The role of CL-11, a novel recognition component of the lectin activation pathway of complement in pneumococcal infection

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "The role of CL-11, a novel recognition component of the lectin activation pathway of complement in pneumococcal infection" is based on work conducted by the author at the University of Leicester mainly during the period between October 2012 and September 2016.

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

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

Signed: Date:

Abstract

The role of CL-11, a novel recognition component of the lectin activation pathway of complement in pneumococcal infection

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The Lectin activation Pathway of complement (LP) is initiated by LP specific recognition molecules, i.e. MBL, CL-11 and ficolins. These subcomponents recognize a wide range of carbohydrates or acetylated structures on microbial surfaces and activate complement via the LP effector enzyme MASP-2. The essential role of the LP in fighting *S. pneumoniae* infections was first shown by Prof. Schwaeble's team using a mouse line deficient of MASP-2 and in mice deficient of the murine LP recognition component Ficolin A. This is the first report showing that CL- 11, an only recently identified recognition subcomponent of the LP, is critical in the innate immune defense against *S. pneumoniae* infections. My *in vivo* work was supported by numerous *in vitro* analyses using native and recombinant murine CL-11 revealing that this recognition subcomponent critically contributes to complement activation the surface of *S. pneumoniae*. So far, I compared the susceptibility of CL-11 deficient mice with that of sex, age and strain matched wildtype control mice and this analysis revealed a dramatically increased mortality in CL-11 deficient mice with a significant increase of bacteraemia in CL-11$^{-/-}$mice. Another aspect of my study was to assess whether any of the SNPs described for the human CL-11 gene pre-disposes for frequent microbial infections. Also the effects of genetic variations (SNPs) in the promoter on CL-11 expression were studied using a set of nested deletions cloned into an *in vitro* reporter vector system.

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List of abbreviations

aa	Amino acid
AP	Alkaline Phosphate
BBS	Barbital Buffered Saline
BHI	Brain Heart Infusion
bp	base pair
BSA	Bovine Serum Albumin
C1-INH	C1-Inhibitor
C1r	Complement component 1r
C1s	complement component 1s
C3aR	C3a Receptor
C4BP	C4b Binding 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
CRD	Carbohydrate Recognition Domain

CRP	C-reactive protein
CUB	Complement C1r/C1s, Uegf, Bone morphogenetic protein
DAF	Decay Accelerating Factor
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol-bis-N,N,Ń,Ń-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Fc	Fragment crystallizable
FCN	Ficolin
GalNAc	N-aetyl-D-glucosamine
GlueNAc	N-aetyl-D-galactosamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HRP	Horse Radish Peroxidase
i.p.	Intraperitoneal
Ig	Immunoglobulin
IGFBP-5	Insulin-Like Growth Factor Binding Protein-5

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl Thiogalactoside
kDa	Kilodalton
KO	Knockout
LB	Luria-Bertani
LDL	low-density lipoprotein
LTA	Lipoteichoic Acid
MAC	Membrane Attack Complex
MAF	Minor allele frequency
MASP	MBL-Associated Serine Protease
MBL	Mannan Binding Lectin
MEM	Minimum Essential Medium
mL	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
3MC	Malpuech, Michels and Mingarelli-Carnevale syndromes
ng	Nanogram
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis

PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
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
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SP-A	Surfactant protein-A
SP-D	Surfactant protein-D
TBS	Tris Buffered Saline
TSS	Transcription start site
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
Tfb	Transformation buffer
v/v	volume/volume
w/v	weight/volume

WT	Wild Type
Xgal	5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μL	Microliter

Chapter One: General introduction

1   Chapter One: General Introduction

1.1   The immune system

The immune system protects the body from invading pathogens via protective mechanisms which cooperate together to defend the body against pathogens and eliminate cellular debris. The immune system is divided into the innate immune system and the adaptive immune system. The innate immune response provides instant and immediate protection, but does not have a memory function for specific antigens, i.e. previous exposure to these antigens does not change the strength of the innate immune response to these antigens. In contrast, the adaptive immune response develops a memory function to antigens which allows an enhanced response following second or sequential exposure to pathogens (Borghesi & Milcarek, 2007; Iwaki *et al.*, 2011). The memory function of the adaptive immune response is driven by specific immune cells, i.e. T cells and B cells that undergo mutational changes during clonal expansion and bear an array of cell surface receptors that control clonal expansion and adaptation. Since the adaptation process requires time, the adaptive immune system is somewhat insufficient to respond at first contact with pathogens.

The innate immune system provides an essential first line of defense during this particular phase of vulnerability towards pathogens against which the body has not yet developed adaptive immunity. Both the innate and the adaptive responses are regulated by the interaction between cellular and humoral elements. The cellular elements of innate immunity involve haematopoietic cells (neutrophils, eosinophils, macrophages, dendritic cells, mast cells, NK cells, and NK T cells) and non-haematopoietic cells, such as epithelial cells (Turvey & Broide, 2010). The humoral elements include complement proteins, cytokines, chemokines and coagulation factors (Takahashi, 2011).

A hallmark of the adaptive immune response is the production of antibodies produced by B lymphocytes which recognise specific pathogen epitopes. The hallmark of cellular adaptive immunity set by the clonal expansion of B cells and T cells that "recognize" specific antigens, and generate antigen specific plasma cells and antigen specific T-lymphocytes, which both contribute to the effective and highly selective clearance of pathogens (Medzhitov, 2007). (Figure 1.1).
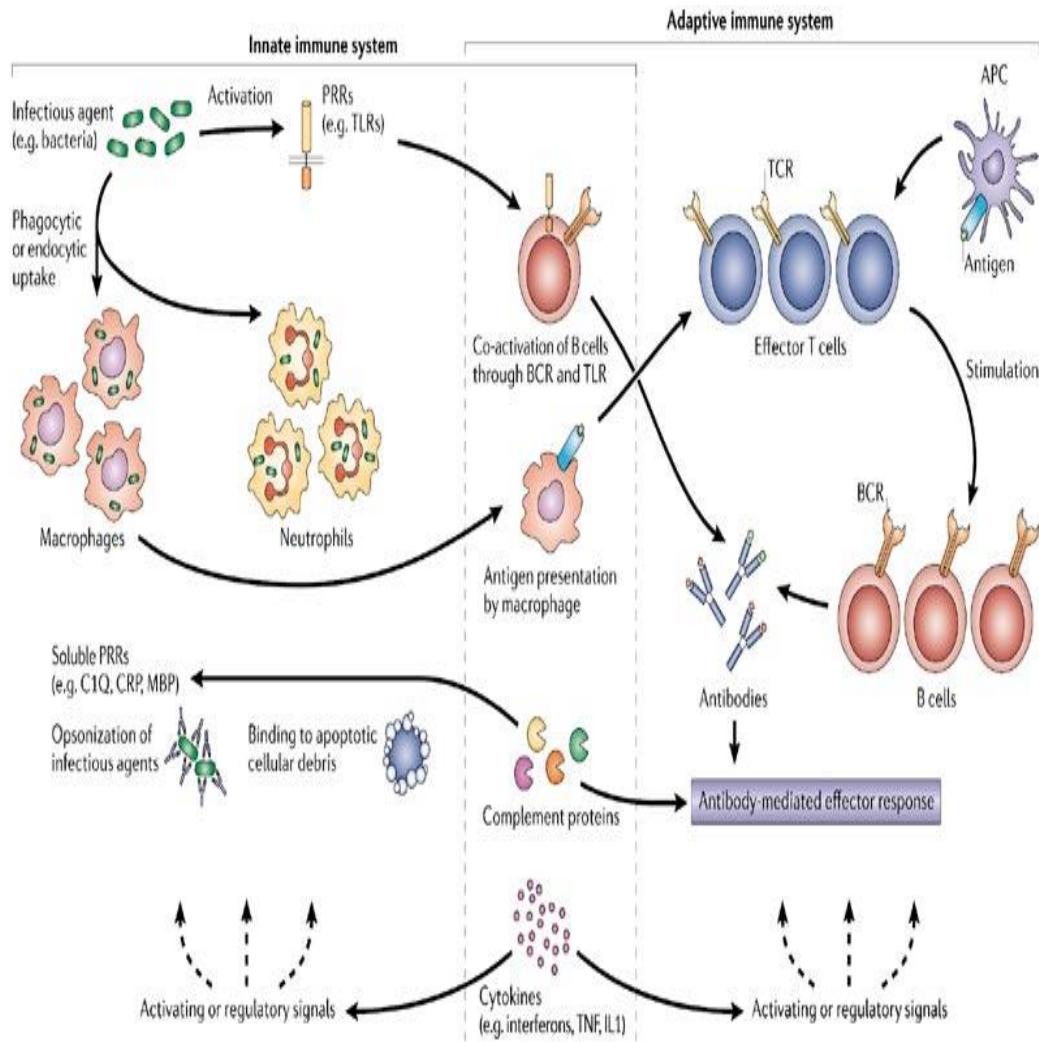
Figure 1-1 The differences between innate and adaptive immune response
 (Gregersen & Behrens, 2006)

1.2   Complement system

Complement was first described by Jules Bordet in 1896 as a heat liable component of the plasma which has a cytolytic activity. The complement system is composed of more than 30 soluble and membrane associated proteins which play a pivotal role in both the innate and the adaptive immune response (Dunkelberger & Song, 2010; Makrides, 1998). The role of the complement system within the host defense is achieved by different processes which include defence against infectious organisms as well as tissue regeneration and the clearing of immune complexes (Degn *et al.*, 2011). Activation of the complement system is initiated by binding interactions of specific recognition subcomponents that act as pattern recognition receptors (PRRs) to specific pathogens associated molecular pattern (PAMPs). These recognition subcomponents include C1q, the recognition subcomponent of the classical pathway of complement activation (CP) that binds to the Fc region of antibodies of the immunoglobulin classes Ig-G1, Ig-G2, Ig-G3 (but not Ig-G4) and Ig-M once these immunoglobulins formed immune complexes binding to their specific antigens. The recognition subcomponents of the lectin activation pathway of complement (LP) are the collectins, Mannan–binding lectin (MBL) and Collectin-11 (also known as CL-K1, since it was first described in the kidney) and ficolins, such as human L-ficolin, M-ficolin and H-ficolin and murine ficolins (ficolin-A, and ficolin B) (Wallis *et al.*, 2010b; Ali *et al.*, 2012). Most of the recognition subcomponents are synthesized in the liver. However, there are also extra-hepatic source of complement biosynthesis, such as monocytes, pulmonary alveolar epithelial cells, osteoblasts and skeletal myoblast (Thomas *et al.*, 2000). Most complement components are present in plasma as zymogens prior to their activation   (Zipfel & Skerka, 2009).

 The complement system can neutralise microbial organisms either by direct lysis or target microbes for elimination by phagocytosis. Activation of complement also triggers cellular activation and chemotaxis through the release of anaphalotoxins including C3a, C4a and C5a which can trigger the adaptive immune response via the complement receptor mediated signaling on leukocytes (Wallis & Lynch, 2007).

The complement system is activated via three different pathways, the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (Endo *et al.*, 2006) (Figure 1.2). All

these pathways lead to the activation of C3 and subsequently to the formation of the membrane attack complex (MAC) (Sarma & Ward, 2011). The C3 convertase of both the classical and lectin pathway (i.e. C4b2a) and that of the alternative pathway (C3bBb) cleave C3 into its activation products C3a and C3b. While C3a is a 9kDa cleavage product that quickly dissociates to bind to specific C3a receptors to either direct chemotaxis or cellular activation in an anaphylatoxin-like manner, the major cleavage product C3b can covalently bind to surface structures through the destabilization of an internal thioester bond. If this binding of C3b occurs in close proximity to the C3 convertase complexes, the accumulation of C3b in proximity to these complexes initiates a substrate switch of these convertase to cleave the complement component C5, forming the C5 convertases C4b2a(C3b)n or C3bBb(C3b)n. With the cleavage of C5, all enzymatic steps of complement activation are completed. C5 is cleaved into the potent anaphylatoxin C5a (which – like C3a quickly dissociates to bind to specific C5a receptors that direct cellular chemotaxis and cellular activation processes that drive inflammatory processes. The major C5 cleavage fragment C5b initiates the activation of the terminal pathway by sequential binding to C6, C7, C8, and C9 to form the membrane attack complex C5b-9 which inserts into the surface of pathogens (Zipfel & Skerka, 2009).
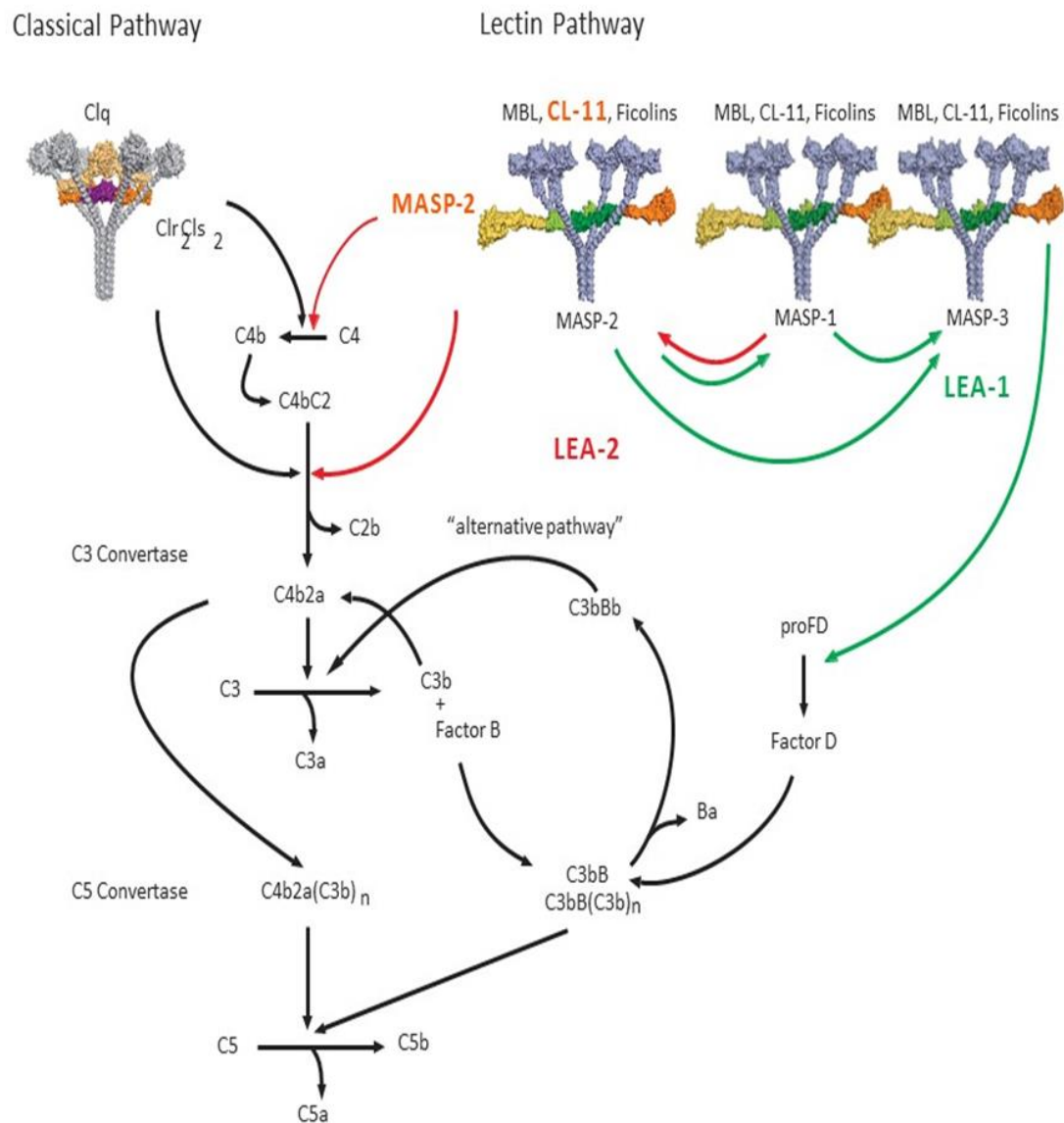
Figure 1-2 A schematic diagram of complement activation: Activation of the classical, the lectin and the alternative pathway.
 (Figure kindly provided by my supervisor Professor Wilhelm Schwaeble).

