversion

percentage was achieved by MASP-2 concentration of 25 $\mu\text{g/mL}$ (1:8) (figure 4-9, lane 2 and figure 4-10) indicating that MASP-2 has higher enzymatic activity on MASP-3 than MASP-1.

The thrombin (figure 4-11) and plasmin (figure 4-12) showed no enzymatic activity towards MASP-3 at all concentrations under the same conditions of digestion as MASP-1 and MASP-2. However, thrombin showed an enzymatic activity on fibrinogen that appears in its structural subunits (α , β and γ chains) (figure 4-11A, lane 8)

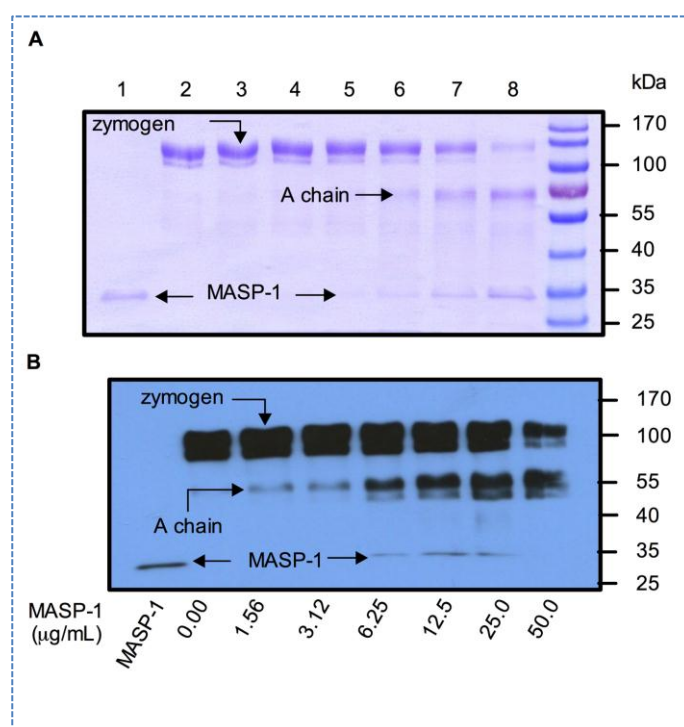


Figure 4-8: SDS-PAGE and Western blotting analysis of MASP-3 activation by MASP-1

The full-length MASP-3 was incubated with different concentrations of MASP-1 (CCP₁-CCP₂-SP) starting at a maximum enzyme substrate ratio of 1:4, MASP-1:MASP-3, (1 μg :4 μg , w/w) for 1 hour at 37°C. MASP-1 enzymatic activity on MASP-3 was analyzed in 10% SDS polyacrylamide gel stained with Coomassie Blue (A). The activated MASP-3 showed a band of approximately 65 kDa (A chain) (lanes 1-6). Polyclonal anti-human MASP-1/-3 (H-260) antibodies were used to visualise MASP-3 heavy chain and CCP₁, CCP₂ fragments of MASP-1 heavy chain (30 kDa) (lane 8) (B). The figure shows a typical experiment of two independent experiments carried out.

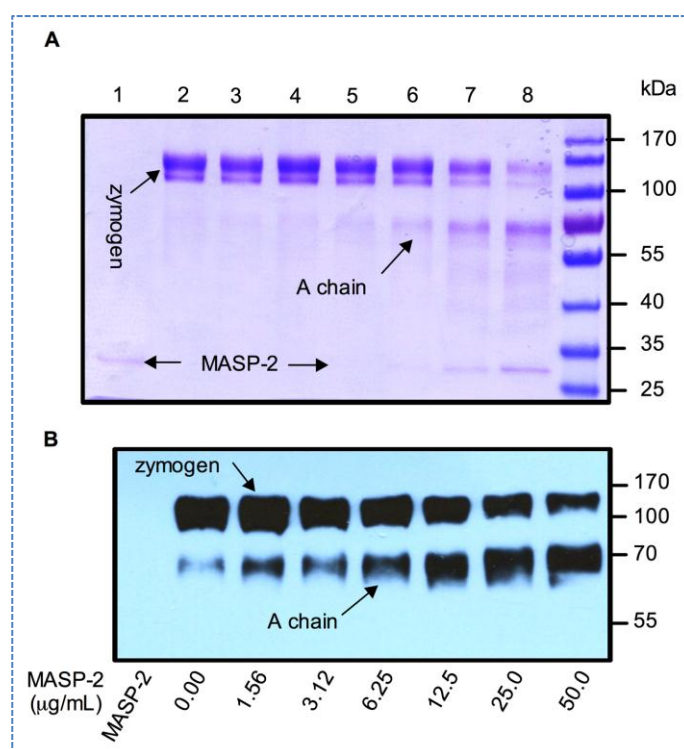


Figure 4-9: SDS-PAGE and Western blotting analysis of MASP-3 activation by MASP-2
 Various concentrations of MASP-2 (CCP₁-CCP₂-SP) were incubated with the full-length human MASP-3 for 1 hour at 37°C starting at a maximum enzyme substrate ratio of 1:4, MASP-2:MASP-3, (1µg:4µg, w/w). MASP-2 proteolytic activity on MASP-3 was analyzed in 10% SDS polyacrylamide gel stained with Coomassie Blue (**A**). The SDS polyacrylamide separated proteins were blotted onto nitrocellulose membrane and polyclonal anti-human MASP-1/-3 (H-260) antibodies were used to detect MASP-3 A chain (65 kDa, lanes 1-6) (**B**). The figure shows a typical experiment of two independent experiments carried out.

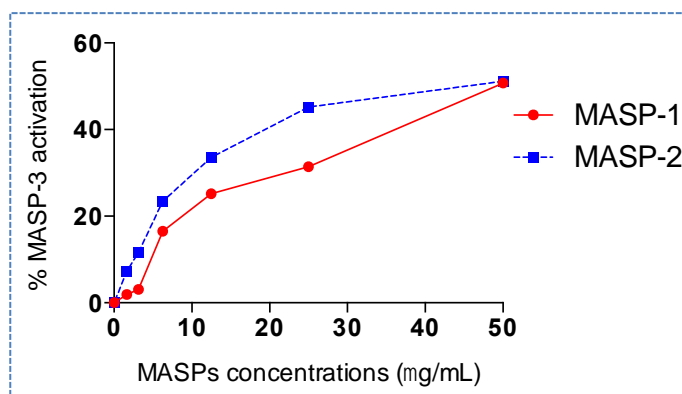


Figure 4-10: the percentage of zymogen MASP-3 conversion into active enzyme by MASP-1 and MASP-2

The intensities of zymogen MASP-3 and heavy chain bands were determined by densitometry and the percentage activation calculated and blotted against MASP-1 and MASP-2 concentrations. Fifty percent MASP-3 conversion is achieved at 50 $\mu\text{g/mL}$ of MASP-1 and 25 $\mu\text{g/mL}$ of MASP-2.

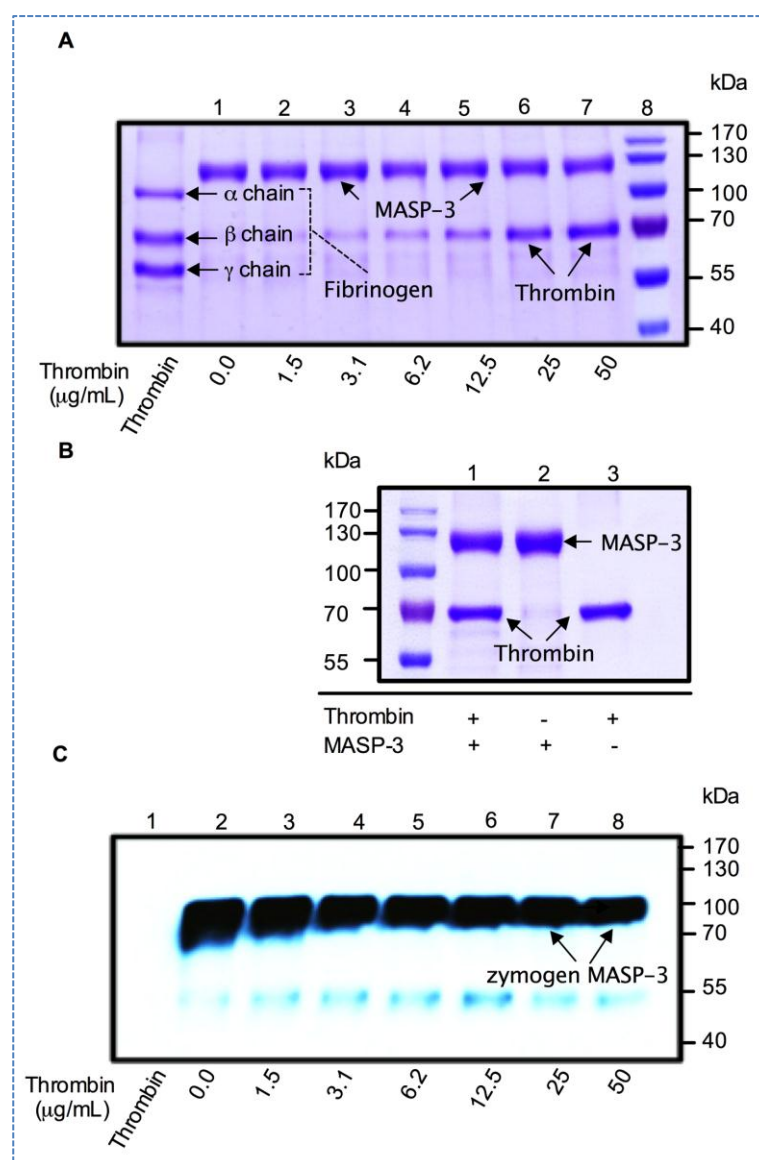


Figure 4-11: SDS-PAGE and Western blotting analysis of MASP-3 activation by thrombin

Recombinant full-length human MASP-3 was incubated with thrombin for 1 hour at 37°C starting at a maximum enzyme substrate ratio of 1:4, thrombin:MASP-3, (1 μg :4 μg , w/w). Thrombin activity was analyzed in 10% SDS polyacrylamide gel stained with Coomassie Blue (A). The zymogen MASP-3 showed a band of approximately 115 kDa and the thrombin showed a band of 70 kDa. MASP-3 is not activated by thrombin at all concentrations used (lanes 1-6) while fibrinogen appears in its structural subunits i.e. α , β , and γ chains (lane 8). MASP-3/thrombin interactions were analyzed in SDS-PAGE to confirm that the lower band 70 kDa is thrombin and not MASP-3 A chain (lane 3) (B). MASP-3 activation by thrombin was also analyzed by Western blotting using polyclonal anti-human MASP-1/-3 antibodies (C). The figure shows a typical experiment of two independent experiments carried out.

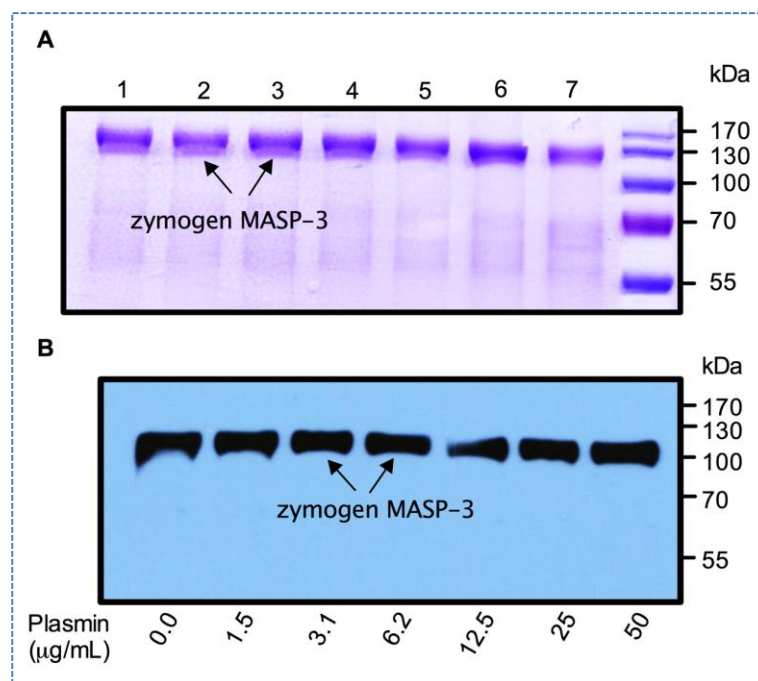


Figure 4-12: SDS-PAGE and Western blotting analysis of plasmin proteolytic activity on MASP-3

Various concentrations of plasmin were incubated with recombinant full-length human MASP-3 at 37°C for an hour. Plasmin/MASP-3 (1µg:4µg, w/w) interactions were analyzed in 10% SDS polyacrylamide gel stained with Coomassie Blue (A). The zymogen MASP-3 showed a band of approximately 115 kDa. (B) Polyclonal anti-human MASP-1/-3 (H-260) antibodies were used to visualize any MASP-3 activation. The figure shows a typical experiment of two independent experiments carried out.

4.2.3: MASP-3 activated by MASP-2 efficiently cleaves the fluorescent substrate VPR-AMC

Amidolytic activity of MASP-3 (CCP₁-CCP₂-SP) on VPR-AMC (Val-Pro-Arg aminomethyl coumarin) substrate was assessed by measuring AMC released upon cleavage.

MASP-3 shows enzymatic activity on the synthetic substrate VPR-AMC only in the presence of MASP-2 (CCP₁-CCP₂-SP) (figure 4-13A). The AMC release is dependent on the concentration of MASP-3. VPR-AMC turnover by MASP-2 alone is negligible (figure 4-13, A and B). These results are consistent with MASP-2 activating MASP-3 (figure 4-9) and subsequently activated MASP-3 releases AMC. MASP-3 alone (without addition of MASP-2) shows weak

activity on VPR-AMC at the higher concentrations used e.g. 100 $\mu\text{g}/\text{mL}$ (figure 4-13, A and B). The reason for this is that MASP-3 preparation contains a very small amount of activated MASP-3 (figure 4-14). The rate (v_i) was calculated at different MASP-3 concentrations and Michaelis-Menten constant (K_m) calculated from the Lineweaver-Burk (figure 4-14D) and the K_m equals 4.3 $\mu\text{g}/\mu\text{L}$.

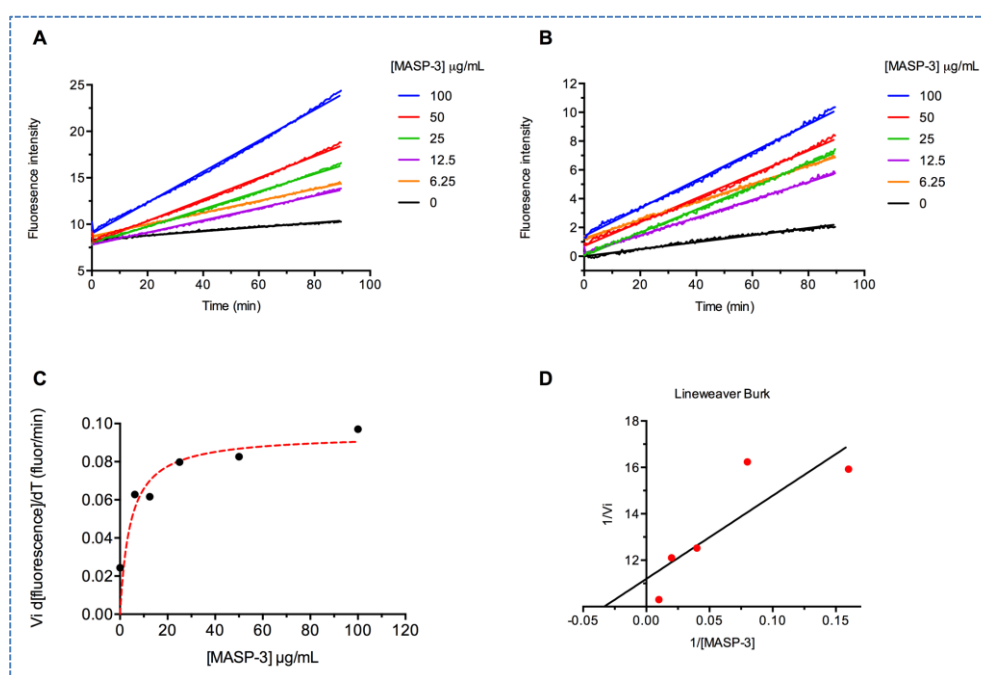


Figure 4-13: MASP-3 activity generated by MASP-2 measured by cleavage of VPR-AMC

Various concentrations of recombinant human MASP-3 (CCP1-CCP2-SP) were mixed with fixed concentration of recombinant human MASP-2 (CCP1-CCP2-SP) (2 $\mu\text{g}/\text{mL}$) and VPR-AMC (0.1 mM final concentration) the fluorescence measured every 30 seconds for 90 minutes. (A) Progress curve of AMC released by indicated concentrations of MASP-3. Data show the MASP-3 activity in presence of MASP-2 and omit the controls (MASP-3 without MASP-2). Black line shows controls without MASP-2. (B) MASP-3 activity on VPR-AMC was calculated by subtraction of the activity of zymogen MASP-3 (MASP-3 and VPR-AMC only) from its corresponding activity in presence of MASP-2. (C) Data from A were fitted by linear regression to the Michaelis-Menten equation. (D) V_i was calculated from A and the values plotted against MASP-3 concentrations.

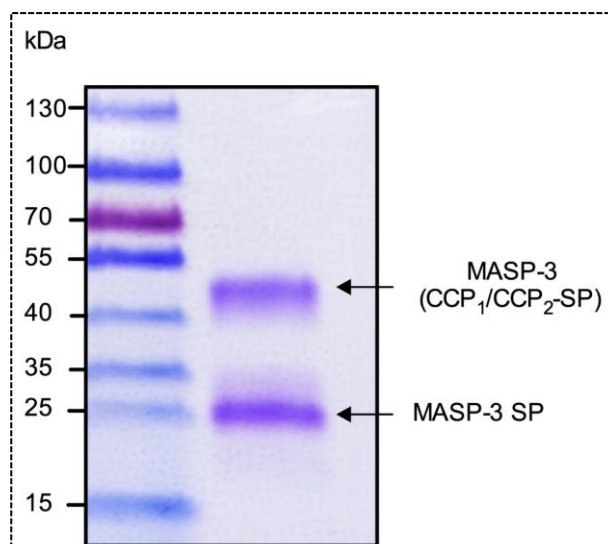


Figure 4-14: SDS-PAGE analysis of recombinant human MASP-3 (CCP1-CCP2-SP)

Five micrograms of recombinant human MASP-3 were reduced and separated in 4–20% Mini-protein® TGX™ gel (Bio Rad). Gel was stained with Coomassie Blue. The recombinant protein shows a band of approximately 50 kDa, CCP1-CCP2-SP domains and another band of approximately 25 kDa, SP domain.

4.2.4: MASP-3 devoid proteolytic activity on FH and C5

The proteolytic activity of MASP-3 catalytic domains (CCP₁-CCP₂-SP) was examined on complement components FH and C5 and on C3 (See chapter three, figure 3-3). The reason for choosing FH to be tested as a natural substrate for MASP-3 is that FH is a major down-regulator of the alternative pathway and MASP-3 might influence the alternative pathway by interfering with a regulator.

FH and C5 were incubated with human recombinant MASP-3 (CCP₁-CCP₂-SP) in presence or absence of the MASP-3 natural activators, MASP-1 and/or MASP-2. The SDS-PAGE analysis shows the single band of FH with no indication of degradation products (figure 4-15A, lanes 1-6). For C5, the alpha and beta chains can be seen at approximately 115 and 70 kDa, respectively. Again with no appearance of degradation products (figure 4-15B, lanes 1-6) indicating that MASP-3 has no enzymatic activity towards FH or C5. It is also

shown in the figure that MASP-1 (CCP₁-CCP₂-SP) and MASP-2 (CCP₁-CCP₂-SP) also do not cleave FH and C5.

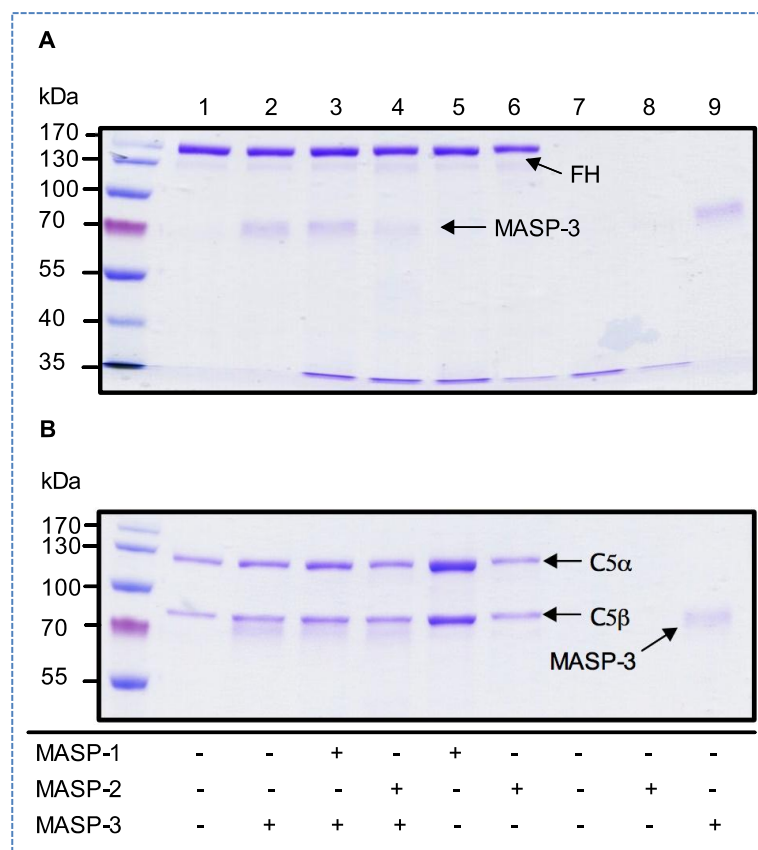


Figure 4-15: SDS-PAGE analysis of MASP-3 activity on FH and C5

Purified human factor H (A) or purified human C5 (B) at 100 µg/mL each were incubated with catalytic fragments (CCP₁-CCP₂-SP) of MASP-3 (25 µg/mL) in presence or absence of 25 µg/mL of MASP-1 (CCP₁-CCP₂-SP) or MASP-2 (CCP₁-CCP₂-SP) for 1 hour. Proteins were separated in 10% SDS polyacrylamide gel and gel was stained with Coomassie Blue. FH shows a single band of 155 kDa (A, lane 1-6) and C5 shows two bands of 115 kDa and 70 kDa (B, lanes 1-6). Recombinant MASP-3 alone is shown in lane 9 on each gel. The figure shows a typical experiment of two independent experiments carried out.

4.2.5: FB, FD, and MASP-3 are essential for effective activation of the alternative pathway

The enzymatic activity of the catalytically active MASP-3 on zymogen FB and pro-FD was investigated by incubating FBdpl (Factor B-depleted) serum or FDdpl (Factor D-depleted) serum or MASP-1/-3 knockout mouse serum on zymosan coated microtiter plates, with or without addition of purified human FB, recombinant human pro-FD, MASP-1 (CCP₁-CCP₂-SP) or MASP-3 (CCP₁-CCP₂-SP) and measuring C3b deposition on the zymosan. The lack of FD or FB leads to complete inhibition of the alternative pathway activity (i.e. complete inhibition of C3b deposition) since the alternative pathway activation is initiated by activated FD that cleaves FB in the C3bB complex resulting in C3bBb (C3 convertase) formation and then Bb in C3bBb cleaves C3 to C3b that binds to the zymosan surface. FBdpl serum shows no alternative pathway activity (figure 4-16, A and B) but that can be restored when FB is added back to the FBdpl serum. To assay potential direct proteolytic activity of MASP-3 towards FB, FB was mixed with MASP-3 (CCP₁-CCP₂-SP) and the mixture was then added to the FBdpl serum. As can be seen in figure 4-16, addition of the pre-incubated FB/MASP-3 mixture makes no difference in alternative pathway activity compared to the addition of FB alone. This indicates that MASP-3 does not cleave FB (and inactivate it) during pre-incubation, nor does it accelerate the conversion of C3bB to C3bBb. Therefore MASP-3 has no direct activity on zymogen FB.

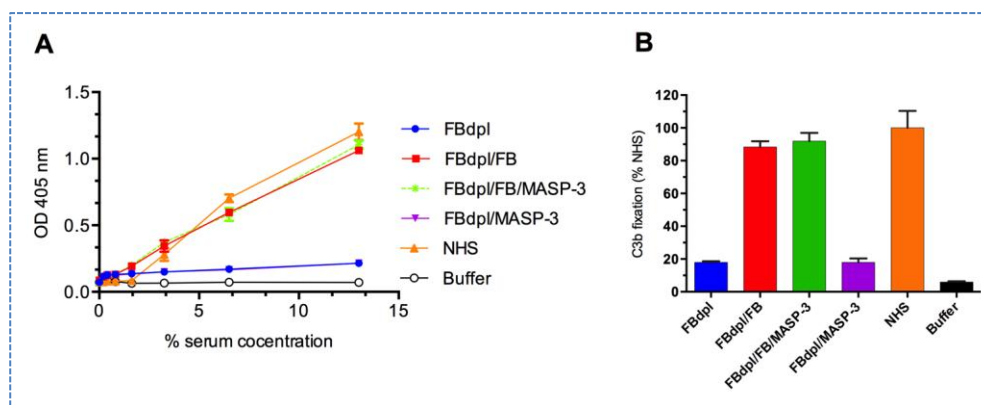


Figure 4-16: The alternative pathway of complement activation in FB depleted serum

(A) Microtiter ELISA plate was coated with zymosan (1 μ g /well). FB depleted serum (FBdpl) was reconstituted with purified human FB (2 mg/mL final concentration in the undiluted serum) in presence or absence of recombinant human MASP-3 (CCP1-CCP2-SP) (6 μ g/mL final concentration in the undiluted serum). Serum was diluted in GVB-Mg²⁺-EGTA buffer and incubated in the zymosan-coated wells. C3b fixation was probed by rabbit anti-human C3c. (B) The percentage of C3 activation was determined by plotting the highest C3 deposition against C3 deposition in normal human serum. This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

Table 4-1: Statistical significance difference (t-test) in alternative pathway activation between FBdpl and NHS

	Means diff.	Sign.? P<0.05	P value
FBdpl vs NHS	0.182	Yes	<0.0001
FBdpl vs FBdpl+FB	0.191	Yes	<0.0001
FBdpl vs FBdpl+M3	-0.002	No	0.940
FBdpl+FB+M3 vs FBdpl+FB	0.006	No	0.920
NHS vs FBdpl+FB	-0.008	No	0.608

FDdpl displays complete deficiency in the alternative pathway (figure 4-17, A and B) and when MASP-3 (CCP₁-CCP₂-SP) added to this serum could not restore the alternative pathway activity but when the serum was reconstituted with recombinant human pro-FD there is a partial restoration in the alternative pathway activity and the reason for that may be that the endogenous MASP-3 (CCP₁-CCP₂-SP) activates pro-FD. The pre-incubation of pro-FD with catalytically active MASP-3 shows a complete restoration of the activity of alternative pathway (figure 4-17) and these results prove that MASP-3 acts with pro-FD.