1.2.1   Classical pathway

The activation of the classical pathway (CP) is initiated by binding of the C1 complex to antibody –antigen complexes. The C1 complex is composed of C1q, C1r and C1s. C1q is a multimeric 460-kDa protein complex composed of six subunits with each subunit being composed of three different, but highly homologous polypeptide chains (A- chain, B-chain, C chain). (Figure1.3) Each chain has an N-terminal domain, a collagen-like domain and a globular domain at the C-terminus (Schwaeble *et al.*, 1995). The binding of C1q to Fc region of immune complexes (IgG (IgG1, IgG2, IgG3, but not IgG4) and IgM) or binding directly to microbial surface leads to activation C1r and C1s. C1r initiates the internal activation of C1s while C1s transfers the signal to cleavage of C4 and C2 and formation of the C3 convertase (Wallis *et al.*, 2010b). C1q can bind to apoptotic cells and clear them by activation of the classical pathway. C1q links the innate and the adaptive immunity by binding to target IgG and IgM and activate the classical pathway (Nayak *et al.*, 2012).
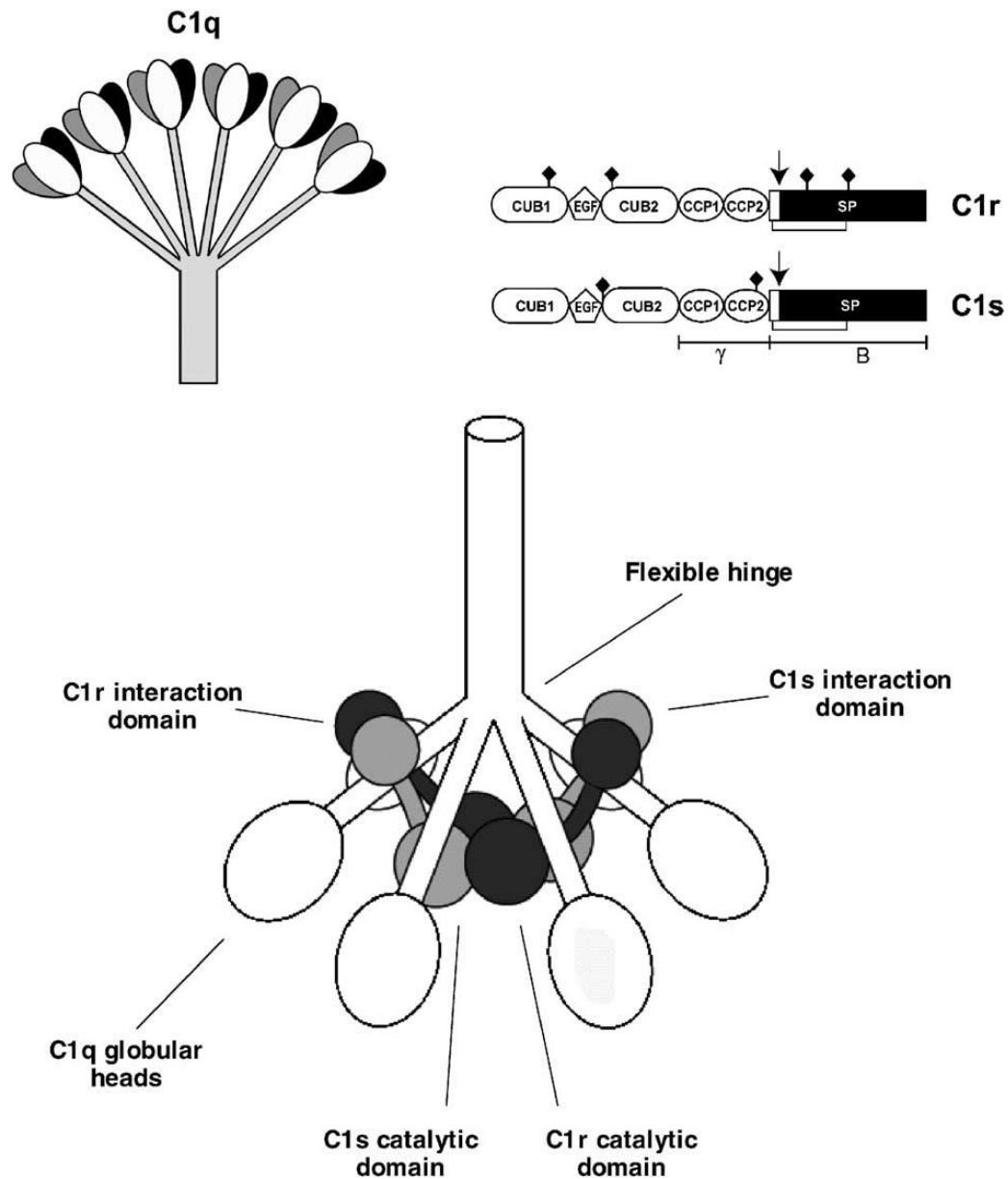
Figure 1-3 The Structure of the C1 complex
The Structure of the C1 complex, which is the first protein in the classical pathway of complement activation containing C1q, C1r, and C1s. C1q is composed of six identical subunits with globular heads and long collagen-like tails (Arlaud *et al.*, 2002).

1.2.2   Alternative pathway

The activation via this pathway is initiated by spontaneous hydrolysis of C3 to form C3 (H_2O). This hydrolysis of C3 changes the conformation of C3 and allows the binding of factor B to form a C3 (H_2O) B zymogen complex. Subsequently factor B can be cleaved by factor D to form the C3 convertase C3 (H_2O) Bb, which converts C3 to C3a and C3b (Thomas *et al.*, 2000) C3b binds to the surface of pathogens where is serves as an opsonin enhancing the uptake of pathogens by C3 receptor bearing cells. Membrane bound C3b can also bind factor B to initiate the generation of a C3 convertase complex. The fact that the cleavage product of C3, C3b can initiate the formation of further C3 convertases accounts for the particular importance of the alternative pathway, since the AP provides an amplification loop to maximize complement activation processes in a physiologically relevant fashion. To prevent un-controlled activation via the AP amplification loop, the AP is carefully controlled by negative regulators such as Factor H, or DAF (alias CD55) and positive regulators like properdin (Carroll & Sim, 2011). The key components of the AP are Factor B (fB), Factor D (fD), C3 and Properdin. Factor B binds to C3b in presence of Mg^{2+} The conversion of the zymogen complex C3bB into its enzymatically active form C3bBb critically requires the cleavage of C3b-bound factor B by factor D to form C3bBb (the C3 convertase of alternative pathway). As described for the CP and LP specific C3 convertase C4b2a, the binding to multiple C3b cleavage products initiates the formation of C5 convertase complexes, since the binding of multiple C3b molecules in close proximity to the C3 convertase switches the substrate specificity of this complex from C3 to C5.

Properdin is a positive regulator of the AP and acts by stabilizing the C3 convertase C3bBb and C5 convertase complexes C3bBb (C3b) n by protecting them from decay by factor H and DAF (Farries *et al.*, 1988; Schwaeble & Reid, 1999). The LP specific serine protease MASP-1 and MASP-3 have recently been identified to play a key role in the activation of alternative pathway since they are required to convert the zymogen form of factor D (i.e. pro-factor D) into its enzymatically active form (Iwaki *et al.*, 2011). Mice deficient in both MASP-1 and MASP-3 were previously shown to be deficient in their AP functional ability (Takahashi *et al.*, 2010).

1.2.3   Lectin pathway:

Lectin pathway is part of the innate immune defense system and plays a key role in the protection against the pathogens (Endo *et al.*, 2006). The activation of this pathway is initiated when the pathogen recognition molecules (PRMs) i.e. the recognition subcomponents of the lectin pathway bind to the pathogen associated molecular patterns (PAMPs) and activate the MBL-associated serine proteases (Gupta & Surolia, 2007). These PRMs include proteins belonging to either the ficolin or the collectin family, which form multimolecular functional lectin pathway activation complexes in plasma and serum with MBL-associated serine protease (MASPs) (Endo *et al.*, 2006).

There are three different MASPs, called in the sequence of their discovery MASP-1, MASP-2 and MASP-3. In addition, there are non-enzymatic, truncated products of MASP- 2 gene and MASP-1/3 gene which are MAp19 and MAp44 respectively (Fujita, 2002). Each MASP is composed of six domains that resemble the domain structure and organization previously described for the serine proteases of the classical pathway called C1r and C1s. Each MASP or C1r or C1s has an N-terminal CUB domain (CUB I), followed an EGF domain, a second CUB domain (CUB II), two complement control protein (CCP) domains and a C-terminal serine protease domain (Gadjeva *et al.*, 2004). Both MASP-1 and MASP-3 are the products of a single structural MASP-1/3 gene and arise from two different RNA transcripts generated by alternative splicing (Dahl *et al.*, 2001; Schwaeble *et al.*, 2002). In contrast to MASP-2 (which is exclusively expressed in hepatocytes), MASP-1 and MASP-3 are also expressed in extrahepatic tissues such as the pancreas, the spleen, the thymus, the brain, the prostate and the ovary (Yongqing *et al.*, 2012)

1.3   The effect of complement activation:

The complement system has different biological activities aiming to protect the host against invading pathogens and helping to eliminate apoptotic cells and necrotic cells (Carroll & Sim, 2011). These activities mainly include opsonisation, chemotaxis, cell lysis and the promotion of pro-inflammatory cell activation. In addition, the complement system plays an important role in activation the adaptive immune response (Fujita, 2002). Opsonisation is one

of the most important functions of the complement system and is initiated upon binding of C3b or iC3b and C4b or iC4b to the surface of the pathogens. This process will facilitate phagocytosis by binding of these components to C3 and C4 receptors such as complement receptors I (CRI, CD35) and CR3 on the surface of macrophages and leukocytes (Aoyagi *et al.*, 2005). In addition, these opsonins, MBL and ficolins may also have a direct role in opsonisation of pathogens (Carroll & Sim, 2011). Through the activation of the complement system, some of anaphylatoxic peptides such as C4a, C3a, and C5a are released. These molecules may attract different types of immune cells and initiate inflammatory activities. They can, for example, induce the release of histamine by mast cells (Sarma & Ward, 2011).

1.4   Regulation of complement system

The host cells are protected from the effects of complement activation by several membrane bound or membrane associated negative regulators (the membrane associated regulators can be fluid phase regulators that show high regulatory activity when associating to host cell membrane structures (Zipfel & Skerka, 2009).

1.4.1   Fluid phase regulators:

Fluid phase regulators play a crucial role in regulating the three pathways of the complement system. They include factor I, C4 binding protein, factor H, C1 inhibitor, Carboxpeptidase N (SCPN), S – protein, Vimentin and Clusterin. Factor I is a glycoprotein, which is present in serum in an average concentration of approximately 35 µg/ml. Factor I controls all the complement pathways by inactivation of C3b and C4b (Davis *et al.*, 2008). C1 inhibitor (C1-INH) regulates both the classical and the lectin pathway by controlling the activation of C1r, C1s and MASP-1 and MASP-2 (but not MASP-3) (Nilsson *et al.*, 2011; Zundel *et al.*, 2004). C4 binding protein (C4bp) is another regulator of the classical and the lectin pathway. It prevents the formation of the CP and the LP C3 convertase by the decay of C4b on its complexes and by serving as a cofactor in the conversion of C4b into haemolytically inactive iC4b. C4bp is involved in the conversion of C4b to iC4b, C4e and C4d as a cofactor to factor I (Hillarp & Dahlback, 1988). Factor H (FH) is a regulator for alternative pathway which

prevents the formation of the C3bBb complex by competition with factor B for binding to C3b (Wu *et al.*, 2009) .

Factor H is the main fluid phase regulator of the complement system and in addition to its fluid phase function, the interaction of factor H with the glycosaminoglycan and or sialic acid of host cells can significantly increase the affinity of Factor H to bind to free and complex bound C3b. Like C4BP, factor H serves as a decay accelerating factor, taking C3b out of its complexes and a cofactor of Factor I, by allowing factor I to cleave C3b into haemolytically inactive iC3b and catalyse the further decay of iC3b to C3dg and C3c via factor I (Schwaeble *et al.*, 1987). Also the anaphylotoxin molecules C5a and C3a are inactivated by Serum carboxpeptidase N (SCPN) which cleaves the C-terminal Arginine and lysine resulting in decrease the activity of these molecules (Kalant *et al.*, 2005). Furthermore, there are other inhibitors of complement activation like for example S – protein, Vimentin and Clusterin which interfere with the formation of the MAC complex by binding with C5b-7 and prevent C8 and C9 to bind (Sarma & Ward, 2011).

1.4.2 Membrane bound regulators:

Membrane bound regulators include proteins or receptors that are involved in controlling the complement system. One of these regulators is complement receptor 1 (CR1) also called CD35 which plays a role as a cofactor for factor I in the process of inactivation of C3b and C4b. In addition, membrane cofactor protein (MCP) or (CD 46) is another regulator that is involved in inactivation of C3b (Whaley & Schwaeble, 1997). CD59 (also called Protectin) is a regulator of MAC formation. CD59 binds to C8 and C9 and prevents them from associating with C5b-7 complexes (Kim & Song, 2006). The surface regulatory component DAF (CD55) dissociates C3 and C5 convertase complexes by binding to C3b and C4b. (Medof *et al.*, 1984) .

1.5   Serine proteases of the lectin pathway

When it became clear that MBL may serve as a recognition subcomponent in complement activating complexes it was first considered that MBL could fulfil this function by associating with the classical pathway serine proteases C1r and C1s (Lu *et al.*, 1990). In 1986, Fujita and coworkers identified a MBL-associated serine protease that they called MASP (for MBL-associated serine protease) with striking similarity to the classical pathway serine proteases C1r and C1s (Matsushita & Fujita, 1992). In 1995, a second MASP, MASP-2 was identified (Thiel *et al.*, 1997), followed by the discovery of a third MASP in 2001 (Dahl *et al.*, 2001).The organization of these serine proteases is reviewed in (Schwaeble *et al.*, 2002). In addition alternatively spliced mRNA transcripts of the two MASP genes encode MBL-associated proteins including MAp19 (or sMAP) encoded by the MASP-2 gene (Stover *et al.*, 2004) and MAp44 (MAP-1) encoded by the MASP1 gene (Skjoedt *et al.*, 2010). Both MAp19 and MAp44 are truncated mRNA transcripts encoding a truncated protein devoid of a serine protease domain with a unique C-terminal sequence encoded by an alternatively spliced exon. MBL binds to all 3 MASPs and MAp19 and MAp44 and the MBL binding site of MASPs is located in the CUB1-EGF region. MAp19 and MAp44 are considered to be regulators of the LP since they bind to the MASPs binding site and act as competitive inhibitors by occupying the MASPs binding sites. The binding of MASPs to the recognition subcomponents is Ca^{2+}-dependent (Wallis & Dodd, 2000; Lynch *et al.*, 2005).

1.5.1   MASP-1

MASP-1 is mainly produced in hepatocytes and detected at a plasma concentration of 1 μg/ml. MASP-1 is encoded by the gene MASP1/3 which located on human chromosome 3q27-q28 or the murine chromosome 16B2-B3 respectively (Yongqing *et al.*, 2012). It is secreted into the circulation as a zymogen that can associate with lectin pathway recognition molecules (MBL, ficolins and CL-11). The role of MASP-1 in the activation of the lectin pathway is limited since it can only cleave C2 to C2b and C2a but cannot cleave C4. In addition, MASP-1 cleaves and activates MASP-2. Recent work showed that MASP-1 may be involved in the conversion of profactor D into active factor D, thus it may have an important role in activation the alternative pathway (Dobo *et al.*, 2009; Yongqing *et al.*,

2012). MASP-1 links the complement system and the coagulation system via cleavage of fibrinogen, thrombin and factor XIII (Hajela *et al.*, 2002).