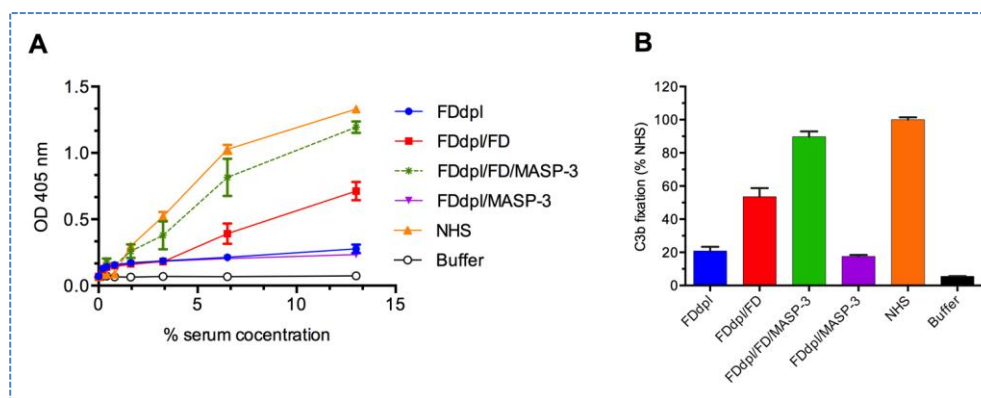


Figure 4-17: The alternative pathway of complement activation in FD depleted serum

(A) Microtiter ELISA plate was coated with zymosan (10 $\mu\text{g}/\text{mL}$). FD depleted serum (FDdpl) was reconstituted with recombinant human pro-FD (2 $\mu\text{g}/\text{mL}$ final concentration in the undiluted serum) in presence or absence of recombinant human MASP-3 (CCP1-CCP2-SP) (6 $\mu\text{g}/\text{mL}$ final concentration in the undiluted serum). Serum was diluted in GVB-Mg²⁺-EGTA buffer and incubated in the zymosan-coated wells. C3b fixation was probed by rabbit anti-human C3c. (B) The percentage of C3 activation was determined by plotting the highest C3 deposition against C3 deposition in normal human serum. This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

Table 4-2: Statistical significance difference (t-test) in alternative pathway activation between FDdpl and NHS

	Means diff.	Sign.? P<0.05	P value
FDdpl vs NHS	0.268	Yes	<0.0001
FDdpl vs FDdpl+FD	0.072	Yes	<0.0039
FDdpl vs FDdpl+MASP-3	-0.011	No	0.671
FDdpl+FD vs FDdpl+FD+M3	0.155	No	0.118
NHS vs FDdpl+FD	0.195	Yes	0.042

MASP-1/-3 KO serum displays a complete defect in the alternative pathway (figure 4-18, A and B). whereas MASP-2 KO mouse serum shows some defect in C3 fixation on zymosan coated surface (figure 4-18). The reason for that is probably that MASP-2 is an essential initiator of MASP-3 (see figures 4-9 and 4-13). C1q KO mouse serum displays higher C3 deposition exceeded that wild type control serum and that is probably due to higher expression of the alternative pathway components to compensate the deficiency of the classical pathway.

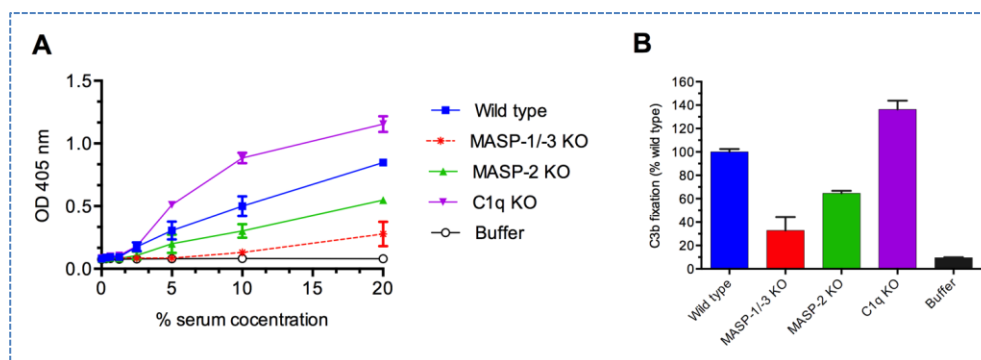


Figure 4-18: The alternative pathway of complement activation in MASP-1/-3 KO, C1q KO and MASP-2 KO mouse sera

(A) The alternative pathway activity was tested in mouse serum with targeted complement defect in C1q, or MASP-1/-3 or MASP-2. Sera were diluted in GVB-Mg²⁺-EGTA buffer and incubated in the zymosan-coated wells. C3b fixation was probed by rabbit anti-human C3c. (B) The percentage of C3 activation was determined by plotting the highest C3 deposition against C3 deposition in wild type mouse serum. This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

Table 4-3: Statistical significance difference (t-test) in alternative pathway activation between wild type vs MASP-1/-3 KO, MASP-2 KO and C1q KO mouse serum

	Means diff.	Sign.? P<0.05	P value
M1/3KO vs WT	-0.157	Yes	<0.0015
M2KO vs WT	-0.088	No	0.212
C1qKO vs WT	0.115	No	0.282

In the other hand, MASP-1/-3 KO mouse serum reconstituted with recombinant human MASP-3 (CCP₁-CCP₂-SP), the alternative pathway activity is restored to the same level as in the control wild type serum (figure 4-19, A and B). Interestingly, addition of catalytically active MASP-1 (CCP₁-CCP₂-SP) to MASP-1/-3 KO serum has no effect on the alternative pathway activity but when MASP-1 and MASP-3 are added together to the serum deficient in MASP-1/-3, the pathway activity exceeded the wild type activity (figure 4-19). The reason for this is likely to be that the MASP-3 activation in MASP-1/-3 KO serum will be initiated by endogenous MASP-2 and addition of the exogenous MASP-1 will enhance MASP-3 activation as both proteins showed proteolytic activity towards MASP-3 (figures 4-8 and 4-9).

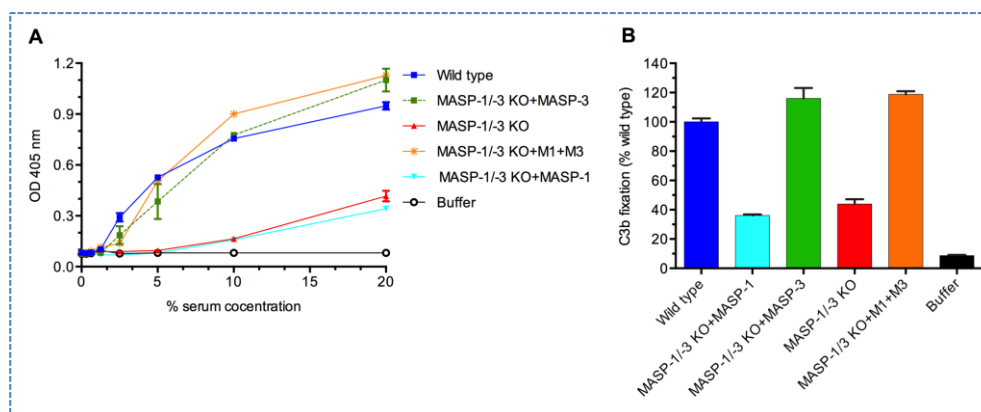


Figure 4-19: The alternative pathway of complement activation in MASP-1/-3 KO sera reconstituted with recombinant MASP-3

(A) Catalytic fragments (CCP1-CCP2-SP) of recombinant human MASP-1 or recombinant human MASP-3 (10 $\mu\text{g}/\text{mL}$ final concentration in the undiluted serum) or both were added to MASP-1/-3 KO mouse serum and serum was diluted in alternative pathway GVB- Mg^{2+} -EGTA buffer and incubated in the zymosan-coated wells. C3b fixation was probed by rabbit anti-human C3c. (B) The percentage of C3 activation was determined by plotting the highest C3 deposition against C3 deposition in wild type mouse serum. This is a typical example representing a series of identical experiments. Data are the means \pm SEM from two independent experiments carried out.

Table 4-4: Statistical significance difference (t-test) between MASP-1/-3 KO mouse serum and wild type serum in alternative pathway activation

	Means diff.	Sign.? $P < 0.05$	P value
M1/3KO vs WT	-0.222	Yes	<0.01
M1/3KO vs M1/3KO +M3	-0.209	Yes	<0.005
M1/3KO vs M1/3KO +M1	-0.018	No	0.611
M1/3KO vs M1/3KO +M1+M3	0.243	Yes	0.003

4.2.6: Effective alternative pathway activation in vitro requires

MASP-3 driven pro-FD activation

To turn over the alternative pathway, C3b or C3(H₂O) is required to bind FB in presence of Mg²⁺ and then the activated FD cleaves FB in C3bB complex producing C3bBb (the alternative pathway C3 convertase).

The alternative pathway protein-protein interactions are shown in figure 4-20. The SDS-PAGE analysis revealed that in the presence of C3b, MASP-3 and FD there is a considerable FB conversion into active form (Bb) (figure 4-17, lane 1). When the C3b is removed from the system, no FB conversion is seen (figure 4-20, lane 2) since FB needs to bind to C3b to be cleaved by FD (Kam et al. 1987). The absence of MASP-3 leads to less conversion of FB to its active form (figure 4-20, lane 3, compared to lane 1) as FD used in this study is not fully activated. However, incubation of purified human FB with purified human C3b (figure 4-20, lane 5) led to unexpected activation of zymogen FB into its structural fragments Bb and Ba. Several different batches of purified human FB and purified human C3 were used and all showed FB activation in presence of C3 by unknown protease/mechanism (See appendices B and C). The SDS-PAGE of human FB showed that purified human FB contains three protein bands with apparent molecular weights of about 100 kDa, 60 kDa, and 50 kDa (figure 4-21). The 100 kDa band was found to be zymogen FB as confirmed with Western blotting analysis (see chapter three figure 3-4) and the amino acid sequences revealed that 60 kDa and 50 kDa bands were complement FB (see appendix D). This activation of zymogen FB in presence of C3b could probably due to contamination with trace amount of FD in FB preparation or C3b preparations. Another possibility is that activated FB, Bb might act as an enzyme to activate its zymogen form. However, the addition of recombinant human pro-FD alone to FB/C3b mixture shows no further activation for FB (figure 4-20, lane 3) and this indicates that there is no further activation for pro-FD in absence of MASP-3. Same result was obtained when MASP-3 alone was added to FB/C3b mixture (figure 4-20, lane 4).

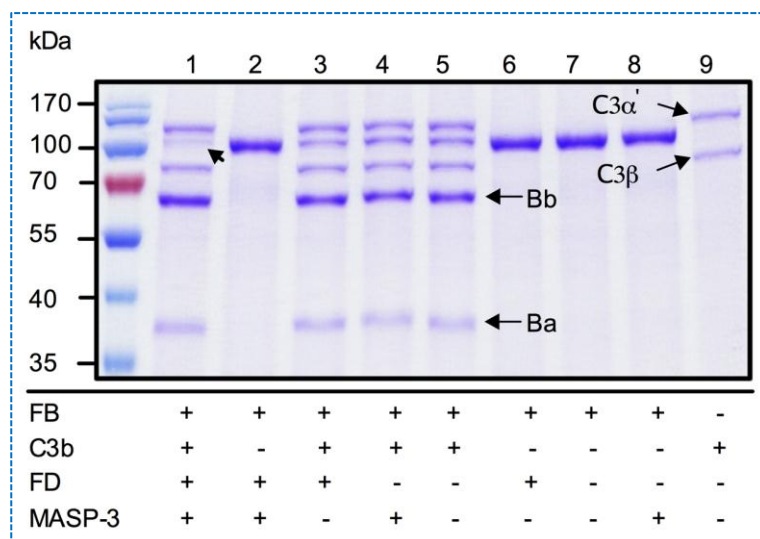


Figure 4-20: SDS-PAGE analysis shows alternative pathway protein-protein interactions

An equal amount of purified human C3b and purified human FB (each of which at 100 µg/mL) were incubated with MASP-3 (CCP1-CCP2-SP) and pro-FD in GVB⁺⁺ at 37°C and the proteins were then separated in 10% SDS polyacrylamide gel and the gel stained with Coomassie Blue. The figure shows a typical experiment of two independent experiments carried out.

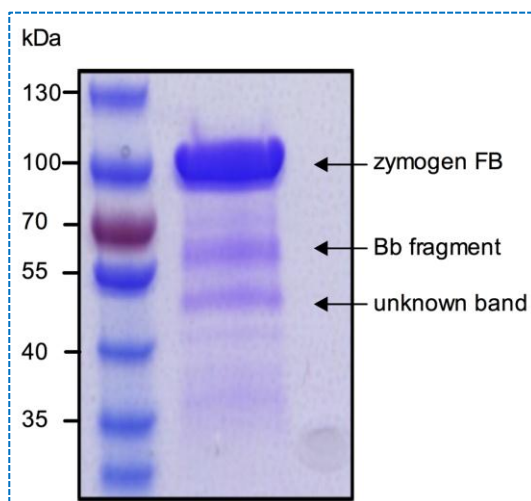


Figure 4-21: SDS-PAGE analysis of human FB preparation

Three micrograms of purified human FB were reduced and separated in 10% polyacrylamide gel and gel was stained with Coomassie Blue. The purified zymogen FB shows a band of approximately 100 kDa and its larger Bb fragment has an apparent molecular weight of approximately 60 kDa. The FB degraded product occurring at about 45 kDa.

4.2.7: MASP-3 efficiently activates pro-FD

The function of FD is to activate FB in C3bB complex. The ability of the catalytic fragments of MASP-3 (CCP₁-CCP₂-SP) to specifically cleave pro-FD was investigated. The assay was performed in presence of various concentrations of MASP-3 starting at a maximum enzyme substrate ratio of 1:10, MASP-3:pro-FD (w/w). The catalytically active MASP-3 cleaves pro-FD at all MASP-3 concentrations (figure 4-22A, lanes 1-4) and 50% pro-FD conversion was achieved by MASP-3 concentration of 3.7 $\mu\text{g/mL}$ (ratio of 1:30) (figure 4-22B).

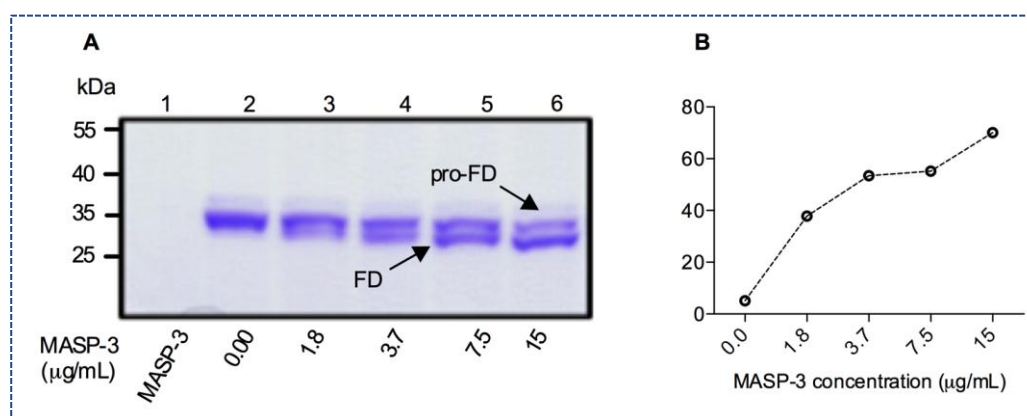


Figure 4-22: Pro-FD conversion to active FD

Various concentrations of catalytically active MASP-3 (CCP₁-CCP₂-SP) were incubated with recombinant human pro-FD in enzyme:substrate starting at maximum ratio of 1:10, MASP-3:pro-FD (0.3 μg :3 μg , w/w) at 37°C for 4 hours. Proteins were separated in a Novex 4-20% Tris-Glycin gel (Life Technologies) and stained with Coomassie Blue (A). Active FD was formed at all MASP-3 concentrations used (A, lanes 1-4). (B) The intensities of FD bands were determined by densitometry and the percentage activation calculated and plotted against MASP-3 concentrations. The figure shows typical experiment of two independent experiments carried out.

4.2.8: MASP-3 reconstitution restores alternative pathway activation in MASP-1/-3 KO mouse serum

The third complement component, C3, is the most abundant complement protein in serum with a median serum concentration of 1.3 mg/mL. Native C3 is composed of two chains, C3 α (110 kDa) and C3 β (75 kDa). Upon activation by C3 convertase, C3b will be generated by releasing C3a (9 kDa) from C3 α chain. C3b undergoes sequential cleavage steps by FI in the presence of cofactors. The first cleavage step is to release C3f (2 kDa) and generate iC3b (174 kDa). iC3b is further cleaved by FI with help of FH into C3dg (37 kDa) and C3c (137 kDa). If the C3b being degraded was originally bound to a surface, C3dg will remain bound and C3c is released into the microenvironment (Perkins and Sim 1986).

An experiment was performed to analyze C3 breakdown products in MASP-1/-3 KO serum in the alternative pathway in presence of heat killed *S. pneumoniae*. C3 breakdown products were analyzed by Western blotting of reduced SDS-PAGE using anti-C3c antibodies (which do not detect C3dg). C3b breakdown to iC3b produces the C3 alpha1 fragment (see figure 4-23) which co-runs on SDS-PAGE (reduced) with C3 beta chain. Further breakdown to C3c and C3dg shows the appearance of the C3c 41kDa alpha chain fragment. MASP-1/-3 KO mouse serum showed little C3 breakdown: the C3 alpha and beta chains are visible and there may be some C3 alpha 1 fragment (figure 4-23A, lane 2). When recombinant full-length mouse MASP-3 was added to the serum (6 μ g/mL final concentration in the undiluted serum), there was much more C3 breakdown, producing the C3c 41 kDa fragment (figure 4-24A, lane 3), comparable to breakdown in the wild type serum (lane 1). Increasing C3c 41 kDa generation was seen as more MASP-3 was added to the KO serum (figure 4-24B, lanes 3-5)

An alternative method was used to assay C3 breakdown in MASP-1/-3 KO mouse serum by measuring C3 bound on the surface of *S. pneumoniae*. MASP-1/-3 KO serum showed lack of C3 deposition on the bacterial surface and when the serum was reconstituted with 6 μ g/mL recombinant full-length mouse

MASP-3, the deposited C3 rises to be more than in wild type serum (figure 4-24C). However, more MASP-3 addition resulted in more C3 deposition (figure 4-24D). This result suggests that MASP-1/-3 KO mice have a high level of pro-FD in the serum and the addition of MASP-3 will convert it to its active form which accelerates the alternative pathway activity. More MASP-3 added to MASP-1/-3 KO serum will accelerate pro-FD conversion.

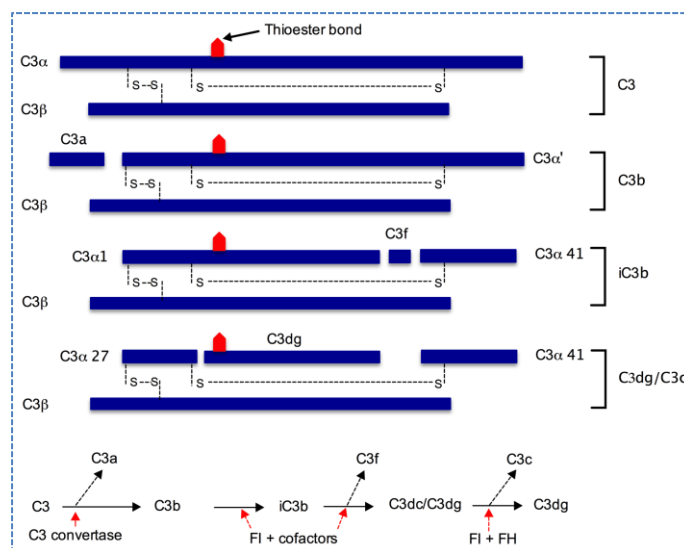


Figure 4-23: Schematic representation of C3 breakdown

Native C3 (185 kDa) is cleaved by C3 convertase into C3b (176 kDa) and C3a (9 kDa). C3b undergoes cleavage by FI and cofactors to generate iC3b (174 kDa) and release C3f (2 kDa). iC3b is further cleaved by FI and FH to generate C3dg (37 kDa) and C3c (137 kDa) (Sim et al. 1981).

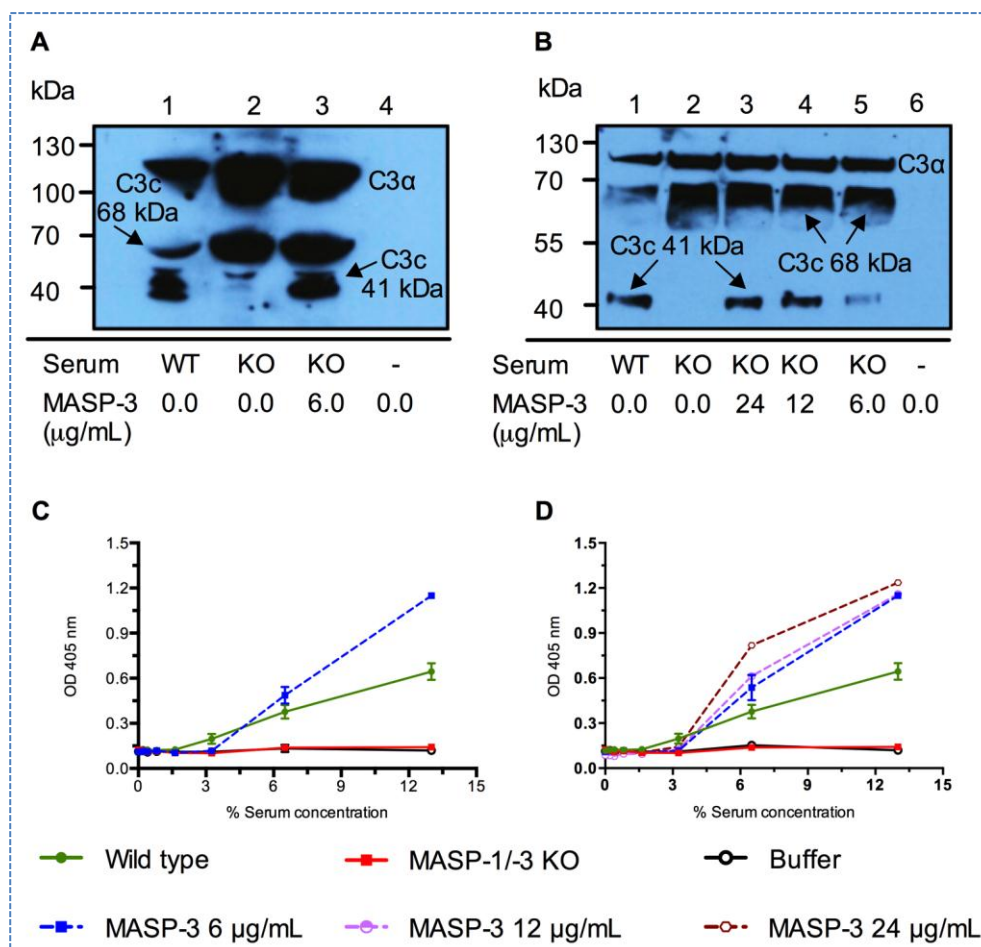


Figure 4-24: Alternative pathway activation assay in MASP-1/-3 KO mouse serum showing C3 breakdown products in the fluid phase and C3 deposited on the surface of *S. pneumoniae*

2×10^8 heat killed *S. pneumoniae* were added to 100 μ L of GVB-Mg²⁺-EGTA buffer containing 10% wild type, MASP-1/-3 KO mouse serum or MASP-1/-3 KO serum reconstituted with 6 μ g/mL of the undiluted serum of full-length mouse MASP-3 (A) or with increasing concentrations of recombinant full-length mouse MASP-3 starting at minimum concentration of 6 μ g/mL of the undiluted serum (B). After incubation at 37°C for 1 hour, bacteria were spun down and the supernatant was boiled in protein loading buffer and separated in 10% SDS polyacrylamide gel and transferred onto nitrocellulose membrane. C3 fluid phase products were visualized by polyclonal rabbit anti-human C3c antibodies. The figure shows typical experiment of three independent experiments carried out. For C and D, 130 μ L of MASP-1/-3 KO mouse serum was reconstituted with recombinant full-length mouse MASP-3 (6 μ g/mL final concentration of the undiluted serum) (C) or with recombinant full-length mouse MASP-3 starting at minimum concentration of 6 μ g/mL of the undiluted serum (D). Serum was diluted in GVB-Mg²⁺-EGTA buffer to a final volume of 1 mL. 100 μ L/well was added to microtiter plate wells coated with formalin fixed *S. pneumoniae*. C3 deposited was probed with polyclonal rabbit anti-human C3c.

4.2.9: Recombinant full-length mouse MASP-3 effectively restores

defective alternative pathway activation in MASP-1/-3 KO mice

A complete understanding of the role of the MASP-3 in initiation or turnover of the alternative pathway requires an animal model that allows complement proteins to interact with each other. The route of MASP-3 maturation *in vivo* was studied by injecting MASP-1/-3 KO mice with 20 µg/mouse of recombinant full-length mouse MASP-3. Since MASP-3 injected is a very small amount and it is difficult to detect the recombinant protein in mouse blood by Western blotting, measuring the alternative pathway activity was used as an indicator of the MASP-3 availability/activity.

MASP-3 enzymatic activity starts very weakly at 24 hours post *in vivo* reconstitution (figure 4-25A) and was improved at 48 hours (figure 4-25B) and after 72 hours post reconstitutions the alternative pathway functional activity is fully restored (figure 4-25, C and D)

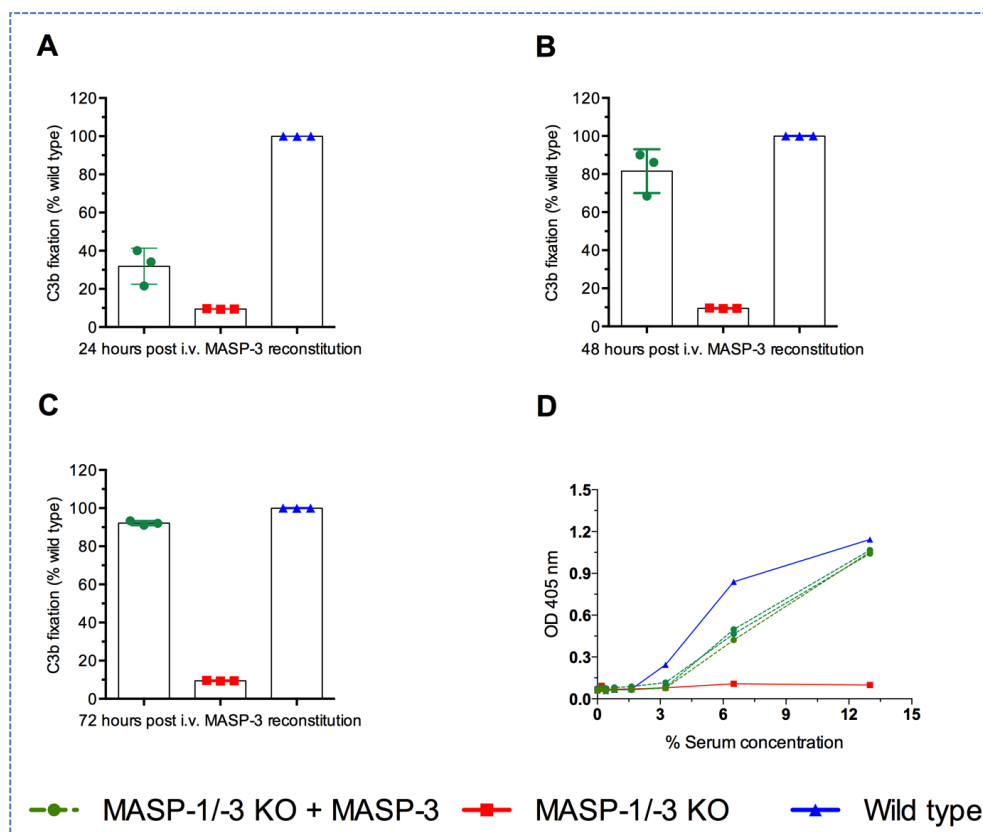


Figure 4-25: Reconstitution of C3 deposition in MASP-1/-3 KO mouse serum with recombinant mouse MASP-3

MASP1/3 KO mice, on C57BL/6 background, were given 20 µg/mouse of recombinant full-length mouse MASP-3 by i.v. injection. Mice were bled 24, 48 and 72 hours post injection and sera were tested for alternative pathway functional activity. Alternative pathway functional activity was determined by measuring the deposition of C3 activation products in an alternative pathway specific ELISA using 13% of mouse serum collected from wild type, MASP-1/-3 KO mice and MASP-1/-3 KO mice after reconstitution with MASP-3 at several time points on zymosan coated ELISA plate in GVB-Mg²⁺-EGTA buffer. The recombinant MASP-3 gradually restores the defective alternative pathway in MASP-1/-3 KO mice approaching wild type levels of alternative pathway functional activity in sera 24, 48 and 72 hours after reconstitution (see A-C). Figure D shows the effect of MASP-3 reconstitution in serum of MASP-3 deficient mice 72 hours after reconstitution as a function of serum dilution.