1.5.2 MASP-2

MASP-2 is produced as a proenzyme encoded by the MASP2 gene on human chromosome 1p36.3-2 or murine chromosome 4 respectively. It is synthesized in hepatocytes (Stover *et al.*, 2001; Stover *et al.*, 2004). As mentioned above, all MASPs are highly similar and composed of the same domain structure as C1r and C1s (i.e. CUB1, EGF, CUB2, CCP1, CCP2, SP) and the binding site of MASP2 is located in the CUB1-EGF region (Figure 1.4) (Stover *et al.*, 2001). Autoactivation of MASP-2 or direct cleavage of MASP-2 by MASP-1 occurs when the lectin pathway subcomponents MBL or CL-11 or ficolins bind to the surface of pathogens and subsequently leads to formation of C3 convertase C4b2a (Heja *et al.*, 2012). MASP-2 has a crucial role in lectin pathway; it cleaves not only C4 to C4b and C4a, but also C2 to C2b and C2a. C3 convertase (C4b2a) is formed by the binding of C4b to C2a. C3 convertase cleaves C3 to C3b and C3a and forms C5 convertase which cleaves C5 to C5b and C5a leading to lysis of microbes by formation of the membrane attack complex (Wallis *et al.*, 2010a).
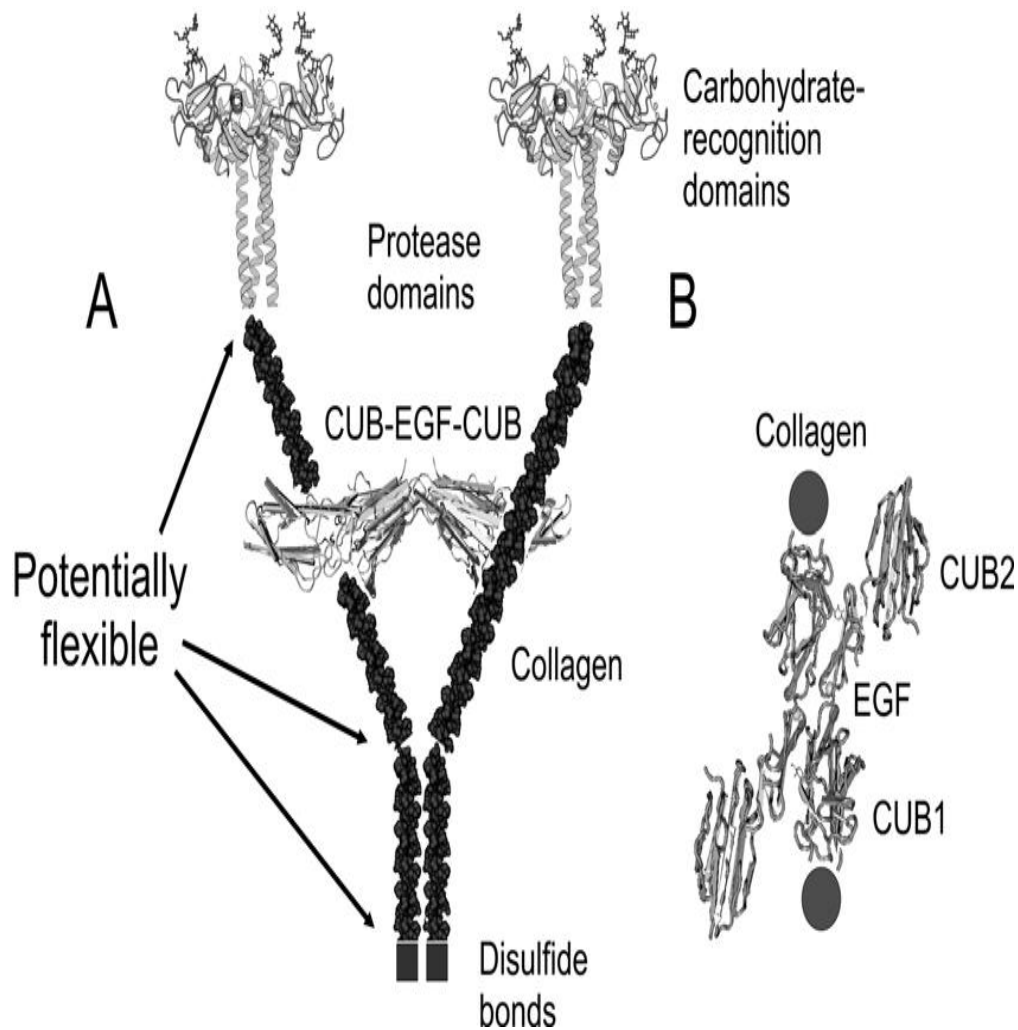
Figure 1-4 The interaction between MBL and MASP-2.
(Wallis & Lynch, 2007).

1.5.3   MASP-3

The gene encoding both MASP-1 and MASP-3 is located on chromosome 3q27-28 and consists of 18 exons. MASP-1 and MASP-3 share an identical N-terminal sequence covering the complete A-chain and including all CUB, EGF and CCP domains. They only differ in their C-terminal domain also known as a light chain (B-chain) or serine protease domain (Paréj *et al.*, 2014; Degn *et al.*, 2010). MASP-3 is expressed in the liver and also in spleen, small intestine, lung, thymus and brain and is secreted to the serum at concentration   6.4

µg/ml (Lynch *et al.*, 2005; Skjoedt *et al.*, 2010). Previous studies showed that MASP-3 is unable to cleave C2, C4 or C3, while more recent reports show that MASP-3 fulfils an important physiological role as a positive regulator of the alternative pathway by converting pro-factor D into its enzymatically active form (Iwaki *et al.*, 2011).

1.6   Carbohydrate recognition molecules of the lectin pathway

The lectin pathway is driven by soluble recognition subcomponents binding to specific pathogen associated molecular patterns (PAMPs). These molecules either belong to a collectin family (characterized by a long collagenous region and the possession of a C-terminal C-type lectin carbohydrate recognition domain (MBL and CL-11) or they belong to the ficolin family characterized by a long collagenous region and a C-terminal fibrinogen–like domain (Fujita, 2002). In man, the lectin pathway recognition molecules include MBL, Collectin-11, FCN1 (M-ficolin), FCN2 (L-ficolin) and FCN3 (H-ficolin), while their murine analogues are MBL-A, MBL-C, Collectin-11, ficolin A and ficolin B (Fujita, 2002; Ali *et al.*, 2012).

1.6.1   Ficolins

 Ficolins play an important role in defending the host against the pathogens. Their ability to activate the lectin pathway via recognition of pathogen associated molecular patterns (PAMPs) on the surface of invading microorganisms widens the spectrum of PAMPs that can be recognized by and can activate the LP (Endo *et al.*, 2011). All ficolins contain a cysteine rich N-terminal domain; followed by a collagen like domain, a neck region and fibrinogen-like domain which is the carbohydrate recognition domain of ficolins (Fig 1-5). It has been found that ficolins associate with the serine proteases via the collagen-like domain (Wallis *et al.*, 2004). Three of these polypeptides bind together in the collagen like domain to form a homotrimer of ficolin. These homotrimric subunits form ficolin polymers through a disulfide bond in the N-terminal domain that allows complexes formed of oligomers of homotrimers. Most ficolins have the ability to bind to N-acetylpolysaccharides such as N-acetylgalactoseamine (GalNAC) and N-acetylglucoseamine (GlcNAC) (Matsushita, 2013).

There are three types of ficolins in human, M-ficolin (ficolin 1), L-ficolin (ficolin 2, P35) and H-ficolin (ficolin 3 or Hakata antigen) (Matsushita, 2013; Endo *et al.*, 2011; Matsushita *et al.*, 1996). M-ficolin is synthesized in lung, spleen and monocytes. This type of ficolin is found in a low concentration in serum in comparison with the other human ficolin types (Honore *et al.*, 2008; Wittenborn *et al.*, 2010; Teh *et al.*, 2000). L-ficolin is produced in the liver and excreted in serum, while H-ficolin is produced in the lung and liver by the epithelial cells of bile duct and secreted into the serum and bile (Endo *et al.*, 2011; Matsushita, 2013). The murine ficolins are called ficolin A and ficolin B. Ficolin A is produced in the liver and spleen and is secreted into the serum while ficolin B is produced in myeloid cells. Ficolin A is considered the mouse orthologue of human L-ficolin and 80% of its amino acid sequence is identical to the amino acid sequences of ficolin L, ficolin M and ficolin B (Endo *et al.*, 2011).
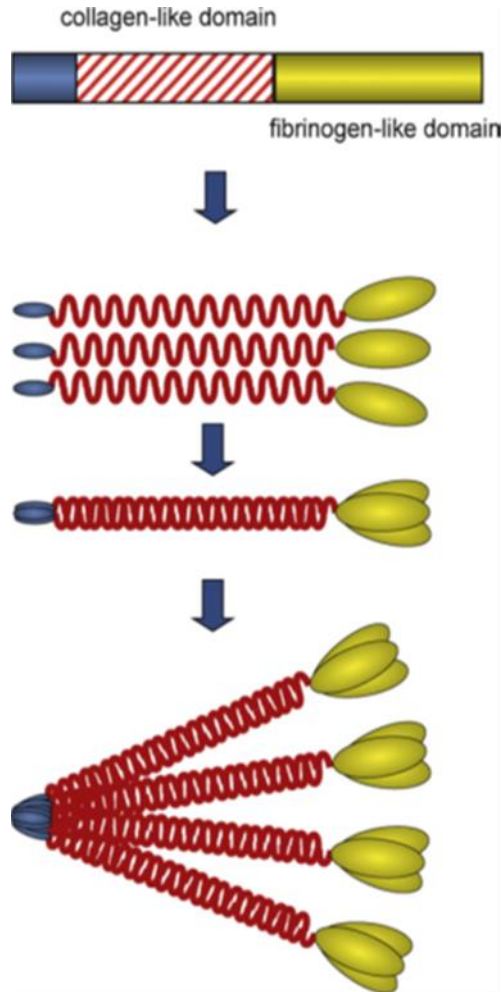
Figure 1-5 Ficolin structure
Ficolins contain a cysteine rich N-terminal domain, followed by a collagen like domain, a neck region and fibrinogen-like domain. Three of these polypeptides bind together in the collagen like domain to form a homotrimer of ficolin. These homotrimeric subunits form ficolin polymers through a disulfide bond in the N-terminal domain that allows complexes formed of oligomers of homotrimers.(Matsushita, 2013).

1.6.2   Collectins

Collectins are calcium dependent carbohydrate recognition molecules which have an important role in activation the lectin pathway. Collectin polypeptides are composed of N-terminal cysteine-rich domain, a collagen domain, neck region and carbohydrate recognition domain (CRD) (Gadjeva *et al.*, 2004). These polypeptides are called monomers and the presence of neck domain may facilitate the forming of homotrimers (Trimeric subunits) by twisting three identical monomers together in the collagen like region. Oligomers are formed as a result of bonding of trimeric subunits by disulfide bonds depending on the type of collectin (Veldhuizen *et al.*, 2011; van de Wetering *et al.*, 2004). The oligomerization state of the collectins, as well as the number of ligands on the surface of pathogens, affect the affinity of binding to the pathogens (van de Wetering *et al.*, 2004). The Collectin family includes mannan binding lectin (MBL), Surfactant protein A and D (SP-A and SP-D), collectin placenta 1 (CL-P1), collectin liver 1 (CL-L1; CL-10) and collectin kidney (CL-K1) or collectin 11(Selman & Hansen, 2012). Of these, only MBL, CL-10 and CL-11 are currently known to be directly involved in complement activation.

1.6.2.1   Mannan binding lectin (MBL)

MBL is a plasma protein which binds not only to mannan but also mannose, fucose and N-acetylglucosamine (Kilpatrick, 2002).It forms oligomers of homotrimeric subunits and each subunit is composed of three identical polypeptide chains twisted to each other (Figure. 1.6). MBL is present as a complex with MASPs and involved in activating these proteins after binding to the carbohydrate patterns (Gadjeva *et al.*, 2004). In the mouse, there are two types of MBL, MBL-A (serum MBL) and MBL-C (liver MBL) while in the human only one MBL (Kilpatrick, 2002). MBL is mainly synthesized by the liver (hepatocytes) and then secreted into the blood. The concentration in the blood varies depending on variation in coding regions and promoter of the MBL gene (Lu *et al.*, 2002). Once MBL binds to carbohydrate patterns on the surface of pathogens, MASP-2 will be activated leading to the activation of the complement cascade via the LP. MBL has the ability to bind to different bacteria, viruses, parasites and fungi such as *Staphylococcus aureus*, *Klebsiella aerogenes*, *Mycobacterium*

avium, *Mycobacterium tuberculosis*, *Neisseria meningitides*, *E.coli*, *Cryptococcus neoformans*, and *Listeria monocytogenes* (Carroll & Sim, 2011).
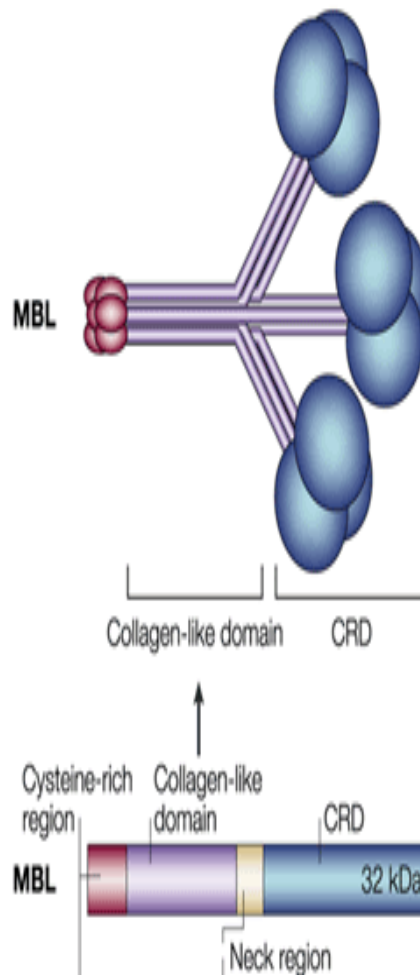
Figure 1-6 The structure Mannan binding lectin MBL
Schematic representation of MBL structure, showing the polypeptide chain subunit and the higher oligomeric forms (Fujita, 2002).

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1.6.2.2   Collectin Kidney CL-K1 (Collectin-11)

Collectin -11 is a secreted protein, which belongs to collectin family. However, it was first called collectin Kidney 1 (CL-K1) when it was identified in 2006 by Kishi *et al*. 2006 as a result of its presence in kidney at a high level (Hansen *et al.*, 2010). Recent research has demonstrated that CL-11 is not only found in kidney tissue (distal tubules, glomerulus and proximal tubules) in murine and human kidneys, but also in the adrenal gland (zona fasciculate, glomerulosa and reticularis) , liver (hepatocytes) , thymus, pancreas, placenta, spinal cord and intestine (Motomura *et al.*, 2008; Hansen *et al.*, 2010; Ohtani *et al.*, 2012). The concentration of CL-11 in human blood is relatively low (approximately 0.34 ± 0.13 µg/ml) in comparison to the serum concentration of MBL (1.2-5 µg/ml) (Yoshizaki *et al.*, 2012). According to HUGO (Human Genome Organization) the human collectin 11 gene *COLEC11* is located on chromosome 2 at position 2p25.3 (Ohtani *et al.*, 2012). The *COLEC11* gene consists of seven exons and 11 isoforms of mRNA transcripts. Only isoform a is a full-length protein, which is detected in circulation, while the other isoforms (isoform c, d, e) were detected and are characterized by the partial absence of coding sequences for the exons encoding the collagen-like region. In addition isoforms (b, f, g, h, i, j) can differ in the transcription start sites and may also differ in the untranslated regions within these exons (Figure 1.7). The mRNA encoding a full-length CL-11 transcript comprises an open reading frame of 813 nucleotides encoding 271 amino acids (Selman & Hansen, 2012). In mouse, The *COLEC11*gene is located on (chromosome 12; region 12 B1). The gene consists of seven exons and one isoform of mRNA transcripts. The mRNA encoding full- length mCL-11 has an open reading frame comprised of 816 nucleotides which codes for a peptide sequence comprising 272 amino acids. (NCBI)

Recent data indicate that there is a polymorphism in the promoter region of *COLEC11* gene 9570 C > T (rs3820897) associated with increased serum concentration levels of CL-11 (an increase of 10%, P = 0.044). However, CL-11 p.His219Arg (rs7567833) in the carbohydrate recognition domain (CRD) (minor allele frequency 0.033) has no effect on the level of CL-11 in serum among healthy Danish Caucasians (Bayarri-Olmos *et al.*, 2015). CL-11 p. His219Arg (rs7567833) has been associated with decreased (a decrease of approximately 40% in serum concentration; P = 0.03) CL-11 level in serum among Nigerian individuals (Antony *et al.*, 2015). The sequence identity at the amino acid level of collectins is ranging

between 68% - 92 % spanning from zebrafish to human (Selman & Hansen, 2012). Collectin polypeptides are composed of N-terminal cysteine-rich domain (cysteine residues participate in disulphide bridging between polypeptides and subunits, a collagen domain, neck region (a C-X-C motif in the beginning of the neck region) and carbohydrate recognition domain (CRD) which is similar to CRDs of the other collectins (short with 4 cysteine residues but without N-linked glycosylation motifs). Collectin 11 tends to form dimers of trimeric subunits which are held together by disulfide bonds (Hansen *et al.*, 2016)(Figure1.7). The molecular weight of recombinant human CL-11 on SDS PAGE is approximately 34 kDa under reducing conditions and 100 – 200 kDa under non - reducing conditions. CL-11 tends to assemble into higher oligomers of the basic homotrimer i.e. dimers (200 kDa), trimers (300 kDa) and multimers of subunits (>300 kDa) in serum. After treating the recombinant CL-11 with collagenase, the polypeptide subunit runs at about 18 kDa on an SDS polyacrylamide gel under reducing conditions and 50 kDa under non-reducing conditions. It is emphasized that the cysteine residues (Cys88 and Cys90) in the neck domain have a role in stabilizing the trimeric subunit by forming disulfide bonds (Hansen *et al.*, 2010; Selman & Hansen, 2012; Hansen *et al.*, 2016). Collectin 11 shows binding to mannose (α 1-2) mannose, -mannose, L-fucose, and GlcNAc. Also, collectin 11 showed strong binding activity to disaccharide D-mannose (1-2) -d-mannose by successful co-crystallization of the neck-CRD fragment (Girija *et al.*, 2015). In addition to carbohydrate binding, CL-11 shows binding to ligands with a negative charge (DNA and RNA) and sulfated compounds (such as heparin). These bindings to carbohydrates and negative charge ligands led to clearance of apoptotic cells (Hansen *et al.*, 2016). Collectin 11 has the ability to bind to *Escherichia coli*, *Klebsiella pneumonia*, *saccharomyces cerevisiae, Candida albicans*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and influenza A virus (Hansen *et al.*, 2010; Troegeler *et al.*, 2015).