**4.2.10: *MASP-1/-3 KO mice are more susceptible to S.
pneumoniae infection***

The role of the MASP-3 in the protection against *S. pneumoniae* infection was studied in MASP-1/-3 KO mice. Five mice each from wild type and MASP-1/-3 KO were infected with 1.5×10^5 CFU of *S. pneumoniae* intranasally. One deficient mouse showed severe symptoms and was euthanized 24 hours post infection. In the next 24 hours two mice each from wild type and MASP-1/-3 KO were euthanized. The experiment was followed for 7 days and the mortality of MASP-1/-3 KO mice was 80% compared with 20% of their wild type littermates (figure 4-26A).

Bacteremia was measured 24 hours post-infection and at the time of death. 80% of MASP-1/-3 KO mice showed bacteria in the blood after 24 hours of infection compared with 20% of wild type mice. MASP-1/-3 KO mice showed higher bacterial burden in blood at 24 hours and at the time of death compared to their wild type littermates (figure 4-26, B and D).

CFU in the lung was measured at time of death (figure 4-26D). As in bacteremia, MASP-1/-3 KO mice showed significantly higher bacterial burden in lungs compare to their wild type control group (figure 4-26C).

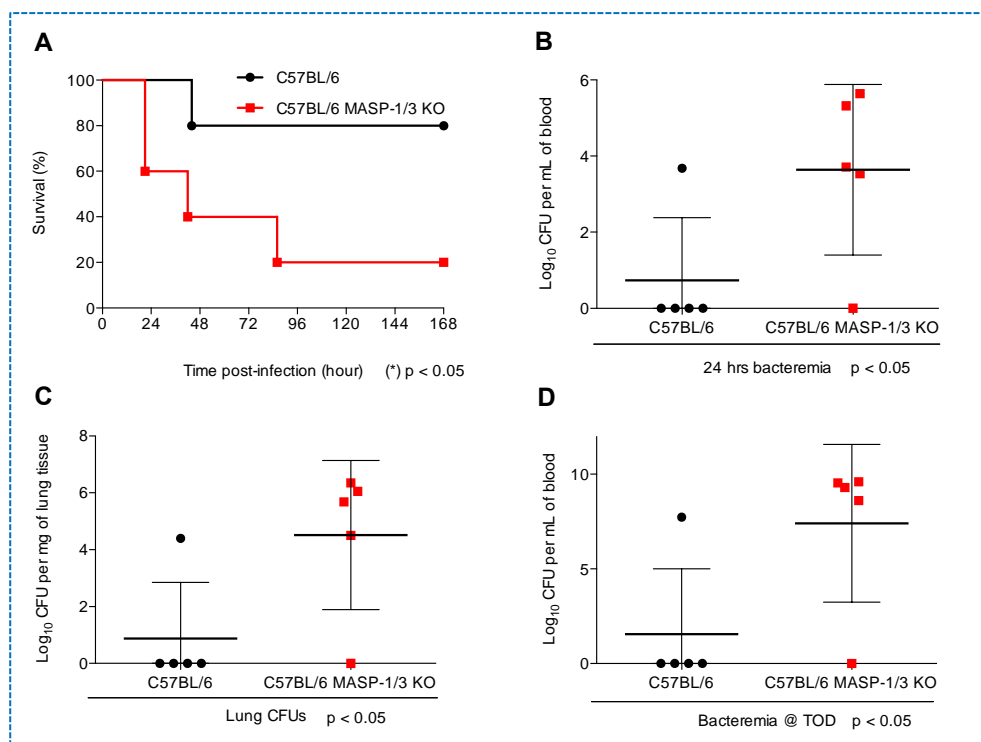


Figure 4-26: Infection of MASP-1/-3 KO mice with *S. pneumoniae*

MASP-1/-3 KO mice were infected intranasally with 1.5×10^5 CFU of *S. pneumoniae* in 50 μL of PBS. Mice were followed for 7 days after infection. (A) A survival curve of MASP-1/-3 KO group and their wild type littermates shows 80% of wild type mice and 20% of the deficient mice survived. Blood was collected from the infected mice and viable count was calculated at 24 hours of infection and at the time of death. The deficient mice developed bacteremia 24 hours post infection (B) and at time of death (D). The viable count in the lungs of infected mice was calculated by plating tissue homogenate into blood agar plates. The deficient mice showed a very high bacterial burden in lung tissues compared to wild type mice (C) * $P < 0.05$ vs. wild type.

4.3: Discussion

The alternative pathway of complement maintains a continuous state of activation of complement and is initiated through hydrolysis of component C3 at low rate (Ricklin et al. 2010). FD is an essential component of the alternative pathway amplification loop and exhibits a unique enzymatic activity against its unique natural substrate zymogen C3b-bound FB (Kam et al. 1987). Early studies on the alternative pathway of complement demonstrated the necessity of C3(H₂O) or C3b to bind to the zymogen FB to be cleaved by FD to convert C3bB complexes into their enzymatically active form through a cleavage and releasing of the 30 kDa N-terminal Ba fragment to form the alternative pathway C3 convertase C3bBb. FD does not cleave the 90 kDa zymogen serine protease FB in serum unless FB is bound to either C3b or C3(H₂O) (Kam et al. 1987, Xu et al. 2001, Milder et al. 2007, Forneris et al. 2010). The binding of FB to C3b or C3(H₂O) only occurs in the presence of metal ions such as Mg²⁺ or Ni²⁺ (Hourcade and Mitchell 2011). Once FB has bound to C3b, FD cleaves FB in this complex into two unequal molecular weight fragments. The larger Bb remains in association with C3b to generate C3bBb and the smaller fragment, Ba, which dissociates into the fluid phase (Mole et al. 1984, Williams et al. 1999). Some plasma serine proteases such as kalikrin and plasmin showed enzymatic activities on zymogen FB but it seems that their activity is physiologically irrelevant (Taylor et al. 1999, Xu et al. 2001) since neither of these serine proteases is capable to initiate the alternative pathway functional activity in mice deficient of FD.

The complement component FD is synthesized as a zymogen by adipocytes and once secreted, pro-FD is activated by an unknown mechanism and circulated in the blood in its enzymatic active form (Yamauchi et al. 1994, Xu et al. 2001). It was, however, generally accepted that pro-FD may be activated by an autocatalytic cleavage, a process that may take place before secretion (Kam et al. 1987). Later research of the same group, however, produced recombinant FD expressed in baculovirus expression system. The recombinant FD was released

in its zymogen form and devoid of any enzymatic activity towards FB unless it was activated by trypsin digestion (Yamauchi et al. 1994). Despite that, this observation indicated the need to search for the physiologically relevant activation mechanism to convert pro-FD into enzymatically active form, the general opinion prevailed that FD is available in the circulation in its active form. This viewpoint was again challenged when Teizo Fujita's team observed that their MASP-1/-3 deficient mouse line was totally deficient of the alternative pathway functional activity (Takahashi et al. 2010). They demonstrated convincingly that the lack of alternative pathway functional activity in MASP-1/-3 deficient mice was due to the absence of active FD in this mouse serum with FD circulating exclusively in its zymogen form. Since the targeting construct used to establish a dysfunctional *MASP1* gene disrupted the coding sequence of the mRNA transcript encoding MASP-1 as well as the coding sequence of the mRNA transcript encoding MASP-3, it remained unclear whether or not the lack of FD zymogen cleavage was due to the absence of MASP-1 or of MASP-3. An *in vitro* assay incubating recombinant MASP-1 with recombinant pro-FD indicated that MASP-1 can convert pro-FD into its enzymatically active form (Takahashi et al. 2010). Reconstitution of MASP-1/-3 deficient mice, however, with recombinant MASP-1 failed to restore deficient alternative pathway functional activity in serum (see figure 4-19).

MASP-1 is a trypsin-like serine protease that has a broad range of substrates including C2, hydrolyzed C3, MASP-2, and MASP-3 (Thiel et al. 1997, Moller-Kristensen et al. 2007, Takahashi et al. 2008, Heja et al. 2012, Megyeri et al. 2014), variety of non-complement substrates, such as fibrinogen and factor XIII (Hajela et al. 2002, Krarup et al. 2008, Megyeri et al. 2009) also MASP-1 cleaves several synthetic substrates such as FGR-AMC, VPR-AMC, FSR-AMC, QGR-AMC, GGR-AMC, VLK-AMC, and LGR-AMC (Presanis et al. 2004). My work assessed the possible role of pro-FD cleavage by MASP-3 under physiological conditions. We wanted to know whether recombinant MASP-3 can restore the

deficient alternative pathway functional activity in MASP-1/-3 deficient mice *in vivo* and *in vitro*.

Human and mouse MASP-3 was expressed in the Chinese Hamster Ovary Cell line (CHO-K1) and purified through its histidine-tag by nickel column chromatography. The characteristics of the recombinant MASP-3 proteins were studied under reducing and non-reducing conditions. The recombinant human and mouse MASP-3 were glycosylated, running as a single band of 115 kDa in molecular weight [N.B. the calculated molecular weight for both is approximately 81 kDa, derived from the amino acid sequence of MASP-3 alone (Dahl et al. 2001)]. The mature human zymogen MASP-3 is a 728 amino acid long polypeptide which exhibits seven N-glycosylation sites and, upon activation is cleaved into a disulphide bridge linked heavy chain and light chain. Of the seven N-glycosylation sites, three are located on the heavy chain (Dahl et al., 2001). Each N-linked glycosylation site adds approximately 2.5 kDa to the molecular weight of the protein (Kornfeld and Kornfeld 1985).

Previous studies have indicated that MASP-3 is not a self-activating enzyme and that the recombinant protein was constantly purified in its pro-enzymatic form (Dahl et al. 2001, Zundel et al. 2004, Skjoedt et al. 2010b, Gaboriaud et al. 2013). This is in contrast to MASP-1 and MASP-2 which are easily activated by autocatalytic cleavage during the purification process (Vorup-Jensen et al. 2000, Thielens et al. 2001, Cseh et al. 2002, Iwaki and Fujita 2005).

First, I tested whether recombinant human MASP-3 can be activated by trypsin cleavage. A typical cleavage site for trypsin is located behind an arginine or lysine residue (i.e. Arg-|- Xaa or Lys-|- Xaa) and according to the derived amino acid sequence, human MASP-3 contains 60 potential cleavage sites for trypsin equally distributed on both chains (GenBank entry number AAK84071.1). Surprisingly, my results showed that MASP-3 is resistant to trypsin proteolytic activity. A possible reason for that is that MASP-3 is a highly

folded protein which offers limited access for trypsin to its potential cleavage sites.

In analogy to what was known about MASP-1 and MASP-2 at the time of MASP-3 discovery, it was anticipated that MASP-3 is also an autoactivating enzyme and there was a report showing that MASP-3 can be converted from its pro-enzyme form into its active form simply by incubating MASP-3 at 4°C for a 4 weeks (Zundel et al. 2004). However, a subsequent study using an identical recombinant protein could not reproduce this experiment suggesting that that this conversion was caused through a trace contamination with an unidentified protease in the cell culture supernatant (Cortesio and Jiang 2006).

In this study, human and mouse MASP-3 were expressed as single chain full-length proteins. These pro-enzyme forms of MASP-3 were resistant to auto-activation. In order to study MASP-3 activation process, the proteolysis activity of several serine proteases was tested on MASP-3. The conversion of MASP-3 to its enzymatically active form was later shown to be achieved by incubating MASP-3 with the recombinant catalytic fragments (CCP₁-CCP₂-SP) of MASP-1 (figure 4-8) as well as of MASP-2 (figure 4-9) while plasmin and thrombin failed to cleave zymogen MASP-3 (figures 4-11 and 4-12). Iwaki et al. also demonstrated that MASP-3 is cleaved and activated by MASP-1 (Iwaki et al. 2011). The physiological processes involved in MASPs activation in the serum are still a matter of debate, as some authors claim that within a single lectin pathway activation complex, dimers of MASP-1 or MASP-2 can interact with each other with MASP-1 cleaving MASP-2 or MASP-1, cleaving MASP-3 (Gal et al. 2005). From the stoichiometry of lectin pathway activation complexes, it is highly unlikely that one single activation complex can contain more than one serine protease dimer (Wallis et al. 2010). Other authors claim that MASPs can activate each other by forming heterocomplexes i.e. MBL(MASP-1:MASP-2) or ficolin (MASP-1:MASP-2) allowing a transactivation of these enzymes within the same complex (Degn et al. 2013).

My work aimed to define to what extent the enzymatic activity of MASP-2 towards MASP-3 is physiologically significant. To study this, I used a synthetic substrate for MASP-3, VPR-AMC to quantify the physiological activity of MASP-3 following activation by MASP-2. The activation of zymogen MASP-3 by MASP-2 was monitored by the release of AMC from the MASP-3 substrate VPR-AMC. In absence of MASP-2, MASP-3 was devoid of any enzymatic activity towards VPR-AMC. The MASP-3 serine protease domains alone exhibits enzymatic activity against several synthetic substrates, including FGR-AMC (a substrate of tissue plasmin activator), PFR-AMC (a substrate of plasmin and kallikreins), and VPR-AMC (a substrate of thrombin, factor XIII, kallikerins) (Gaboriaud et al. 2013).

Interestingly, as shown in figure (4-13), higher concentrations of my recombinant zymogen MASP-3 (exposed in absence MASP-2 to the VPR-AMC substrate) showed a weak activity towards VPR-AMC. This background activity is most likely caused by trace contaminations of my recombinant zymogen with a very small amount of possibly hydrolyzed active MASP-3 or by a residual activity of the zymogen towards VPR-AMC (see figure 4-14). MASP-2, however, has no enzymatic activity towards VPR-AMC (Presanis et al. 2004). The increase in fluorescence following VPR-AMC cleavage was therefore mediated through MASP-2 dependent activation of MASP-3.

Identifying a physiological substrate for MASP-3 within the process of complement activation has been a matter on great interest since the discovery of MASP-3. MASP-3 was shown to have no enzymatic activity towards C2, C3 and C4 (Dahl et al. 2001). Amongst other potential substrates, I studied whether MASP-3 and activated MASP-3 shows any proteolytic activity towards the complement components FH, C5 and C3. As shown in figure (3-3 and 4-15), MASP-3 showed no enzymatic activity towards these components, even after MASP-3 was activated in presence of MASP-1 or MASP-2. The same assay also showed that neither MASP-1 nor MASP-2 were able to cleave FH or C5. The inability of MASPs to cleave C5 is well in line with a previously published

report showing the lack of the enzymatic activity of MASP towards C5 (Matsushita and Fujita 1995).

The following part of my work addressed the involvement of MASP-3 in the activation of complement via the alternative pathway.

My results confirmed that the absence of the alternative pathway complement components FD or FB leads to a total loss of the alternative pathway functional activity since this pathway totally depends on the availability of enzymatically active FD to cleave FB within the C3bB complex in order to generate the alternative pathway C3 convertase C3bBb.

In both FB depleted serum and FD depleted serum, no alternative pathway functional activity was seen (see figures 4-16 and 4-17). Reconstitution of FB depleted human sera with purified human FB restored the alternative pathway functional activity. The addition of recombinant pro-FD to FDdpl increased C3 deposited on zymosan to some extent, probably because of the presence of endogenous MASP-3 in the FDdpl serum which can convert pro-FD into its active form. Incubation of zymogen FB with recombinant human MASP-3 had no influence on C3 fixation by the alternative pathway, while addition of MASP-3 to FDdpl serum in absence of proFD also failed to restore the alternative pathway functional activity indicating that MASP-3 has no direct activity on zymogen FB during pre-incubation, nor does it accelerate the conversion of C3bB to C3bBb. These results agree with the report by Matsushita and Fujita (1995) stating that MASPs have no enzymatic activity towards zymogen FB and contradict the proposal in Iwaki et al. (2011) that postulates that MASP-3 potentially cleaves and activates zymogen FB directly.

Pre-incubation of pro-FD with recombinant MASP-3 increased the C3 fixation on zymosan coated ELISA plates to the level seen in normal human serum. My *in vitro* results prove that MASP-3 is required to activate pro-FD into its enzymatically active form in serum.

Next, I analyzed the serum of MASP-1/-3 deficient mice to assess alternative pathway functional activity in absence of both MASP-1 and MASP-3. My results are in complete agreement with the previous studies by Teizo Fujita's research team (Takahashi et al. 2010, Iwaki et al. 2011) and show that the alternative pathway of complement activation is totally deficient in MASP-1/-3 deficient (figure 4-18). In order to assess whether this phenotype is either caused by the absence of MASP-1 or the absence of MASP-3 or the absence of both, I reconstituted MASP-1/-3 deficient mouse serum with an enzymatically active recombinant fragment of human MASP-3, composed of the three C-terminal domains CCP₁-CCP₂-SP. As shown in figure (4-19), addition of these MASP-3 fragments restored the alternative pathway functional activity in a concentration dependent manner.

Interestingly, MASP-3 reconstitution succeeded to restore alternative pathway activity in MASP-1/-3 deficient mouse serum, while the previous attempts by Teizo Fujita's team to restore the alternative pathway functional activity by reconstitution of MASP-1/-3 deficient serum failed to show this direct repair of alternative pathway activity following reconstitution of this serum with recombinant MASP-1 (Takahashi et al. 2010).

I further analyzed alternative pathway functional activity in mouse sera with targeted deficiencies in other complement components. My results showed that C3 deposition was higher in C1q deficient mouse serum than the wild type control serum. That is perhaps due to higher expression levels of lectin pathway or alternative pathway components to compensate the absence of classical pathway functional activity. The higher alternative pathway functional activity in absence of C1q may explain the predisposition of C1q deficient individuals to develop inflammatory pathologies together with the loss of C1q-mediated scavenger functions, such as the clearance of immune complexes.

In contrast, the lack of MASP-2 in MASP-2 deficient mouse serum showed a lower degree of C3 deposition via the alternative pathway. My results strongly support that MASP-2 plays a physiologically relevant role in the activation of

pro-enzyme MASP-3 as previously postulated by Teizo Fujita's group (Iwaki et al. 2011). It is very likely that MASP-2 plays a synergistic role in the MASP-1 mediated activation of MASP-3.

The cleavage and activation of zymogen FB was then analyzed using purified components under serum free conditions. The result showed a considerable conversion of zymogen FB into its active form (Bb) in presence of C3b, MASP-3 in conjunction with FD. In the absence of MASP-3, the conversion of zymogen FB by FD was dramatically reduced, indicating that a major part of FD is present as pro-FD which requires to be activated in a MASP-3 dependent fashion (see figure 4-20). My results also show that MASP-3 exhibits no direct proteolytic activity towards FB revealing that MASP-3 is devoid of any direct enzymatic activity towards zymogen FB. These results fully agree with my serum analysis (see figure 4-16).

Incubation of native purified C3 with purified serum FB alone resulted in some degree of C3 activation. This cleavage is pH dependent and the optimal pH for cleavage was determined to be greater than pH 7.0 (see appendix C). I have tried different batches of C3 (see appendix B) together with different batches of purified human FB and all showed a spontaneous degree of FB activation in the presence of C3. When the FB preparation was analyzed on SDS-PAGE there was a slight activation of FB in that preparation and in presence of C3(H₂O) in C3 preparation resulted C3 convertase formation (C3(H₂O)Bb) which immediately cleaves native C3.

The direct activity of MASP-3 on pro-FD was also tested under the serum free conditions. The results shown in figure (4-22) clearly demonstrate that MASP-3 efficiently cleaves pro-FD, revealing that the cleavage of pro-FD is likely to be a major physiological function of MASP-3.

Based on the results presented in this thesis, the sequence of lectin pathway dependent activation events required to effectively drive the alternative pathway of complement activation are as follows: Zymogen MASP-3 is converted into its active form by either MASP-1 or MASP-2 in a lectin pathway

dependent fashion, subsequently, active MASP-3 converts zymogen Pro-FD into its active form to convert enzymatically inactive alternative pathway zymogen complexes C3(H₂O)B or C3bB into their enzymatically active form to generate the potent C3 convertase C3bBb and C5 convertase C3bBb(C3b)_n subsequently (see diagram 4-27 below).

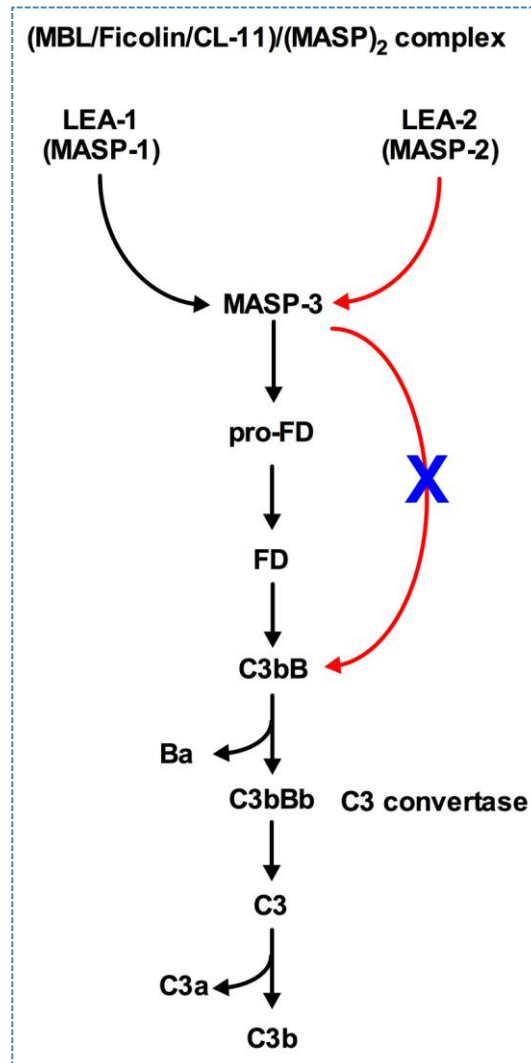


Figure 4-27: The sequence of the alternative pathway dependent the lectin pathway activation

This diagram illustrates the contribution of the lectin pathway towards the early steps of alternative pathway activation. MASP-1 and MASP-2 activate zymogen MASP-3 which in turn cleaves pro-FD and converts it into its active form. Active FD cleaves C3b-bound zymogen FB generating the alternative pathway C3 convertase (C3bBb). The red arrow refers to the result of my study that showed the previously postulated direct cleavage of FB by MASP-3 (Iwaki et al. 2011) cannot be observed under any of the chosen conditions.

A further point of my PhD project was to study C3 activation by the alternative pathway on the surface of *S. pneumoniae*. The incubation of heat killed *S. pneumoniae* in MASP-1/-3 deficient mouse serum revealed a severely compromised degree of C3 cleavage on the bacterial surface and the loss of the 41 kDa alpha chain fragment C3c in MASP-1/-3 deficient serum (see figure 4-24). The reconstitution of MASP-1/-3 deficient serum with full-length recombinant mouse MASP-3 restored alternative pathway dependent C3 activation on the bacterial surface leading to the reappearance of the 41 kDa C3c fragment seen in wild type serum (see figure 4-24, A and B). The increase of the 41 kDa C3c activation fragment is correlated directly with the amount of recombinant MASP-3 added to the MASP-1/-3 deficient mouse serum. The complete conversion of C3 into its breakdown products leads to the generation of membrane bound and fluid phase products including C3a, C3f and C3c (Perkins and Sim 1986).

Alternative pathway dependent C3 fixation on the surface of *S. pneumoniae* was also assessed in MASP-1/-3 deficient mouse serum. The addition of recombinant mouse MASP-3 to the MASP-1/-3 deficient mouse serum significantly increased C3 fixation on the surface of *S. pneumoniae* (see figure 4-24, C). Similar to the C3 breakdown assay, C3 deposition was increased in direct correlation with the amount of recombinant full-length mouse MASP-3 added to the MASP-1/-3 deficient serum (see figure 4-24, D). Interestingly, the degree of C3 fixation in MASP-3 reconstituted serum exceeded the levels seen in wild type serum which could be explained by the presence of high levels of pro-FD in the MASP-1/-3 deficient mice serum, as reported in human MASP-1/-3 deficient (Takahashi et al. 2014) and addition of MASP-3 will accelerate pro-FD conversion.

As noted above, the mouse MASP-3 was expressed as a single chain pro-enzyme protein. Therefore, the activation of pro-enzyme MASP-3 in MASP-1/-3 deficient serum is most likely mediated by endogenous MASP-2 (see diagram 4-27). A recently published observation that recombinant human C1r can

cleave MASP-3 encourages to study the role of the classical pathway in the activation of human MASP-3 (Wijeyewickrema et al. 2013).

The prominent physiological role of MASP-3 in driving the alternative pathway was clearly demonstrated by the successful *in vivo* reconstitution of alternative pathway functional activity in MASP-1/-3 deficient mice following a low dose (20 microgram/mouse) single dose administration of recombinant mouse MASP-3 as shown in figure (4-25). Alternative pathway functional activity was assessed in serum of MASP-1/-3 deficient mice collected at time points 24, 48 or 73 hours after a single dose i.v. injection of 20 microgram of recombinant full-length mouse MASP-3. As shown in figure (4-25), the total lack of alternative pathway functional activity seen in MASP-1/3 deficient mouse serum control was gradually restored over time with serum samples taken at time point 24 hours reaching approximately a third of the levels of C3 deposition via the alternative pathway (compared to the wild type controls), which increased to approximately 60% of the activity of the wild type control in reconstituted MASP-1/-3 deficient serum taken at time point 48 hours after reconstitution and reached nearly the level of C3 activation at time point 72 hours after MASP-3 reconstitution of the MASP-1/-3 deficient serum (see figure 4-25) .

The role of the complement system in fighting against *S. pneumoniae* infection was extensively studied using mouse line with targeted deficiency in one or more complement component (Brown et al. 2002, Yuste et al. 2008, Ali et al. 2012, Ali et al. 2014). Within my project, I studied the susceptibility of MASP-1/-3 deficient mice to *S. pneumoniae* infection. The result showed a very high degree of mortality in MASP-1/-3 deficient mice compared with their wild type littermates (see figure 4-26). Moreover, the bacterial burden observed in blood and in lung tissue was higher than their wild type littermates. This result underlines the critical role of MASP-1 and MASP-3 in protection against *S. pneumoniae* infection.