Recently, CL-11 was found in the circulation as heteromeric complexes with another collectin, collectin L1 (CL-L1, CL-10) and these complexes are named CL-LK (Henriksen *et al.*, 2014). These heteromers (CL-LK) formed complexes with MASP-1 and MASP-3 and MASP-2 and activated the complement system by interacting with MASP-2 and MASP-1. The activation of complement is depended on oligomerization of CL-LK complex (Hansen *et al.*, 2016; Kjaer *et al.*, 2016). CL-11 has a role not only in complement activation but also

in developmental processes. Recent researches indicate that mutations in *COLEC11* and MASP-1 genes may cause a rare autosomal disorder named Carnvale,Mingarelli Malpuech and Micheles Syndrome (3MC syndrome) (Rooryck *et al.*, 2011). This syndrome includes different symptoms such as a facial cleft, and abnormalities of the renal system, male genitalia, skull growth and learning disability. Therefore, collectin 11 and MASP-3 may play a major role during embryogenesis (Ohtani *et al.*, 2012). So far, five mutations in *COLEC11* were associated with the 3MC syndrome. Three mutations are located within the CRD region (one deletion mutation deleting Ser217 and two substitutions. i.e. Gly204 Ser, and Ser169Pro) and two mutations in collagenous domain (frameshift mutations Gly101Val and Phe16 Ser). The mutations within the *MASP1* gene include substitutions in exon 12 (which lead to a substitution in the serine protease domain of MASP-3 (Cys630Arg, His497Tyr and Gly666Glu) (Rooryck *et al.*, 2011). Recent research showed there is a relation between CL-11 and coagulation. High levels of CL-11 (median plasma concentration increased approximately 70%) were observed among patients with disseminated intravascular coagulopathy (DIC) which resulted from a dysregulation of the coagulation system which causes bleeding and thrombosis at the same time (Takahashi *et al.*, 2014). Low serum levels of CL-11 were observed amongst patients with systemic lupus erythematosus (SLE) (average plasma concentration was decreased by approximately 20%) (Troldborg *et al.*, 2015). While the complement system fulfills an important role to protect the host from infectious disease and to scavenge debris and dead cells, under pathophysiological conditions, it can also cause damage to host cells like for example by promoting ischemia/reperfusion injury in ischaemic tissues (Wallis & Lynch, 2007). It was shown that CL-11 expression increases in renal tubular cells during the post-ischaemic period and drives the inflammatory response via CL-11 mediated lectin pathway activation (Distressed renal tubule cells drive CL-11 dependent and MASP-2 dependent activation by binding to altered pattern of fucosylated ligands)(Farrar *et al.*, 2016).

Also, high serum levels of CL-11 were observed amongst patients with schistosomiasis caused by Schistosoma haematobium and there is a correlation between variant in exon 8 of the CL-11 gene with urinary schistosomiasis (Antony *et al.*, 2015). Although, the serum

levels of CL-L and CL-K were reduced in patients infected with Mycobacteria tuberculosis, there is no difference in susceptibility to *Mycobacteria tuberculosis* in mice deficient in CL-11 (Troegeler *et al.*, 2015). Moreover, murine and human collectin 11 binds to *Streptococcus pneumoniae* and tends to complex with MASP-2. Thus, collectin 11 may have a critical role in driving the activation of the lectin pathway on the surface of *Streptococcus pneumoniae* (Ali *et al.*, 2012) .
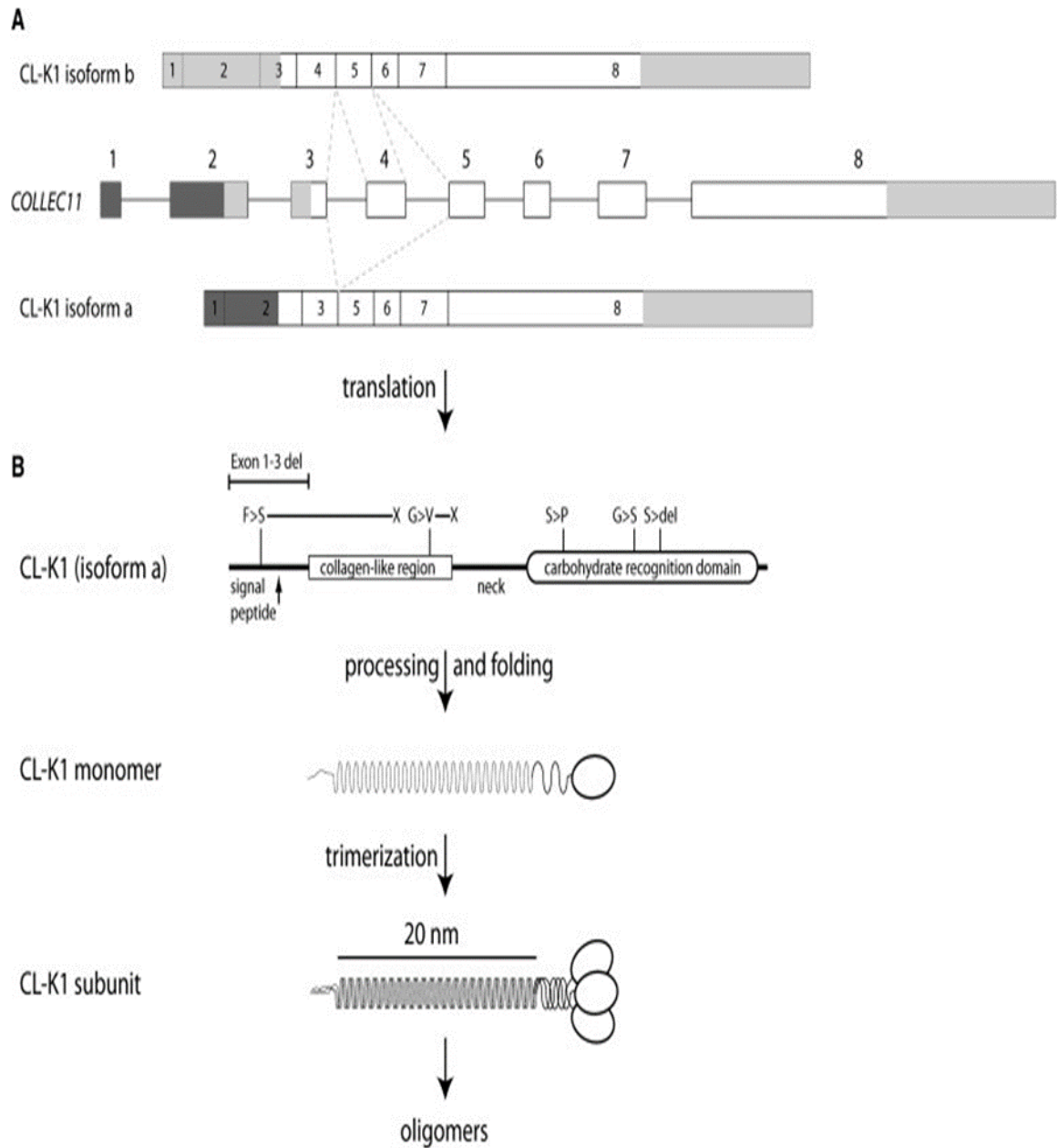
Figure 1-7 Figure Schematic representation the Gene Structure and Protein Products of COLEC11
(Degan et al., 2011)

1.6.2.3   Collectin Liver 1 (CL-L1, CL-10)

Collectin of Liver (CL-L1; also known as CL-10) belongs to the family of collectins. It was initially described as an intracellular hepatic protein due to its non –classical signal peptide, but later it was discovered that CL-L1 is also found in serum with concentration 3µg/ml (Henriksen *et al.*, 2013). CL-L1 is encoded by a *COLEC10* gene on chromosome 8 (q23-24.1 which consists of six exons and the mRNA encodes an ORF of 277 amino acids. CL-L1 is mainly synthesized in the liver, adrenal glands and placenta (Bayarri-Olmos *et al.*, 2015). CL-L1 was found to bind to D-mannose, N-acetylglucosamine, D-galactose, D-fucose, L-fucose (Ohtani *et al.*, 1999; Axelgaard *et al.*, 2013). CL-L1 has two N-linked glycosylation motifs in the CRD and EPS motif which also is found in chicken SP-A (Hansen *et al.*, 2016). A recent study shows that CL-L1 level was 20% lower (P > 0.001) in patients with systemic lupus erythematosus (SLE) compared to healthy controls (Troldborg *et al.*, 2015). There is a polymorphism in the *COLEC10* gene Arg125Trp detected among healthy Danish Caucasians, which is correlated with increasing the concentration of CL-L1 in the serum 40% (P = 0.0478) (Bayarri-Olmos *et al.*, 2015). Recently, as described above, CL-L1 was found in circulation as hetromeric complexes with CL-11 named CL-LK which related to the complement system (Henriksen *et al.*, 2013).

1.6.2.4   Collectin placenta (CL-P1, CL-12)

CL-P1 is an insoluble collectin founds on the cell membrane of endothelial cells (van de Wetering *et al.*, 2004). The gene *COLEC12* encoding CL-P1 localises in human chromosome 18p11.32 and the open reading frame of 2226 base pairs encoding 742 amino acids and mRNA encoding CL-P1 have been detected in the placenta, the lung and the heart (Ohtani *et al.*, 2001).The polypeptide chains are composed of a collagenous region, carbohydrate recognition domain (CRD), coiled-coil domains, intracytoplasmic domain and transmembrane domain. The structure of CL-P1 resembles that of scavenger receptors SR-AI and SR-A3 in replacing the cysteine-rich domain by a CRD. It can bind to *Escherichia coli*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* and also with oxidized low density lipoprotein (OxLDL) but not with acetylated LDL (AcLDL) (Ohtani *et al.*, 2012). Recently, it has been discovered that CL-P1 binds to C-reactive protein (CRP) and activates

the classical complement pathway through C1q and an alternative pathway via properdin (Roy *et al.*, 2016) . Also, it was found that CL-P1 in soluble form can activate an alternative pathway by binding *Aspergillus fumigatus* and interacting with properdin. (Ma et al., 2015). More recently, researches have been published showing that CL-P1 is associated with different diseases such as Alzheimer's disease, rheumatoid arthritis, diabetic retinopathy, breast and thyroid cancer (Nakamura *et al.*, 2006; Srivastava & Chen, 2009; Peng *et al.*, 2015; Espinal-Enríquez *et al.*, 2015; von der Heyde *et al.*, 2015).

1.7   The *Streptococcus pneumoniae*

 Streptococcus pneumoniae is a Gram-positive bacterium, that presents as diplococcic or short chains and is characterized by being non-spore forming, non-motile, capsulated and facultative anaerobic, growing at 5-10% CO_2. The identification and diagnosis of *S. pneumoniae* depends on the morphology of its colony, alpha-hemolytic activity, optochin sensitivity and bile solubility. The clinical importance of *S. pneumoniae* is due to its ability to cause mortality to one million people every year, especially for children with age under 5 years (Werno & Murdoch, 2008). This bacteria colonises the human nasopharynx and may cause invasive or non –invasive diseases. Non –invasive diseases include otitis media and sinusitis, while the transfer of this bacteria to sterile sites such as blood, pleural space and cerebrospinal fluid as a result of invasion from the epithelium cells of the respiratory tract lead to diseases such as pneumonia, septicemia, osteomyelitis, endocarditis, peritonitis, cellulitis and meningitis (Weiser, 2010; Dockrell *et al.*, 2012).

The pathogenicity of *S. pneumoniae* has been related to different virulence factors which include capsular polysaccharides (CPS), cell wall, cell surface protein, neuraminidase enzyme, and pneumolysin (Figure 1.8) (Mitchell & Mitchell, 2010; Mitchell, 2003).
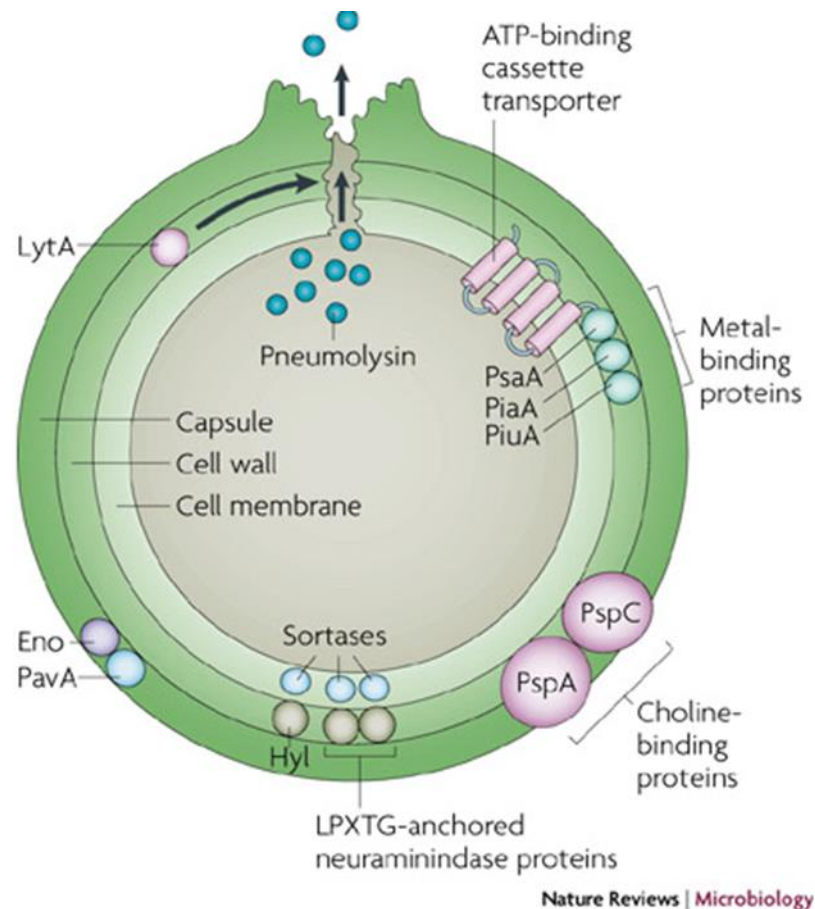
Figure 1-8 virulence factors of *Streptococcus pneumoniae*
(Kadioglu *et al*., 2008)

These factors help the *S. pneumoniae* to colonize and spread to the respiratory tract and cause the invasive or non –invasive diseases. The importance of these virulence factors differs during the infection, but they are combined together to give the full virulence to the *S. pneumoniae*. The first barrier of the immune system that fails to resist the *S. pneumoniae* is the mucus of the respiratory system. This happens with the polysaccharide capsule (CPS) which is one of the most important virulence factors of *S. pneumoniae,* which attaches to the bacterial cell wall as a 100-400nm thick shell (Petersen *et al.*, 2014). The negative charge of

sialic acid residues of the mucous is repulsed by the negative charge of the capsule and this enables the *S. pneumoniae* to escape from the entrapment by the mucus.