Chapter five: Conclusion and future directions

5.1: Conclusion

The central event in the activation of the complement cascade is the conversion of the abundant plasma protein C3 into C3a and C3b. While the anaphylatoxic small cleavage fragment C3a is released, the major fragment C3b can bind covalently through nucleophilic surface structures through a reactive thioester bond.

The key biological functions of C3b and/or its further cleavage products are:

- i) that C3b and its further degradation product iC3b bind to the activator surface and facilitate phagocytosis by opsonizing particles for the uptake by C3-receptor bearing cells of the reticuloendothelial system;
- ii) that C3 cleavage and activation products critically modulate the activation state of immune cells;
- iii) that C3b is essential for the formation of the alternative pathway C3 convertase C3bBb by binding to the zymogen FB. This zymogen FB can only be converted by FD into its active form when complexed with C3b and a main limiting step of this conversion is the availability of the specific enzyme for this conversion, the serine serum protease FD. A major part of my work (see 4.2.6 and 4.2.7) focused on the molecular process leading to the conversion of FD from its zymogen precursor pro-FD into its enzymatically active form;
- iv) the C3b deposition dependent switch of substrate specificity of the C3 convertase complexes of the lectin pathway and the classical pathway (i.e. C4bC2a), and the alternative pathway (i.e. C3(H₂O)Bb or C3bBb) from cleaving the substrate C3 to cleave the substrate C5. The switch of a C3 convertase complex to a C5 convertase complex depends on the binding of multiple C3b molecules in close proximity to initial C3 convertase complex.

My work has delivered strong evidence for the existence of two novel cascade events that both result in the activation of this central complement component C3:

5.1.1: A MASP-2 dependent, lectin pathway mediated C4-bypass

activation of C3

Several independent lines of evidence have demonstrated that complement C3 can be activated in a lectin pathway dependent fashion. This lectin pathway mediated C4-bypass activation of C3 was shown to be MASP-2 dependent and explained, for example, the severe phenotype of MASP-2 deficiency in models of *Streptococcus pneumoniae* infection (Ali *et al.* 2012). Here, this C4-bypass activation of C3 was shown to be responsible for the lectin pathway mediated C3b/iC3b opsonization of pneumococci, since the protective strategy of these pathogens to decay any C4 that binds to the pathogen surface before the lectin pathway C3 convertase, C4bC2a can form failed to avoid C3b/iC3b opsonization, since a MASP-2 dependent C4-bypass route opsonized these bacteria and rendered them susceptible for phagocytosis even though no C4bC2a complexes were detectable on the activator surface. This lectin pathway mediated C4-bypass activation of C3 was lost in absence of MASP-2 and explained the high susceptibility to and severity of *Streptococcus pneumoniae* infections in MASP-2 deficient mice. The second surprising phenotype that lead to the postulation of a C4-bypass activation route was the demonstration that ischaemia-reperfusion injury involves C3 cleavage in a lectin pathway and MASP-2 dependent fashion, a C3 cleavage which does not require the presence of the lectin pathway C3 convertase, C4bC2a since complement C4 deficient individuals are not protected from ischaemia-reperfusion injury (Schwaeble *et al.* 2011, Farrar *et al.* 2012, Gorsuch *et al.* 2012).

Therefore, the first part of my work aimed to identify how MASP-2 could activate C3 in absence of C4.

In the first instance, I revisited previously published studies that assessed the possible direct cleavage activities of any of the three different MASPs against C3. I have used commercially available preparations of human C3 and first examined these preparations for any possible contamination with either C4 or complement FB. Subsequently, I assessed the proteolytic activity of enzymatically active recombinant fragments of the lectin pathway serine proteases MASP-1 (CCP₁-CCP₂-SP), MASP-2 (CCP₁-CCP₂-SP), and MASP-3 (CCP₁-CCP₂-SP) towards different highly pure C3 preparations from human blood. While MASP-3 did not C3 cleavage C3 at all, both recombinant truncated MASP-1 and MASP-2 cleaved human C3 at a low, but concentration dependent fashion at neutral pH with the highest cleavage activity seen with recombinant MASP-2.

In order to assess the specificity of C3 cleavage by the different sources of MASPs tested and exclude the remote possibility that trace contaminating proteases could account for the cleavage of C3 activation, I repeated the *in vitro* C3 cleavage assay in presence of the natural inhibitor for MASP-1 and MASP-2 as well as in presence of the MASP-2 specific inhibitory antibody OMS721 HG4. My results showed that C1-INH efficiently blocked C3 cleavage by MASP-2, while OMS721 HG4 efficiently blocked MASP-2 proteolytic activity towards C3 showing that the C3 cleavage seen was solely a function of the recombinant lectin pathway convertases MASP-1 and MASP-2.

Since previous work has shown that MASP-1 preferentially cleaved hydrolyzed C3 (C3H₂O) (Hajela et al., 2002), I designed an assay to discriminate whether or not MASP-1 and or MASP-2 can also cleave native C3 where the reactive internal thioester is still intact and can allow the covalent binding of the C3 activation product, C3b to the activator surface, a function that the cleavage product of hydrolyzed C3 cannot fulfil. Moreover, I wanted to study whether not just active recombinant truncated fragments of MASP-1 and MASP-2 can cleave C3, but MASPs as parts of lectin pathway activation complexes as they occur in serum.

In order to achieve this, I harvested MBL(MASP)₂ complexes from different mouse and human serum sources through their affinity to bind to mannan coated surfaces as described in (3.2.8). After allowing the MBL(MASP)₂ serum complexes to bind to mannan-coated plates, the plates were washed three-times to only allow the MBL(MASP)₂ complexes to remain stuck to the plates while all unbound serum components were discarded.

Subsequently, purified C3 was added to the plates as a substrate for the lectin pathway activation complexes attached to the plates and the assay developed by quantifying the amount of C3b deposition to the ELISA plate (as a reference for the cleavage of native C3) as described in (3.2.8). Using mouse sera of wild type, MASP-1/-3 deficient, C4 deficient, C1q deficient and C3 deficient mice clearly revealed activation of native C3 as validated by the dose dependent deposition of C3b. Surprisingly, absence of MASP-1 and MASP-3 did not affect the relative efficacy of C3 cleavage of these complexes since they were exclusively loaded with MASP-2. However, lectin pathway complexes harvested from MASP-2 deficient mouse serum showed no detectable C3b deposition whatsoever, supporting the previously published observation that the MASP-1 enzyme contained in this complexes cannot cleave native C3 and can therefore not deposit C3b on the activator surface (Hajela et al., 2002). The observation that similar quantities of C3b were deposited when lectin pathway complexes were harvested from serum of C4, C1q and C3 deficient mice clarify that the cleavage activity towards the substrate C3 cannot be explained by any contaminating traces of C3 convertases of the classical, the lectin or the alternative pathway sticking to the ELISA plate.

The results presented in part (figures 3-9 to 3-13) of my thesis strongly support the conclusion that MASP-2 can directly cleave native C3 to deposit C3b on activator surfaces, while MASP-1 is unable to mediate this function.

If this C4-bypass activation route in fact presents the postulated pathophysiological activation path responsible for the mediation of ischaemia-reperfusion injury, the results of this analysis would not only explain the

absence of a protective phenotype in C4 deficiency (Schwaebble et al. 2011, Farrar et al. 2012), but moreover the unexpected phenotype that MASP-2 deficient mice are significantly protected from ischemia-reperfusion injury, while MASP-1/-3 deficient mice are not (Gorsuch et al. 2012).

5.1.2: A MASP-3 dependent, lectin pathway mediated activation of the alternative pathway C3 convertase

The present text book viewpoint is that the alternative pathway of complement is initiated by spontaneous hydrolysis of C3 into C3(H₂O) followed by FB binding to generate a C3(H₂O)B complex. In this complex, FB undergoes cleavage by FD resulting in C3(H₂O)Bb, the C3 convertase of the alternative pathway. This part of my thesis work focuses on a previously postulated axillary function of the lectin pathway towards the molecular activation events of the alternative pathway and possible physiological role of the lectin pathway in the initiation of complement activation via the alternative pathway. When I started my work in 2011, Teizo Fujita's team had described that their MASP-1/-3 deficient mouse line had a defective alternative pathway (Takahashi et al. 2010). This key paper postulated that it was in fact the absence of MASP-1 that caused this deficiency after showing that these MASP-1/-3 deficient mice had exclusively zymogen pro-FD in their blood which was not able to cleave and activate the zymogen alternative pathway convertase C3bB. They showed that recombinant MASP-1 can cleave pro-FD into its active form, while addition of recombinant MASP-1 failed to restore alternative pathway functional activity in MASP-1/-3 deficient mouse serum. In addition, when I started my work, my supervisor's laboratory had shown that human serum deficient of MASP-3, but sufficient of MASP-1 had also a defective alternative pathway with the loss of alternative pathway dependent serum lytic activity of complement towards *Neisseria meningitides*. In order to generate the key reagents to analyze the possible role of MASP-3 in the initiation of alternative pathway activation, I expressed recombinant full-length human and mouse MASP-3 in CHO-K1 cells

as His-tagged proteins to purify them by Ni²⁺ affinity chromatography. The recombinant proteins were analyzed by SDS-PAGE analysis under reducing and non-reducing conditions and confirmed by Western blotting analysis. Recombinant human MASP-3 showed very high susceptibility to cleavage by trypsin.

Natural activators for MASP-3 were investigated by screening the proteolytic activity of several serum serine proteases towards recombinant MASP-3. The proteolytic activity of recombinant human MASP-1 (CCP₁-CCP₂-SP) and recombinant human MASP-2 (CCP₁-CCP₂-SP) on recombinant full-length human MASP-3 was studied using SDS-PAGE and Western blotting analysis. Both enzymes were able to activate MASP-3. Interestingly, less MASP-2 was required to achieve a 50% conversion of the MASP-3 zymogen compare to the quantity of MASP-1 needed. In order to assess whether the cleavage of MASP-3 by the enzymatically active recombinant MASP-2 fragment (CCP₁-CCP₂-SP) triggers MASP-3 functional activity towards VPR-AMC, the turn-over of the MASP-3 substrate VPR-AMC was measured in presence or absence of the MASP-3 cleaving MASP-2 fragment. Only MASP-2 cleaved MASP-3 was able to turn-over the VPR-AMC substrate as monitored by the release of AMC.

The enzymatic activity of two other serum serine proteases, i.e. thrombin and plasmin, was tested towards recombinant full-length MASP-3. After incubation, MASP-3 cleavage was monitored by analyzing the reaction on SDS-PAGE and by Western blotting. Both thrombin and plasmin showed no enzymatic activity towards MASP-3 indicating that the MASP-1 and MASP-2 proteolytic activities towards MASP-3 were relatively specific and that only specific serine proteases are able to cleave MASP-3.

Furthermore, I studied which of the following complement components can be cleaved of activated MASP-3. For this study, I exposed the purified serum components FH, zymogen FB, zymogen C5, and recombinant zymogen FD (pro-FD) with the enzymatically active recombinant MASP-3 fragment composed of the N-terminal domains CCP₁-CCP₂-SP. While active MASP-3

showed no proteolytic activity towards FH, FB and C5, MASP-3 effectively converted pro-FD into its enzymatically active form.

I then revisited the roles of FB and FD in the alternative pathway by testing FB depleted serum and FD depleted serum. Alternative pathway functional activity was undetectable in both depleted sera, but adding either purified human FB or recombinant human pro-FD back to the depleted corresponding serum restored alternative pathway functional activity. Pre-incubation of purified FD with MASP-3 enhanced the restoration of alternative pathway functional activity in FD depleted serum.

Subsequently, I investigated to what extent recombinant MASP-3 can restore alternative pathway functional activity in MASP-1/-3 deficient mouse serum. Although Takahashi *et al.* provided a clear explanation for the absence of alternative pathway activity in MASP-1/-3 deficient mice by demonstrating that these mice have nearly exclusively pro-FD in their plasma and demonstrated that recombinant MASP-1 can convert pro-FD into its enzymatically active form, reconstitution of MASP-1/-3 deficient serum failed to restore alternative pathway functional activity (Takahashi et al. 2010).

In my first set of experiments, I have added increasing concentrations of enzymatically active recombinant human MASP-1 and MASP-3 (both composed of the N-terminal domains CCP₁-CCP₂-SP) to MASP-1/-3 deficient mouse serum and measured C3b/iC3b deposition on zymosan under alternative pathway conditions. While no alternative pathway functional activity could be detected in either non-reconstituted or MASP-1 reconstituted MASP-1/-3 deficient serum, addition of truncated MASP-3 fully restored alternative pathway functional activity *in vitro*. Adding both MASP-1 and MASP-3 revealed synergistic activity in restoring alternative pathway functional activity.

I then studied the cleavage activity of MASP-3 using its enzymatically active truncated recombinant N-terminal fragments under serum free conditions towards recombinant pro-FD. MASP-3 converted pro-FD into its enzymatically

active form. The observation that this cleavage is mediated by MASP-3 underlines my conclusion from other experiments that MASP-3 and not MASP-1 is the prominent and physiologically most relevant enzyme mediating pro-FD conversion into its active form. This viewpoint is strongly supported through the analysis of human serum from a patient presenting with a developmental defect known as Carnevale, Mingarelli, Malpuech and Michels syndrome. This patient has a homozygous Single Nucleotide Polymorphism (SNP) in exon 12 of the *MASP1* gene, the exon encoding the serine protease domain of MASP-3. This SNP renders the MASP-3 dysfunctional, while MASP-1 is present in this patient's serum levels and fully active (Professor Wilhelm Schwaeble, personal communication).

The analysis of this patient's serum carried out in the lab 231 revealed that it is deficient in alternative pathway functional activity despite having normal levels of MASP-1. This result strongly underlines the conclusions drawn from my other analyses indicating that MASP-3 and not MASP-1 is the critical lectin pathway component responsible for the conversion of pro-FD into its active form and that MASP-3 deficiency is the primary cause of alternative pathway deficiency in MASP-1/-3 deficient mice.