The capsular polysaccharide of *S. pneumoniae* is the basis for classifying it into 46 serogroups and 90 serotypes according to antibodies which are produced due to response to antigenic determinants on the capsule (Shapiro *et al.*, 1991; Henrichsen, 1995).The capsule is the main virulence factor of *S. pneumoniae*, the importance of it is due to its ability to prevent phagocytosis by preventing the interaction between iC3b or the Fc of immunoglobulins and the receptors of phagocytic cells. Also, it helps to prevent the mechanical removal of *S. pneumoniae* by mucus (Mitchell & Mitchell, 2010).

Pneumolysin (ply) is another virulence factor of *Streptococcus pneumonia,* which has a catalytic activity. This protein is expressed during the late log phase and release as monomers and form pores through the membrane of the host cells (Tilley *et al.*, 2005). Subsequent studies revealed that pneumolysin induces a proinflammatory response and increases the recruitment of cells, especially neutrophils, in mouse lungs after infection with PLY$^{-/-}$ pneumococci. Moreover, it has been shown that low concentrations of pneumolysin can induce a proinflammatory response by the interaction of pneumolysin with the receptor Toll-like receptor 4 (TLR4) leading to activate the NF-κB and the expression of proinflammatory cytokines (Mitchell & Mitchell, 2010). Pneumolysin may contribute to activation of the classical pathway without the presence the antibodies that are specific to pneumolysin and lead to stimulate the inflammatory response (Kerr *et al.*, 2005; Paterson & Orihuela, 2010).

S. pneumoniae has proteins in the cell wall which contribute to its pathogenicity. One of these proteins is pneumococcal surface protein A (PspA). This protein is located in the cell wall of all the strains of *Streptococcus pneumoniae* and protects it from phagocytosis by interfering with complement activation and C3, C4 deposition (Jedrzejas, 2001; Li *et al.*, 2007). It has been shown that mice infected with PspA deficient *S. pneumoniae* showed a reduction in their virulence in comparison to mice infected with the PspA sufficient *S. pneumoniae* (Ren *et al.*, 2004; Ren *et al.*, 2012).

The other cell wall proteins are pneumococcal surface protein C (PspC) also called choline binding protein A (CbpA) which has an important role in colonization and adherence of the

bacteria with the epithelial cells of lung and the nasopharynx. In addition, PspC binds to immunoglobulin A (IgA) , C3, and factor H. The binding to factor H may cause down regulation of the alternative pathway (Quin *et al.*, 2007; Ogunniyi *et al.*, 2007).

1.8 The role of complement system in pneumococcal infection

The role of complement pathway activation in defence against *S. pneumoniae* has been described recently in terms of the three complement pathways. This was performed by using mice deficient in one or more of the complement pathway components. It has been observed that human patients with C3 deficiency have sensitivity to recurrent infection by *S. pneumoniae*, *Neisseria meningitidis*, *Escherichia coli*, *Haemophilus influenzae*, *Streptococcus pyogenes* and *Staphylococcus aureus* (S Reis *et al.*, 2006). Circolo *et al.* (1999) used C3 deficient mice infected with *S. pneumoniae* and found that the bacteraemia increased 200 fold in comparison to the wildtype controls. C1q deficient mice showed a high susceptibility to pneumococcal infection in comparison to wild type mice (Yuste *et al.*, 2008) Also IgM deficient mice showed a high susceptibility to infection by *S. pneumoniae*. Brown *et al* 2002 speculated that the alternative pathway may be less important than the classical pathway in fighting *S. pneumoniae*. However, patients deficient in factor B and Factor I have increased susceptibility to *S. pneumoniae* and *N. meningitidis*. This may be as a result of the inability of those patients to regulate the alternative pathway either by being deficient of the AP or by causing a secondary C3 complement deficiency through consumption in the absence of factor I (S Reis *et al.*, 2006). Also, several studies showed that factor B deficient mice are highly susceptible to pneumococcal infection in mouse models of pneumonia, systemic and middle ear model (Tong *et al.*, 2010). The central role of the lectin pathway in fighting pneumococcal infection has been established using MASP-2 deficient and ficolin deficient mouse strains (Ali *et al.*, 2012). MBL appears to play only a minor role in the LP dependent defense against *S. pneumoniae*.

1.9   Aims and Objectives

1- Investigate the role of lectin activation pathway recognition molecule CL-11 in fighting *S. pneumoniae* D39.

2- Assess the susceptibility of CL-11 deficient C57BL/6 mice to experimental infections with *S. pneumoniae* D39.

3- Study the effect of genetic variation in the promoter on the level of CL-11 expression, and assess whether any of the SNPs of the human CL-11 gene correlates with a clinically established pre-disposition for frequent microbial infections

Chapter Two: Materials and methods

2   Chapter Two: Materials and methods

2.1   Material

2.1.1   Chemical materials

1kb plus DNA ladder	Invitrogen
Acetic acid glacial	Fisher scientific
Acrylamide -bis acrylamide	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Ammonium persulphate	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Barbital	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Brilliant Blue R-250	Fisher scientific
Bromophenol Blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Deoxyadinosine triphosphate (dATP)	Thermo scientific
Deoxyribo nucleotide PCR grade (dNTPs)	Promega
Dialysed fetal bovine serum	Invitrogen
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich

Dimethyl sulfoxide (DMSO)   Sigma-Aldrich

Divinyl sulfone   Fluka

Ethanol   Fisher Scientific

Ethidium bromide   Sigma-Aldrich

Ethylene   glycol-bis-N,N,Ń,Ń-tetraacetic   Sigma-Aldrich
acid (EGTA)

Ethylenediaminetetraacetic acid (EDTA)   Sigma-Aldrich

Foetal calf serum   Harlan

Gelatin   BDH

Glycerol   Fisher scientific

Glycine   Fisher scientific

HEPES   Sigma-Aldrich

Horse blood   Oxoid

Isopentane   Fisher Scientific

Isopropanol   Fisher Scientific

Magnesium chloride   Sigma-Aldrich

Mannan   Sigma-Aldrich

Mannose   Sigma-Aldrich

N-acetyl BSA   Promega

N-acetylcysteine   Promega

Oligo (dT)23 anchored primers   Sigma-Aldrich

Oligonucleotides   Eurofin

Phosphate Buffered Saline   Oxoid

Proteinase K   Qiagen

RNAse A solution   Promega

RNase H   Promega

RNasout   Promega

Sigma Fast p-Nitrophenyl Phosphate tablet   Sigma-Aldrich

Sepharose-6B   Sigma-Aldrich

Skim milk   Sigma-Aldrich

Sodium azide   Sigma-Aldrich

Sodium bicarbonate   Sigma-Aldrich

Sodium carbonate an hydrous   Fisher scientific

Sodium chloride   Fisher scientific

Sodium dodecyl sulphate (SDS)   Sigma-Aldrich

Superscript II reverse transcriptase   Invitrogen

T4 DNA ligase   New England Biolabs

T4 DNA ligase 10X buffer   New England Biolabs

Taq DNA polymerase   Thermo

Tetramethylethylenediamine (TEMED)   Sigma-Aldrich

Thermoprime plus DNA polymerase   Thermo scientific

Triazol   Invitrogen

Tris-HCl   Sigma-Aldrich

Triton X-100   BDH laboratories

Trizma base   Sigma-Aldrich

Tween 20   Sigma-Aldrich

Xhol I restriction enzyme   New England Biolabs

Zymosan   Sigma-Aldrich

β-mercaptoethol   Sigma-Aldrich

Power SYBER®Green PCR Master Mix   Applied Biosystems

Hind III restriction enzyme   New England Biolabs

High fidelity polymerase   New England Biolabs

Tris Base   Fisher Scientific

Penicillin/Streptomycin   Sigma

1X-Trypsin-EDTA solution   Sigma

Hygromycin B   Invitrogen

Methotrexate (MTX)   Sigma-Aldrich

Lipofectamine® LTX Reagent   Invitrogen

2.1.2   Kits

pGEM-T easy vector   Promega

QIAquick gel extraction kit   Qiagen

Wizard genomic DNA purification kit   Promega

Wizard   plus   SV   minipreps   DNA   Promega
purification kit

dual luciferase assay   Promega

TURBO DNA-freeTM kit   Invitrogen.

SuperScript II Reverse Transcriptase   Invitrogen

2.1.3   Media

F-12 Nutrient Mix   Gibco

CHO-S-SFM II   Gibco

Minimum Essential Medium (MEM) alpha   Sigma-Aldrich

(-ribonucleosides, -deoxyribonucleosides)

Opti-MEM® I Reduced Serum   Invitrogen

Agar   Oxoid

Blood Agar Base   Oxoid

Brain heart infusion (BHI) medium   Oxoid

Minimal   Essential   Media   α   with   Gibco
nucleosides (MEM α+)

Minimal   Essential   Media   α   with   Gibco
nucleosides (MEM α-)

CHO-S-SFMII   Gibco

DMEM serum media   Gibco

2.1.4   Antibodies

Mouse anti-poly-histidine-tag	Sigma
Rabbit polyclonal Anti-human C3c IgG	Dako
Anti-rabbit polyclonal IgG1 antibody	Sigma
Anti-chicken polyclonal antibody	Sigma
Chicken anti-human C4c	Immunsystem AB
Rabbit anti mouse collectin 11	Proteintech Europe
Anti-rabbit IgG HRP	Dako

2.2   Methods

2.2.1   In *vitro* studies

2.2.1.1   *S. pneumoniae* D39 culture

S. pneumoniae D39 was cultured from the frozen stock on blood agar plate (4% blood agar base (w/v) prepared in 1 L of distilled water and autoclaved then supplemented with 5%(v/v) horse blood).

2.2.1.2   Optochin sensitivity test

S. pneumoniae D39 was cultured on blood agar plate and optochin disks were added and then incubated in 5% CO_2 overnight at 37°C.

2.2.1.3   Preparation of formalin fixed *S. pneumoniae*

The fixation of *S. pneumoniae* D39 was done after inoculating the bacteria from blood agar plate in 10 ml of brain heart infusion agar and incubating overnight at 37°C. After that bacteria were spun at 3000 rpm for 10 minutes ,the pellet was washed three times with PBS pH 7.4 and suspended with 0.5% formalin in PBS at room temperature for1- 3h. Following

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the fixation, bacteria were washed two times with PBS and re-suspended in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and the OD550nm was adjusted to be 0.6.

2.2.1.4   Preparation of mouse sera

Blood was collected from mice by cardiac puncture and transferred immediately on ice for 3 hours to prevent complement activation. Then the blood was spun down using a refrigerated centrifuge at 7000 rpm for 7 minutes. The serum was separated and stored in -80˚C.

2.2.1.5   Complement pathway specific Enzyme Linked Immunosorbent Assays (ELISAs)

2.2.1.5.1 CL-11 binding assays

Microtitre ELISA plates were coated with formalin - fixed *Streptococcus pneumoniae* (OD_{550nm}=0. 6), or with 10 µg/ml zymosan, or 10 µg/ml mannan, or 10 µg/ml N-acetyl BSA, or 10 µg/ml N-acetyl cysteine or 10 µg/ml DNA, then incubated overnight at 4 ˚C. All these ligands were suspended or dissolved in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and 100 µl was added to each well. After that the protein-binding site were blocked using 300 µl of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 10 mM Tris HCl, 140 mM NaCl, pH 7.4),and the plate incubated for two hours. Then it was washed three times with 250 µl of washing buffer (TBS with 0.05%, Tween 20 and 5 mM $CaCl_2$) and serial dilutions of mouse serum in Barbital buffer saline (BBS) (4 mM barbital, 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween-20 pH 7.4) were added in duplicate to the plate starting from 1/40. In order to detect rCL-11 binding to those ligands, rCL-11 was diluted in a serial dilution with Barbital buffer and added to the wells instead of serum. The plate was incubated at 37 ˚C for 60 minutes. 100 µl Rat anti mouse collectin 11 1:500 (Kindly provided by Soren Hansen) was added after washing the plates three times and incubated for 90 minutes. The wells were washed three times and 100 µl of alkaline phosphatase conjugated goat anti -rat antibodies (Sigma-Aldrich) diluted 1:10000 was added and the plates incubated at room temperature for 90 minutes. The plates were washed again three times and 100 µl of the Colorimetric substrate pNPP (Sigma-Aldrich) was added and the absorbance at 405 nm was measured by BioRad microtitre plate reader.

2.2.1.5.2 C3 deposition assay

Microtitre ELISA plates were coated with formalin - fixed *Streptococcus pneumoniae* (OD $_{550}$ =0. 6), or 10 µg/ml zymosan, or 10 µg/ml mannan, or 10 µg/ml N-acetyl BSA, or 10 µg/ml N-acetyl cysteine or 10 µg/ml DNA then incubated overnight at 4˚C, as above. After that the protein-binding sites were blocked using 300 µl of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 10 mM Tris, 140 mM NaCl, pH 7.4) and the plate incubated for two hours. Then it was washed three times with washing buffer (TBS with 0.05%, Tween 20 and 5 mM CaCl2) and serial dilutions of mouse serum were prepared in Barbital buffer saline (BBS) (1 mM MgCl2, 5 mM CaCl2, 4 mM barbital, 145 mM NaCl, 0.1% gelatin, pH 7.4) starting with 1/40. The plate was incubated at 37 ˚C for 90 minutes. 100 µl rabbit anti C3c (Dako) diluted 1:5000 in wash buffer was added after washing the plates three times and incubated for 90 minutes at 37 ˚C. The wells were washed three times and 100 µl of alkaline phosphatase conjugated goat anti -rabbit antibody diluted 1:10000 in washing buffer was added and the plates incubated at room temperature for 90 minutes. The plates were washed again three times and 100 µl of the Colorimetric substrate pNPP was added and the absorbance at 405 nm was measured by BioRad microtitre plate reader.

2.2.1.5.3 C4 deposition assay

Microtiter ELISA plates were coated and blocked as previously described. The wells were washed with washing buffer (TBS containing 0.05% tween-20 and 5 mM CaCl2), then mouse serum dilution in MBL binding buffer (10 mM CaCl2 1M NaCl, 20 mM Tris-HCl, pH 7.4) starting with 1/40 dilution was added to the wells and incubated for 1h at 37 ˚C . Then, Plates were washed again three times and 100 µl of chicken anti-mouse C4 diluted 1:500 in wash buffer was added and incubated at room temperature for 90 minutes. After washing the wells three time, 100 µl of AP conjugated goat anti-chicken diluted 1:10,000 in wash buffer and incubated at room temperature for 90 minutes. After washing the wells, the substrate p-nitrophenyl phosphate (pNPP; Sigma fast p-nitrophenyl phosphate tablets, Sigma-Aldrich) was added and absorbance at 405 nm was measured in BioRad ELISA micro-titre plate reader.

2.2.1.5.4 CL-11 reconstitution

Microtiter ELISA plates were coated, blocked and washed as previously described in (2.2.1.5.1). rCL-11 was prepared in a serial dilution with Barbital buffer saline (BBS) (4 mM barbital, 145 mM NaCl, 1 mM MgCl2, 2 mM CaCl2, 0.1% gelatin, pH 7.4) starting with concentration 50 µg/ml, and 100ul added per well. The plate was incubated at 37 ˚C for 90 minutes, then washed three times with washing buffer. 2.5% of CL-11 $^{-/-}$ serum were prepared in Barbital buffered saline (BBS) (1 mM MgCl2, 5 mM CaCl2, 4 mM barbital, 145 mM NaCl, 0.1% gelatin, pH 7.4) and 100ul added per well. The plate was incubated at 37 ˚C for 30 minutes. 100 μl rabbit anti C3c (Dako) diluted 1:5000 was added after washing the plates three times and incubated for 60 minutes. The wells were washed three times and was added 100 μl of alkaline phosphatase conjugated goat anti -rabbit antibodies (Sigma-Aldrich) diluted 1:10000 in washing buffer and the plates incubated at room temperature for 60 minutes. The plates were washed again three times and 100 μl of the colorimetric substrate pNPP (Sigma-Aldrich) was added and the absorbance at 405 nm was measured by BioRad microtitre plate reader.

2.2.2 In *vivo* studies

2.2.2.1 Mice

Two mouse genotype female were used; CL-11 deficient mice and Wild type mice of C57BL/6 background. Female C57BL/6 wild type mice were obtained from Charles River Laboratories, UK and CL-11 deficient mice on C57BL/6 background were purchased from Mutant Mouse Resource and Research Center (MMRRC) and bred in house. Mice ranging in age from 8 to 10 weeks old were used in all *Streptococcus pneumoniae* D39 infection studies. All procedures used were under the project licence 60/4327 following the guidelines of the Animal Scientific Procedure Act 1986 of the UK Home Office.