The prominent role of MASP-3 in securing the presence of a physiologically effective alternative pathway amplification loop is highlighted by the successful reconstitution of alternative pathway functional activity in MASP-1/-3 deficient mice through a single dose injection of full-length recombinant mouse MASP-3. The fact that it can take up to 3 days for the reconstitution to fully restore alternative pathway functional activity implies that zymogen MASP-3 requires to be activated, most likely in a lectin pathway and MASP-2 dependent fashion and since MASP-1 is absent in MASP-1/-3 deficient mice.

Finally, I assessed the susceptibility of MASP-1/-3 deficient mice to infection in an established mouse model of *S. pneumoniae* infection. First, I compared the degree of C3 deposition on the surface of *S. pneumoniae* D39 bacteria by ELISA assay and Western blotting analysis between wild type and MASP-1/-3 deficient mouse serum. Compared to wild type serum, the amount of C3

cleavage products deposited on the surface of *S. pneumoniae* D39 was significantly reduced in MASP-1/-3 deficient mouse serum. However, reconstitution of MASP-1/-3 deficient mouse serum with recombinant full-length mouse MASP-3 restored this C3 opsonization defect.

This defect in C3 opsonization of MASP-1/-3 deficient mouse serum was also reflected by a highly increased susceptibility of MASP-1/-3 deficient mice to *S. pneumoniae* D39 infection. The infectious dose was adjusted to induce approximately 20% mortality in wild type C57/BL6 mice. MASP-1/-3 deficient mice of the same genetic background and same age and sex infected in parallel showed a mortality of 80% with significantly increased numbers of bacteria in blood and lung tissue.

This clearly highlights the physiological importance of this novel link between the lectin pathway and the alternative pathway where the lectin pathway critically contributes to an effective alternative pathway response through a predominantly MASP-3 mediated activation of pro-FD.

This relevant link feeds new substance to the historical debate to what extent alternative pathway activation of complement involves pathogen specific recognition mechanisms.

5.2: Future directions

I would very much love to continue and expand my study to exploit the very promising avenues of research that the results of my PhD project opened up:

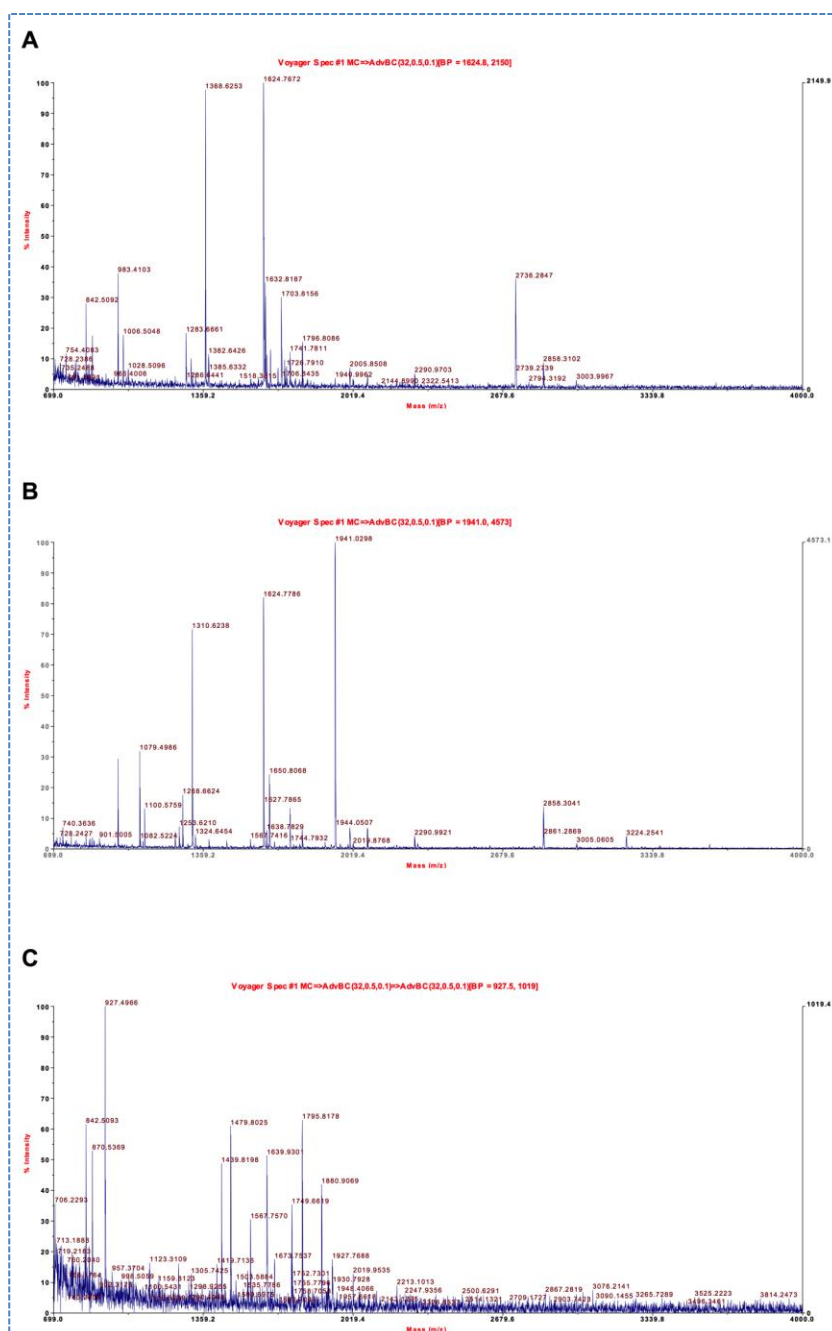
- i) Since I have shown that recombinant MASP-3 can restore the deficient alternative pathway in MASP-1/-3 deficient mice, I would love to assess the efficacy of this restoration in a physiological context and repeat my *S. pneumoniae* D39 infection study and analyze to what extent MASP-3 reconstitution can restore the compromised immune response of MASP-1/-3 deficient mice against this pathogen.
- ii) The longstanding collaboration of my supervisor's laboratory and the research group of OMEROS Corporation in Seattle, USA established a MASP-3 specific recombinant therapeutic inhibitor. This reagent would allow me to study the efficacy of therapeutic MASP-3 inhibition in experimental models of disease where alternative pathway functional activity significantly contributes to pathology. This includes models of haemolytic disease, such as Paroxysmal Nocturnal Haemoglobinuria (PNH), models of collagen-induced arthritis and Experimental Autoimmune Encephalopathy (EAE).
- iii) A recent report described that the classical pathway serine protease C1r is also capable to cleave MASP-3. I would love to study, if classical pathway activation can also initiate or augment alternative pathway activation via C1r mediated conversion of zymogen MASP-3. It is, after all, a long accepted concept in complement research that erythrocytes can be made susceptible to serum lytic activity by being "sensitized" to lysis by coating with

antibodies. If the cytotoxic effects of sensitisation can be abolished by blocking MASP-3 functional activity and control alternative pathway activation, then blocking MASP-3 could potentially open up a widely effective therapeutic concept to treat the cytotoxic effects of auto-reactive antibodies in autoimmune disease.

Appendices

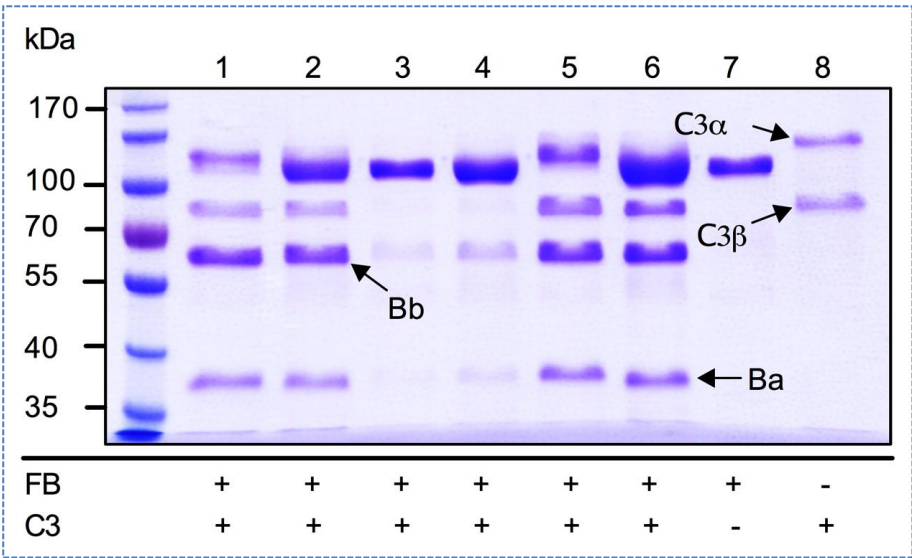
6.1: Appendix A: Peptide sequencing of recombinant human and mouse MASP-3

The recombinant human (A) and mouse (B) MASP-3, and BSA control (C) were sequenced (MRC Toxicology Unit, Leicester, UK). Recumbent proteins identity was confirmed as a complement human and mouse MASP-3



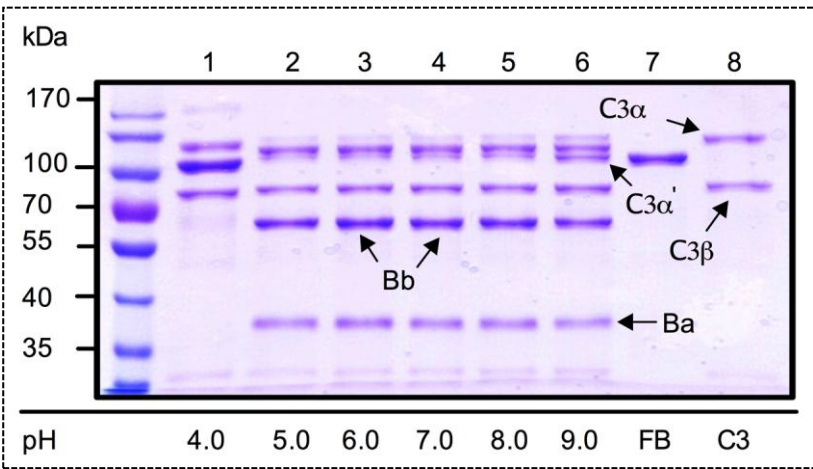
6.2: Appendix B: FB and C3 interaction

Different batches of purified human C3 and purified human FB to avoid FB activation in presence of C3. The figure shows FB activation in all batches tested.



6.3: Appendix C: Effect of pH on FB activation

The effect of pH on FB activation in presence of purified human C3. FB is activated by unknown mechanism/protease in presence of native C3 and the subsequently activated FB cleaves C3 into C3α'. The pH value greater than 7.0 seems to be optimal for C3 activation in this preparation.



6.4: Appendix D: Peptide sequencing of purified human FB preparation

The SDS-PAGE analysis (figure 4-21) revealed the presence of two bands of approximately 60 kDa and 50 kDa in FB preparation. The two bands were peptide sequenced (MRC Toxicology Unit, Leicester, UK) and the result confirmed that these bands as complement FB.

A

Probability Legend:

- over 95%
- 80% to 94%
- 50% to 79%
- 20% to 49%
- 0% to 19%

Bio View:

12 Proteins in 12 Clusters

#	Visible?	Starred?	Description	Accession Number	Molecular Weight	Protein Grouping Ambiguity
1	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement factor B OS=Homo sapiens GN=CFB PE=2 SV=1	B4E1Z4 (+1)	141 kDa	32 35
2	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Kallistatin OS=Homo sapiens GN=SERPINA4 PE=1 SV=3	P29622	49 kDa	8 7
3	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Carboxypeptidase B2 OS=Homo sapiens GN=CPB2 PE=1 SV=2	Q96IY4	48 kDa	6 5
4	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement factor I heavy chain OS=Homo sapiens GN=CFI PE=...	E7ETH0 (+4)	67 kDa	4
5	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement component C8 alpha chain OS=Homo sapiens GN=...	P07357	65 kDa	2
6	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=...	P13645	59 kDa	2
7	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Serum albumin OS=Homo sapiens GN=ALB PE=4 SV=1	B7WNRO (+7)	56 kDa	1 1
8	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Uncharacterized protein OS=Homo sapiens GN=ITIH1 PE=2 SV=1	B7Z549 (+5)	76 kDa	1
9	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=4 SV=...	F5H0Q5 (+1)	47 kDa	1
10	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement component C8 beta chain OS=Homo sapiens GN=...	P07358	67 kDa	1 1
11	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Hepatocyte growth factor-like protein beta chain OS=Homo sapi...	F5H6L8 (+4)	18 kDa	1
12	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Keratin, type II cytoskeletal 3 OS=Homo sapiens GN=KRT3 PE=1...	P12035 (+2)	64 kDa	1

B

Probability Legend:

- over 95%
- 80% to 94%
- 50% to 79%
- 20% to 49%
- 0% to 19%

Bio View:

6 Proteins in 6 Clusters

#	Visible?	Starred?	Description	Accession Number	Molecular Weight	Protein Grouping Ambiguity
1	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement factor B OS=Homo sapiens GN=CFB PE=2 SV=1	B4E1Z4 (+1)	141 kDa	32 35
2	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Kallistatin OS=Homo sapiens GN=SERPINA4 PE=1 SV=3	P29622	49 kDa	8 7
3	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Carboxypeptidase B2 OS=Homo sapiens GN=CPB2 PE=1 SV=2	Q96IY4	48 kDa	6 5
4	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement factor I heavy chain OS=Homo sapiens GN=CFI PE=...	E7ETH0 (+4)	67 kDa	4
5	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Complement component C8 alpha chain OS=Homo sapiens GN=...	P07357	65 kDa	2
6	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=...	P13645	59 kDa	2

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