2.2.2.2   Streptococcal infection study

2.2.2.2.1   Preparation of non-passaged bacteria

Streptococcus pneumoniae D39 was cultured in 10ml BHI broth (Oxoid) for 18 hours at 37 °C in CO_2 gas jar .Next day, bacteria were pelleted and re-suspended in 1ml of BHI with 20% v/v heat inactivated foetal calf serum. 700 µl of re-suspended bacteria was inoculated into 10 ml of BHI with OD550 of 0.6 and incubated at 37 C until bacterial growth reached OD550 = 1.6. The bacterial culture was distributed in 500 µl aliquots and then stored in -80 °C. Viable count of the bacteria was determined by Miles and Misra count after 24 hours of storage at -80 ºC (Miles *et al.*, 1938).

2.2.2.2.2   Preparation of passaged bacteria

Non-passaged *Streptococcus pneumoniae* was inoculated in 10 ml BHI and incubated overnight at 37ºC.Next day, the bacteria were pelleted and washed 3 times with phosphate buffer saline (PBS, pH 7.4). Then, the pellet was re-suspended in 5ml PBS and 50 µl of the suspension was injected intraperitoneally in 8-10 week old MF1 mouse strain purchased from Harlan Olac Ltd. When the symptoms reached the ++ starry coat, a blood sample was collected by cardiac puncture after anaesthetising the mice with 5% v/v isoflurane and 1.6-1.8 L O_2 /min. 50 µl of blood sample was inoculated into 10 ml of BHI and incubated overnight at 37 °C. Next day, the bacterial growth was centrifuged at 3000rpm for 10 and the pellet re-suspended into 30 ml of BHI with 20% foetal calf serum and incubated at 37°C with initial OD500 of 0.6.When the OD500 of bacterial growth reached 1.6, 0.5 ml aliquots of the culture were made and stored at -80 ° C. The next day, the viability of the bacteria was determined by the viable count (see section 2.2.2.4). In order to test the virulence of the passaged *Streptococcus pneumoniae,* one aliquot tube was thawed and centrifuged for 2 minutes at 13000 rpm. Then the pellet was suspended in 500 µl of PBS and adjusted to 1×10^6 CFU/50 µl. 10 MF1 mice were intranasally infected with 50 µl of *Streptococcus pneumoniae* 1×10^6 CFU. Then the mice were culled when they became lethargic. The dose was considered virulent when 80% or more of mice succumbed to infection during 48-72hrs.

2.2.2.2.3 Infection of mice

The infection experiment was carried out in 8-10 week old female CL-11$^{-/-}$ and CL-11 $^{+/+}$ mice to detect the survival of these mice after infection with *Streptococcus pneumoniae*. Mice were anaesthetised with 2.5% (v/v) fluothane over oxygen (1.5 to 2 litre/min).Then, *S. pneumoniae* infection doses (1x10^6 CFU/50 μl) were administered into the nostrils of the mice. Mice were monitored every three hours for illness symptoms (hunched appearance, starry coat, lethargy, moribund). When the mice were showed symptoms (lethargic ++), they were culled by cervical dislocation.

2.2.2.2.4 Determination of blood and lung bacterial burdens

Following infection of CL-11 $^{-/-}$ and CL-11 $^{+/+}$ mice, a number of randomly selected mice from each group were culled by cervical dislocation and blood was collected immediately afterwards by cardiac puncture at pre-chosen time points (12, 24, 36, 48 and 60-hour post infection). Lungs were removed into 5 ml of sterile PBS, weighed, and homogenised by Ultra-Turrax T10 basic IKA- Werke Homogenizer. Viable counts in blood and lungs were determined by 10 folds serial dilution in sterile PBS. Then, 60 μL were plated from each dilution onto blood agar plates supplemented with 5% (v/v) horse blood (oxoid) and incubated overnight at 37°C under anaerobic conditions. The numbers of colonies were counted and CFU/ml was calculated. CFU/mg was calculated by following equation:

CFU/mg = ([No. colonies x dilution factor x 1000 x volume of homogenising solution] /volume plated in μL) /organ weight in mg.

2.2.2.2.5 Freezing of lungs for mRNA extraction

For total RNA extraction of lungs, pre-cooled isopentane (Fisher Scientific) on dry ice was used for flash freezing of the lungs which were then stored in -80°C.

2.2.3   Molecular Biology techniques

2.2.3.1   DNA methods

2.2.3.1.1   Screening of CL-11 deficiency in mice

2.2.3.1.1.1 Isolation of genomic DNA from mouse ear snips

Wizard genomic DNA purification Kit (Promega) was used to isolate genomic DNA from the mouse ear snips. DNA extraction was achieved by incubating 0.3cm of ear snips overnight in 60 μl of 0.5M   EDTA, 250 μl of the Nucleic Lysis Solution and 10μl of proteinase K (Qiagen). The next day, 1.5 μl RNAse A solution (4mg/ml) was added and incubated at 37˚C for 30 minutes, followed by addition 100μl of protein precipitation solution (Promega). The samples were centrifuged for 10 minutes at 13000 rpm and the supernatant that contains the DNA was transferred to new 1.5 ml Eppendorf tubes and the DNA was added 300 μl Isopropanol. After centrifugation at 13000 rpm for 20 minutes 300 μl ethanol 70% was added to the pellet and centrifugation was done again at 13000 rpm .The ethanol was discarded and the pellet was dried, then 50 µl of the Nuclease –free water was added and the solution stored at 4˚C.

2.2.3.1.1.2 Genotyping of the mice

The Genotyping of mice was performed using the multiplex PCR for identification of CL$^{-/-}$ mice before using them in infection study. The PCR mix for the reaction was 15μl containing the reagents and primers in tables 2-1 and 2-2. This method includes three steps: Denaturation, Annealing and Extension. The temperature for denaturation was 95 ˚C, while for Annealing and Extension they were 62 ˚C and 72 ˚C respectively (table 2-3)

Table 2-1 Standard PCR reaction mixture used for genotyping

Reaction mixture contents	Volume per reaction
Genomic DNA diluted 1:10	1.5 µl
MgCl2 (2.5mM)	1.5 µl
dNTP mix. (10 mM)	0.3 µl
wto-F1	1.5 µl
scr-R1	1.5 µl
Neo3a	1.5 µl
Taq-DNA polymerase	0. 2 µl
Nanopure distilled water	5 µl

Table 2-2 The primers used for genotyping of CL-11 KO mice using thermocycler

Primer name	Primer sequence 5′ → 3′
CL-11 –wto-F1	CAGATTCTTGTCCCTGGCCTCA
CL-11–scr R1	CTCAGTGTCAGCTGAATAAATGCCA
Neo3a	GCAGCGCATCGCCTTCTATC

Table 2-3 The program used for genotyping of CL-11 KO mice using thermocycler

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	90 seconds	
Denaturation	95 ˚C	15 seconds	
Annealing	62 ˚C	30 seconds	
Elongation	72 ˚C	30 seconds	35
Final elongation	72 ˚C	5 minutes	
Hold	4 ˚C	∞	

2.2.3.1.2 Polymerase chain reaction of murine CL-11:

Multiplex PCR was used to produce large quantities of collectin 11 DNA from murine cDNA using the following primers and protocol (Table 2.4, Table 2-5 and table 2-6):

Table 2-4 The recipes for PCR reaction for amplifying DNA of mCL-11

Reaction mixture contents	Volume per reaction
10 ng mCL-11 cDNA	1 µl
High fidelity phusion buffer (5x)	5 µl
dNTP mix. (10mM)	0.5 µl
High fidelity-DNA phusion polymerase (5U/µl)	0.25 µl
Forward primer (5 µM)	2.5 µl
Reverse primer (5 µM)	2.5 µl
Nanopure distilled water	13.25 µl

Table 2-5 The program   used for amplifying DNA of mCL-11 using thermocycler

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	90 seconds	
Denaturation	95 °C	15 seconds	
Annealing	70 °C (-0.8C/cycle)	30 seconds	
Elongation	72 °C	40 seconds	15
Denaturation	98°C	15 seconds	
Annealing	58°C	30 seconds	
Elongation	72°C	40 seconds	25
Final elongation	72 °C	5 minutes	
Hold	4 °C	∞	

Table 2-6 The primers   used for amplifying DNA of mCL-11 using thermocycler   with his tag (N or C-terminal) and without his tag

Primer	Sequence (5' → 3')
mCL-11 *Hind III* F	AAG CTTGCCACCATGATG ATGAGGGACCTGGCTC
mCL-11 *Xho1* R	CTCGAGTCACAAGTTCTCTTTGTCAAACTCGCAGAG
mCL-11 *pst1* F	GACATACTGCAGGCCACCATGATGATGAGGGACCTGGCTCTT
mCL-11 *Ecor1* R	GCTAGAGAATTCTCACAAGTTCTCTTTGTCAAACT
mCL-11 C-HIS R	ACGTGAATTCAGTGATGGTGATGGTGATGCAAGTTCTCTTTGTCAAAC
mCL-11 N-HIS F	CTGCCATCTGGATGTCCTGCACATCACCATCACCATCACCAGCAGA CCACAGAGGAC
mCL-11 N-HIS R	GTCCTCTGTGGTCTGCTGGTGATGGTGATGGTGATGTGCAGGAC ATCCAGATGGCAG

 Sequences are the restriction sites (Blue), Kozak sequence (Red)

mCL-11 Hind III F primer and mCL-11 Xho1 R primer were used to amplify the full length of mCL-11and then cloning into pSecTag 2/ Hygro B vector. In order to amplify the full length CL-11 with C-terminal his tag or N-terminal his tag and then cloning into pED4 vector, the primers in (table 2-6) were used. For C- terminal his tag, mCLl-11 pst1 Forward primer and mCL-11 C-HIS Reverse primer were used. There were three PCR steps for adding his tag to N-terminal. In the first PCR step mCL-11 pst1 Forward primer and mCL-11 N-HIS Reverse primer were used, then in the second step mCL-11 N-HIS Forward primer and mCL-11 Ecor1 Reverse primer were used. The PCR product from first PCR   and second PCR were separated on an agarose gel and purified using the QIAquick Gel Extraction Kit

(Promega).50 ng from each PCR product were mixed together and used to amplify the full length of CL-11 N-terminal his tag.   Also mCL-11 pst1 Forward primer and mCL-11 Ecor1 Reverse primer were used to amplify the full length of CL-11 without his tag.

2.2.3.1.3 Purification of DNA from agarose Gel (band-prep)

Purification of DNA bands from agarose gel was carried out using QIAquick Gel Extraction Kit (Promega). This was performed by cutting the agarose containing DNA fragments with clean and sharp scalpel. 3 volumes of buffer QG were added to 1 volume of the gel and incubated at 50˚C for 10minutes until completely dissolved, then it was transferred to the QIAquick spin column and centrifuged for 1minute. After that, 500µl of buffer QG were added and centrifuged for 1minute and the column was washed by 750µl of washing buffer PE and centrifuged for 1minute. The column was centrifuged for another 1 minute, then the DNA was eluted by adding 50µl of nuclease free water.

2.2.3.1.4   A tailing of PCR products

Prior to ligation into TA-cloning vector pGEM-T easy vector that has 3' thymine overhangs in both ends, a 3´ terminal A overhang was added to the PCR products according to the following protocol (table 2.7):

Table 2-7 Protocol used for A-tailing of the purified PCR product

Material	Amount
100mM dATP	0.5µl
Gel extracted DNA	7µl
Taq-polymerase reaction buffer 10x (containing MgCl2)	2µl
DNA Taq-polymerase	0.5µl

The reaction was incubated at 70˚C for 25 minutes.

2.2.3.1.5   Cloning of PCR product into pGEM-T easy vector

The DNA construct was ligated into pGEM-T easy victor (Promega) according to the following protocol (figure 2.1 and table 2.8):
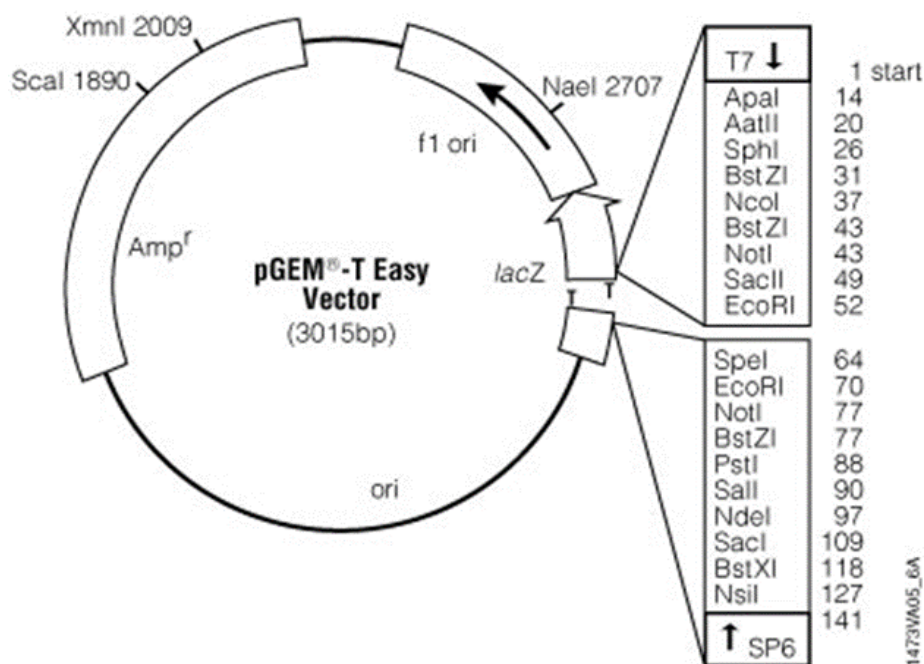
Figure 2-1 A diagram shows the restriction sites of restriction enzymes.
Adapted from the technical manual protocol of pGEM Easy Vector Systems. Promega

Table 2-8 Protocol used for cloning PCR product into pGEM-T easy vector

Material	Amount
2X rapid ligation buffer	5µl
pGEM-T easy vector (50ng)	1µl
PCR product	3µl
T4 DNA ligase	1µl

The reaction was incubated at 4˚C for overnight.

2.2.3.1.6   Preparation of chemically competent cells

In order to prepare competent cells Top 10 *E. coli* from the frozen stock were cultured onto LB agar plate. The plate was incubated for 16h at 37°C and one colony was transferred to 5ml of LB medium containing 20mM $MgSO_4$ and incubated overnight at 37°C. After incubation, 1ml of the overnight culture was transferred into 100ml of LB broth and incubated at 37°C for 2.5 to 3h until the OD550nm was 0.7- 0.8. Then, cells were spun for 10 minutes at 2000xg and re-suspended in 30ml of a sterile ice-cold of TFB1 buffer (K-acetate 3mM, $MnCl_2$ 50mM, KCl 100mM, $CaCl_2$ 10mM, Glycerol 15%) and incubated on ice for 5-30 min. After that, cells were harvested by centrifugation for 10 minutes at 2000xg at 4°C and re-suspended into 4ml of TPF II ($CaCl_2$ 75mM, Na-Mops 10mM, KCl 10mM, Glycerol 15%, pH 7.4), Then distributed in aliquots of 50 µl each   and stored at – 80 °C.

2.2.3.1.7   Transformation of chemically competent *E. coli*

 The chemically competent bacterial cells were thawed on ice and 2µl of the ligation reaction were added to 50µl of the bacterial stock, then incubated on ice for 20 minutes then exposed to heat shock in water bath at 37˚C for 4 minutes. After that, the tube was transferred again into ice for 2 minutes before transferring the reaction mix into 450µl of LB and incubating for 1 hour at 37˚C with gentle shaking. Then 50µl and 200 µl of this culture were plated on LB plate containing 50µg/1ml ampicillin and incubated overnight at 37˚C.

2.2.3.1.8   Isolation and purification of plasmid DNA

5 ml of LB medium containing ampicillin (100µg/ml) was inoculated with a single colony from the overnight culture and incubated overnight at 37˚C with shaking. Plasmid DNA purification was performed by using Wizard plus SV Minipreps DNA Purified System (Promega). The overnight bacterial culture was harvested for 10 minutes at 13000 xg and the cells resuspended with 250 µl of the Cell Resuspension Solution mixed by vortexing. After that, 250µl of Cell Lysis Solution were added and mixed by inverting the tube four times and incubated for 2 minutes at room temperature followed by the addition 10µl of Alkaline Protease Solution for 5 minutes. The cell lysate was neutralized by adding 350µl of neutralization solution and mixed and centrifuged at 13000 rpm for 10 minutes. Then   the supernatant of bacterial lysate was transferred to spin column and centrifuged for 1 minute followed by column washing with 750µl and 250µl of the Column Wash Solution. The purified plasmid DNA was eluted by adding 50µl of Nuclease Free Water.

2.2.3.1.9   Restriction digestion of Plasmid DNA

Purified plasmid DNA was digested using restriction enzymes as the following:

Table 2-9 The protocol of restriction digestion reaction of the DNA construct of CL-11 in pGEM-T-Easy vector.

Material	Amount
DNA sample 1µg	3µl
Restriction enzyme buffer 2 10X	2µl
BSA 10µg/µl	2µl
Restriction enzyme 1 10µg/µl	1µl
Restriction enzyme 2 10µg/µl	1µl
Deionized distilled water	11µl

The reaction mixture was incubated at 37 °C for 2 hours.
Note: Restriction enzymes are *Hind III* and Xho1 or *pst1* and *Ecor1*.

2.2.3.1.10   Sub-Cloning into expression vector pSecTag 2/ Hygro B

DNA construct was cloned into pSecTag 2/ Hygro B after digestion of the construct and the expression vector by the same restriction enzymes and were analysed by agarose gel. They were ligated by the following protocol:

Table 2-10 The protocol of sub-cloning of CL-11 into pSecTag 2/ Hygro B vector

Material	Amount
pSecTag 2/ Hygro B	1µl
10X ligase Buffer	1µl
Purified DNA	3µl
T4 DNA ligase	1µl
Deionized water to a final volume of	10µl

The mixture was transformed into competent cells after incubation overnight at 4°C.

Figure 2-2 A diagram showing the pSecTag2/Hygro vector circle map.
Adapted from the technical manual protocol of pSecTag2/Hygro Vector. Invitrogen

2.2.3.1.11 Sub-Cloning into expression vector pcDNA 3.1 /Hygro (+)

DNA constructs in vector pSecTag2/ Hygro B and pcDNA 3.1 vector were digested with the same restriction enzymes and cloned according to the same previous protocols.
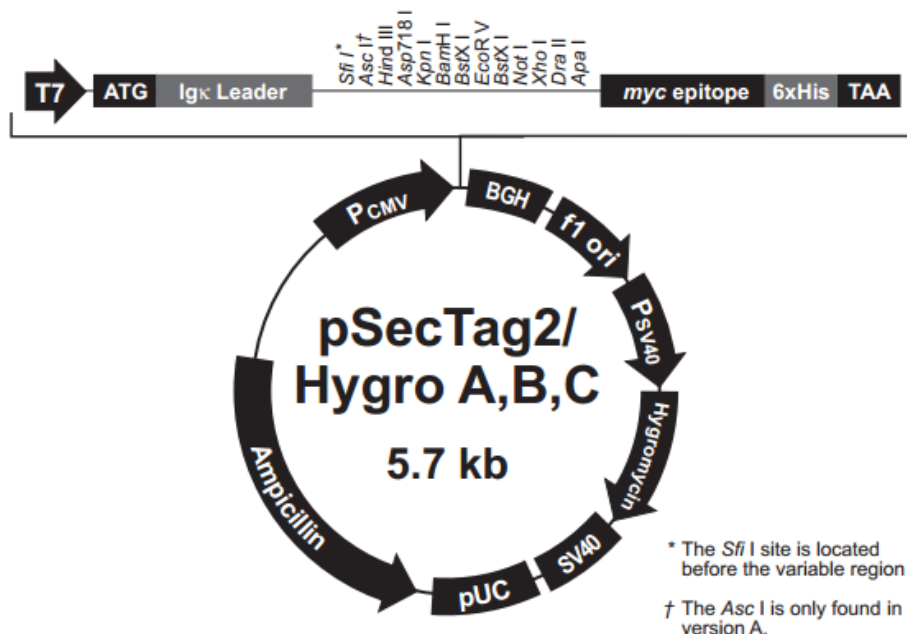
Comments for pcDNA3.1/Hygro (+):
   5597 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2100
Hygromycin resistance gene: bases 2118-3141
SV40 early polyadenylation signal: bases 3154-3526
pUC origin: bases 3786-4456 (complementary strand)
Ampicillin resistance gene: bases 4601-5461 (complementary strand)

Figure 2-3 A diagram showing the pcDNA 3.1 /Hygro(+)vector circle map.
   Adapted from the technical manual protocol of pcDNA 3.1 /Hygro (+) Vector. Invitrogen

2.2.3.1.12   Sub -Cloning into expression vector pED4

After cloning the PCR product of full length CL-11 with C-terminal his tag or N-terminal his tag or without his tag to pGEM-T easy vector, DNA constructs were cloned into pED4 vector by firstly, digesting the construct DNA and pED4 vector by the restriction enzymes *Hind III* and Xho1 according to the same previous protocols in (Table 2-7, Table 2-8, Table 2-9 and Table 2-10)
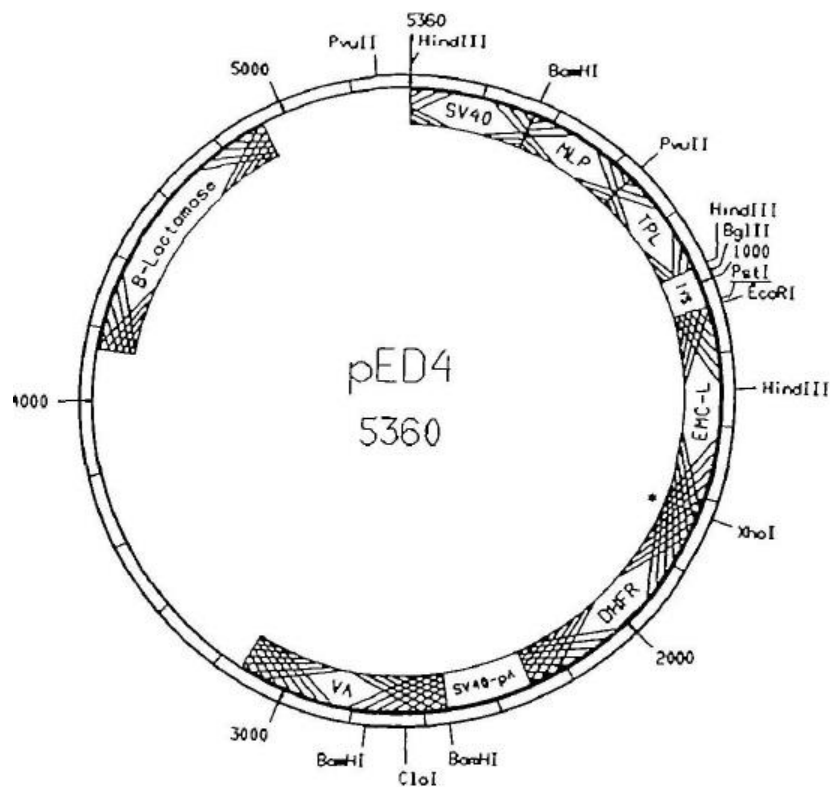
Figure 2-4 pED4 expression vector.
The gene is cloned into the *Pst1* and *Eco*RI sites (Kaufman *et al.*, 1991)

2.2.3.1.13 Gene reporter assays

2.2.3.1.13.1 Primer Design

Constructs containing fragments from the 5' flanking region of the human CL-11 gene were generated by PCR and ligated upstream of the luciferase reporter gene. The primers were used as follows:

Table 2-11 The primers used in Gene reporter assays

Primer	Sequence (5' → 3')
-125 –F Xho1	5' CTCGAGGATGTTCGGGTTGGAGAGTTAGCA
-251–F Xho1	5'CTCGAGGTCTGATTGGTGCCAGGTGATGA
-557– F Xho1	5'CTCGAGCACCTTTGAACCTAGCTTCTTATGA
R Hind III	5'AAGCTT GAGCACGCGCTAGGCGAAC.

2.2.3.1.13.2 Cloning PCR product into Gene Reporter Vectors

The insert fragments were cloned into pGL3-basic and pGL3-enhancer vectors and pGL3-control vector were used as positive controls and pRL-SV40 vector was used as the internal control vector. All these vectors were from Promega and the maps of these reporter vectors can be found in the Figure 2.5, Figure 2.6, Figure 2.7 and Figure 2.8.
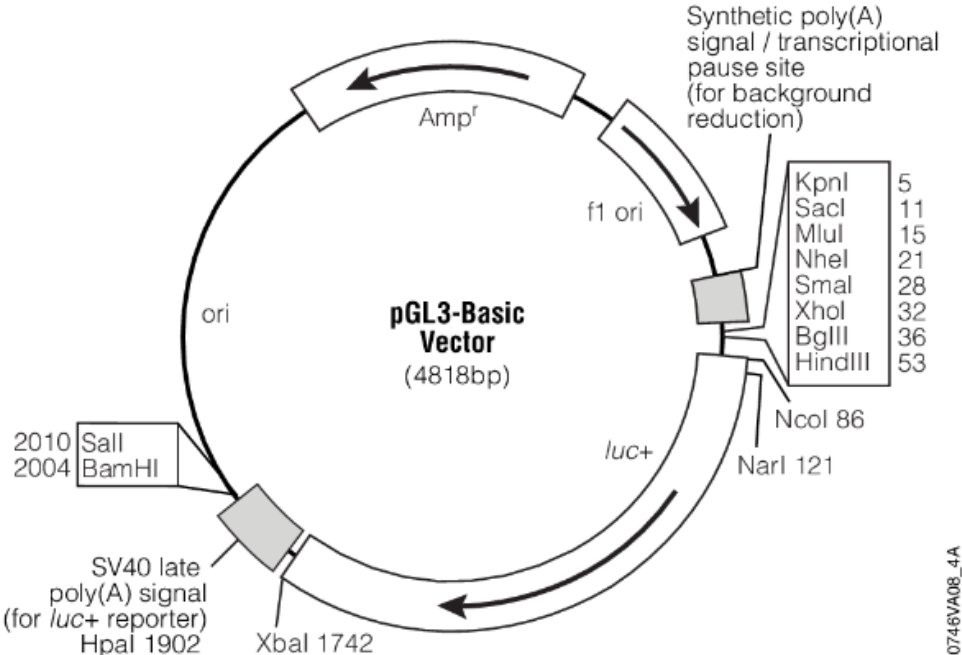
Figure 2-5 pGL3-basic vector map
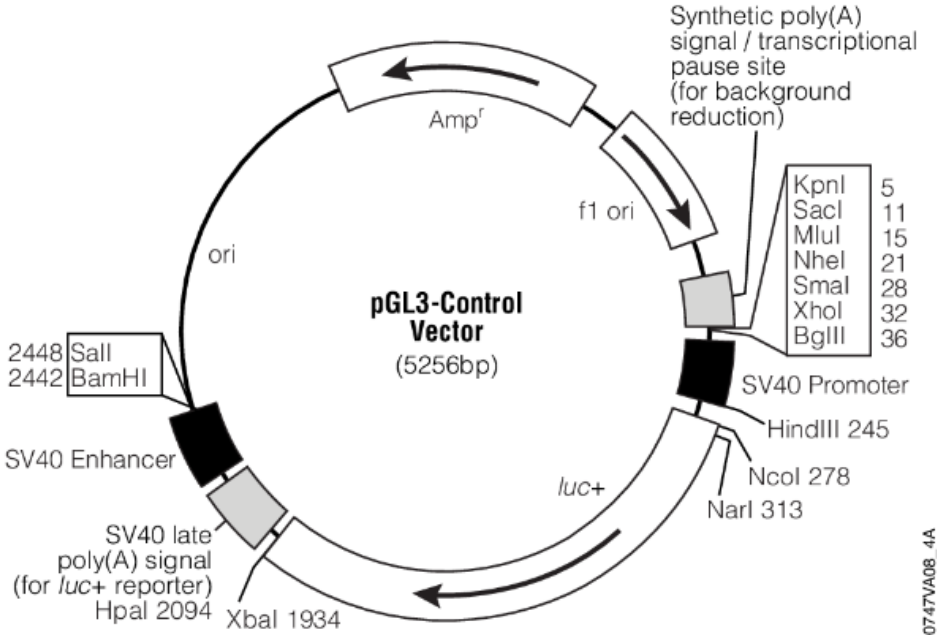
Figure 2-6 pGL3- control vector map
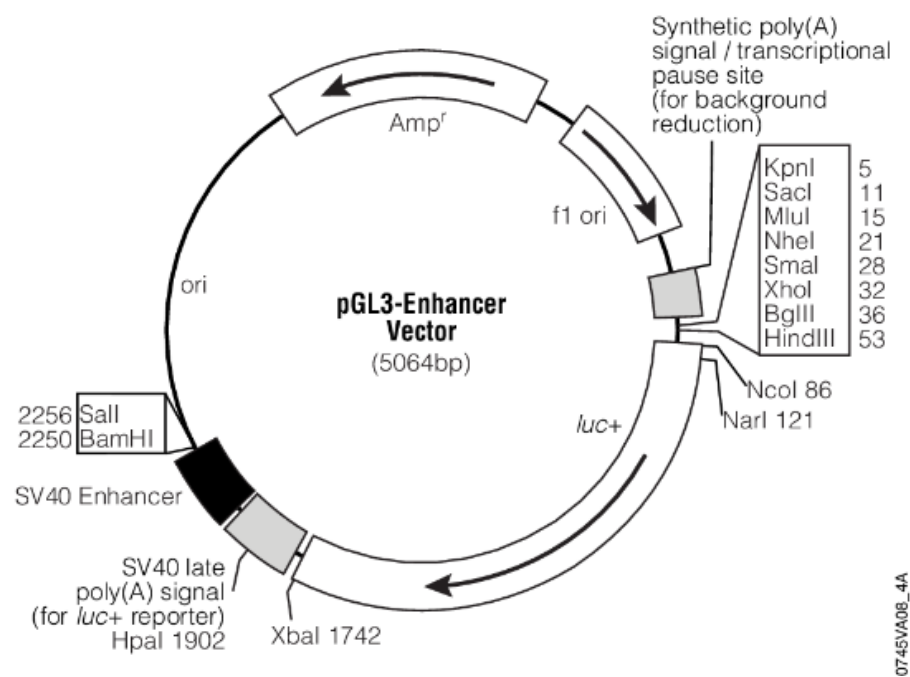
Figure 2-7 pGL3- enhancer vector map
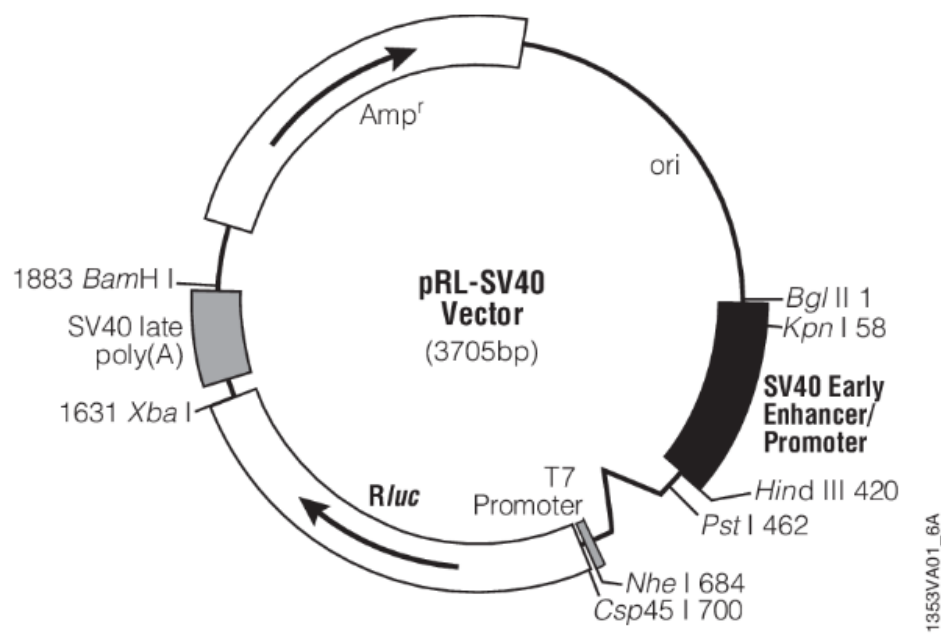
Figure 2-8 PRL-SV40 vector map

2.2.3.1.13.3 Dual Luciferase Reporter Assays

A dual luciferase assay was performed to measure the firefly- luciferase and the Renilla luciferase activity within the transfected cells following the Promega Dual Luciferase System kit protocol.   After 48hr of transfection, growth media was removed and the cells were washed with 100 µl of 1X PBS. After washing, cells were incubated with 100 µl of 1X Passive Cell Lysis Buffer for 20 min at room temperature to lysis the cell. Cell lysates were frozen at -80ºC until luciferase evaluation. 20 µl of the lysate were transferred to luminometer plates and   Luciferase assay is performed using a luminometer. 50 µl of Luciferase Reagent II was injected into the wells and luciferase activity was measured for 10 Sec. Then, 50 µl of *Stop and Glo*® reagent was injected into the wells to stop the luciferase activity and catalyse the Renilla reaction, incubated for 1.6 Sec and then Renilla activity was measured for 10 Sec.

2.2.3.1.14 Allelic Discrimination Analysis

Quantitative Polymerase Chain Reaction was performed for genotype and allelic discrimination by using the Taqman SNP Assay for amplifying and detecting specific CL-11 SNP COLEC11 His219Arg (rs7567833) (Applied Biosystem) in purified genomic DNA samples of 155 premature neonates (97 Polish and 58 British) . Fluorescent dyes was used in this assay to detect DNA binding. VIC (green dye) indicates the fluorescence of the A allele and FAM (blue dye) indicates the fluorescence of the G allele (Figure 2.9).
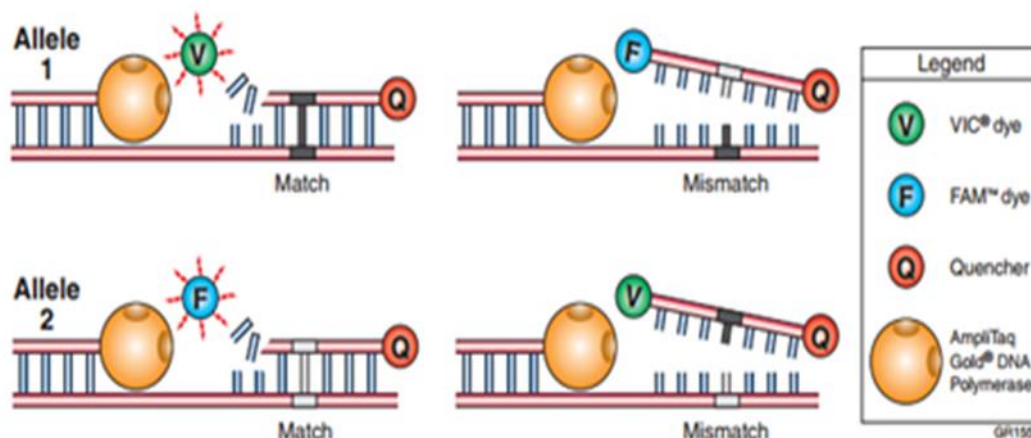
Figure 2-9 TaqMan SNP genotyping assay

To prepare the reaction mix, 1.25 of 20X working stock of SNP Genotyping Assay (40X stock) were added to 12.5 μl of 2X Tagman master mix. After that, 13.75 μl of the mixture were transferred into each well of Optical 96-well reaction plate (Applied Biosystems) before adding 11.25(20 ng) of wet genomic DNA. A negative control without DNA was included on each plate. The plate was inserted into the 7500 fast real time PCR system (Applied Biosystems). Firstly PCR temperature was kept on an initial holding stage to denature the DNA and activate Taq polymerase which is 50.0°C for 2 minutes then 95.0°C for 10 minutes. After that followed by a Cycling stage which 40 cycles for 15 seconds at 95.0°C followed by 1 minute at 60°C. Then, the allelic discrimination post read was carried out and the analysis was performed using the 7500 software.

2.2.3.2   RNA methods

2.2.3.2.1   Extraction of total RNA

In order to analyse the expression of different inflammatory cytokines during the course of infection, total RNA was extracted from frozen lungs. 50-100 mg of the mouse lung was cut and homogenised in 1 ml of TRIzol reagent (Invitrogen) using Ultra-Turrax T10 basic IKA-Werke Homogenizer (Germany). Then, lung homogenate was left at room temperature for 5 minutes and centrifuged at 12000 xg for 10 minutes at 2-8 °C. The supernatant was collected and transferred to RNase-free Microcentrifuge tube and 200 µL of chloroform added with vigorous shaking for 15 seconds and incubated for 2-3 minutes at room temperature. After the incubation, the homogenate was centrifuged at 12000 xg for15 minutes at 2-8 °C. An upper aqueous layer containing RNA was transferred to RNase-free Microcentrifuge tube and precipitated by adding 500 µl of isopropanol and incubating for 10 minutes at room temperature. RNA was centrifuged at 12000 xg for 10 minutes and the RNA pellet was washed with 1ml of 75% ethanol and centrifuged at 7500xg. The RNA pellet was air dried and dissolved in 100 µl of DEPC-treated water. RNA purity was measured by measuring the absorbance ratio A260/A280 using NanoDrop 3300 spectrophotometer. RNA was considered pure when A260/A280 was above 1.8.

2.2.3.2.2   Purification of RNA

To remove contaminating DNA, the extracted RNA sample was treated with DNase using Ambion TURBO DNA-freeTM kit (Invitrogen). In a 0.5 ml Eppendorf tube, 20 µl of total RNA (~ 1.5-2 µg) was mixed with 5 µl of 10X TURBO DNase Buffer, 2 µl of TURBO DNase (2 U/µl) and 23 µl of nuclease-free water. The sample tube was incubated in a water bath at 37°C for 30 min. The reaction was stopped by adding 5 µl of DNase Inactivation Reagent and incubating at room temperature for 5 min. The reaction mixture was then centrifuged at 10000 x g for 1.5 min, and the aqueous phase containing RNA was transferred to a fresh tube and stored at -80°C until further use.

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

In order to synthesise DNA from total RNA, RT-PCR was performed. 10.5 μl of RNA solution equivalent to 1 μg of RNA was added to 1 µl of Oligo (dT) 23 anchored primers (Sigma O4387). The reaction mixture was incubated at 70°C for 10 minutes.   Then, the temperature was decreased to 45°C and 1 μl of SuperScript II Reverse Transcriptase (Invitrogen), 2 μl of 10x reverse transcriptase buffer (Invitrogen), 2 μl of 25 mM MgCl2 (Invitrogen), 2 μl of 0.1 M DTT (Invitrogen), 1 μl of 10 mM deoxyribonucleoside triphosphate mixture (dNTPs) (Promega) and 0.5 μl of RNaseOUT (Promega) were added to each sample and incubated for 60 minutes. In order to inactivate the reverse transcriptase enzyme, the temperature of reaction mixture was raised to 70°C for 10 minutes and then was dropped to 4°C. Finally, 1 μl RNase H (Promega) was added to the cDNA and incubated the reaction tubes at 37°C for 30 minutes. Volume of cDNA was completed to 50 µl with sterile nano-pure water and stored at -20 °C.

2.2.3.2.4   Quantitative Real Time-PCR (qRT-PCR)

Quantitative real time PCR was used to measure the expression levels of different cytokines, including TNF-α (tumor necrosis factor-alpha), IFN-γ, (gamma-interferon), IL-1ß and IL-10. Applied Biosystems 7500 Fast Real-Time PCR (Applied Biosystems, Life Technologies) was used to analyse cDNA by using fluorescent DNA-binging dye; SYBR green (Applied Biosystems, Life Technologies), which binds to double strand DNA and emits fluorescence. Amplification of cDNA was monitored by measuring the increase in the fluorescence throughout the amplification cycles. 1µl of the prepared cDNA was added to 12.5µl of Power SYBER®Green PCR Master Mix (Applied Biosystems) and 1µl of Forward and Reverse primers (or GAPDH qPCR primer as a house keeping gene) (Table 2.12) to the total volume 24µl by adding nano-pure water. Relative expression was presented as (2-ΔΔCT) after normalization of the relative amounts of mRNA to the GAPDH signal for each sample. (Livak & Schmittgen, 2001).

Table 2-12 Sequence of primers used for cytokine expression levels in qRT-PCR.

Primer	Sequence	Amplicon size
TNF _F1	CCTCACACTCAGATCATCTTCTCA	237 bp
TNF _R2	GTGGGTGAGGAGCACATAG	
IFNγ_F	CCTGCGGCCTAGCTCTGA	81bp
IFNγ_R	CAGCCAGAAACAGCCATGAG	
IL-1 _F	CACTCATTGTGGCTGTGGAGA	247 bp
IL-1 _R	AGGTGGAGAGCTTTCAGCTCA	
IL10_F	CTTGCACTACCAAAGCCACA	86 bp
IL10_R	TAAGAGCAGGCAGCATAGCA	
GAPDH_F2	GTGCTGCCAAGGCTGTG 3	211bp
GAPDH_R1	AGACAACCTGGTCCTCAGTGTA	

2.2.4   Cell Culture techniques

2.2.4.1   Transfection of Chinese hamster ovary (CHO-K1) Cells

CHO-K1 cells were cultured in F12 nutrient mixture (Ham) with GlutaMax (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 100U penicillin/0.1 mg/ml Streptomycin (Sigma-Aldrich), then incubated at the 37°C CO_2 incubator until they became 80% confluent, the cells were sub-cultured in 6 well plate and incubated at 37°C to become 50-80% confluent. 3µl of the Lipofectamine® LTX Reagent and 2µl of plasmid DNA were added into 100µl Opti-MEM® I Reduced Serum Medium, mixed gently and incubated for 25 minutes. After that, 100 µl of the mixture was added to each well then the plate placed in a CO_2 incubator at 37°C. Next day, the transfection mixture was replaced with Ham's F-12 Nutrient Mix serum medium, the cells were washed with the PBS after 48h of transfection,

then 100µl of Trypsin-EDTA was added to each well and incubated at 37°C for 1 minute. Then cells were re-suspended in MEMα media and distributed into 96 well plates. After 2-3 weeks of incubation, the colonies were picked and transferred to separate 25 cm2 flasks containing MEMα-.

2.2.4.2   Transfection of Human Embryonic Kidney (HEK 293) Cell Line

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and high glucose concentration (Sigma). This medium was supplemented with 10% Heat Inactivated Fetal Bovine Serum FBS (GIBCO) and 100U penicillin/0.1 mg/ml Streptomycin (Sigma-Aldrich), Cell were incubated at the 37°C CO2 incubator until they became 80% confluent, the cells were sub-cultured in 6 well plate and incubated at 37°C to become 50-80% confluent. The same procedure for CHO-K1 cells was followed for DNA plasmid transfection using Lipofectamine® LTX Reagent. After that, the transient HEK293 cell   line was trypsinised using 1ml 1X-Trypsin (Sigma), and then cells were re-suspended in DMEM serum media containing a selection agent of 300µg/ml HygromycinB (Invitrogen) by which the non-transient cells were killed. Then, the cell suspension was transferred in 24-well plates, and incubated until the cells were confluent. The expression of recombinant CL-11 was screened by using Dot blot and confirmed by Western blot.

2.2.4.3   Transfection of Chinese hamster ovary (CHO- DXB11) cell line

CL-11 was transfected in a Chinese hamster ovary (CHO) cell line called DXB11, which is deficient in the dihydrofolate reductase (DHFR) gene The cells were grown in Minimal Essential Media α with nucleosides (MEM α+) supplemented with 10 % dialyzed, heat treated fetal calf serum (DHFCS) and 100U penicillin/0.1 mg/ml Streptomycin. Then cells were incubated at the 37°C CO_2 incubator until they became 80% confluent, then sub-cultured in 6 well plate and incubated at 37°C to become 50-80% confluent. 3µl of the Lipofectamine® LTX Reagent and 2µl of plasmid DNA were added into 100µl Opti-MEM® I Reduced Serum Medium, mixed gently and incubated for 25 minutes. After that, 100 µl of the mixture was added to each well then the plate placed in a CO_2 incubator at 37°C. Next day, the transfection mixture was replaced with fresh MEMα+ serum medium, the cells were

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washed with PBS after 48h of transfection then 100µl of Trypsin-EDTA was added to each well and incubated at 37°C for 1 minute. Then cells were re-suspended to MEMα- media and distributed into 96 well plates. After 2-3 weeks of incubation, the colonies were picked and transferred to separate 25 cm2 flasks containing MEMα-.

2.2.5   Protein methods

2.2.5.1   Dotblot method

In order to identify cells producing collectin 11, the cell culture supernatant was analyzed using the Dotblot method which detects the positive clones that express the specific protein. This method was performed by adding 30µl of the culture supernatant as a spot on a nitrocellulose membrane and allowing to dry. Then the membrane was blocked with 5% skim milk in PBS for 1h with shaking. A rabbit anti mouse collectin 11 (Proteintech Europe) diluted 1:5000 or monoclonal mouse anti-polyhistidine HRP conjugated antibody in blocking buffer were added and incubated at room temperature for 1h with shaking followed by washing three times with PBS 0.05% Tween 20. For the blots probed with rabbit anti mouse collectin 11, an anti-rabbit IgG HRP conjugate (Dako) diluted 1:2000 in blocking buffer was added and incubated 1h with shaking. Subsequently, washing three times with PBS 0.05% Tween 20, the Luminata Crescendo Western HRP Substrate was added onto nitrocellulose membrane surface for one minute then the membrane was exposed to an autoradiography film (Fuji film) for 1 to 30 minutes.

2.2.5.2   Protein Expression

2.2.5.2.1   Large scale production of mCL-11 in Human Embryonic Kidney (HEK 293) Cells

The positive clones found by Dotblot method were grown in large triple flasks in DMEM media supplemented with 10% Fetal Bovine Serum (FBS), 10U/ml ampicillin /0.1mg/ml streptomycin; 300µg/ml Hygromycin B. When the cells were 80% confluent, the culture medium was replaced with DMEM serum free media 10U/ml ampicillin /0.1mg/ml streptomycin; 300µg/ml Hygromycin B and incubated at 37˚C for two days.

2.2.5.2.2   Large scale production of mCL-11 in Chinese hamster ovary (CHO-DXB11) cell line

The positive clones found by Dotblot method were transferred to a triple layer flask containing 150 ml MEMα-,100U penicillin/0.1 mg/ml Streptomycin and 0.5 μM MTX .When the cells were 80-90% confluent, Serum medium was replaced with CHO-S-SFM II (Gibco) containing 100U penicillin/0.1 mg/ml Streptomycin, 0.5 μM MTX and 50 mM HEPES, pH 7.55. The protein expression was detected by dot blot and confirmed by western blot technique after 48h of incubation at 37˚C.

2.2.5.3   Protein purification

2.2.5.3.1   mCL-11 purification on mannose-sepharose column

50 ml of Sepharose-6B (Sigma-Aldrich) was washed with 2-3 litres of nano-pure water using a large filter and transferred into a beaker containing 50 mL of 0.5 M sodium bicarbonate buffer pH 11 and 5 ml of divinyl sulfone (DVS) (Fluka) and incubated with stirrer   for 70 minutes. Then, the Sepharose was washed again with 2-3 litres of nano-pure water and added to 50 ml 0.5 M sodium bicarbonate buffer pH 10 containing 20 % (w/v) of mannose and incubated with stirrer overnight. After that, the Sepharose was washed with 2-3 litres of nano-pure water and incubated with 50 mL of 0.5 M sodium bicarbonate buffer pH 8.5 and 1 mL of 2-mercaptoethanol (Sigma-Aldrich) for 2 hours. The mannose Sepharose was washed with 2-3 litres of nano-pure water and stored in nano-pure water containing 0.05% sodium azide (Sigma-Aldrich) at 4˚C.

The serum free media were collected and centrifuged at 3000xg for 10 minutes to remove the cell debris ,then diluted with an equal volume of High salt Binding buffer (1.25M NaCl, 50mM Tris-HCl, 25mM CaCl2, pH 7.4) and the pH was adjusted to 7.4. After that, the mixture was applied to the mannose-Sepharose column, then the column was washed with 50ml of High salt Binding buffer (1.25M NaCl, 50mM Tris-HCl, 25mM CaCl2, pH 7.4),50ml low salt Binding buffer (150mM NaCl, 50mM Tris-Hcl, 5mM CaCl2, pH 7.4) and the

recombinant protein was eluted   by using eluted buffer   (150mM NaCl, 50mM Tris-Hcl, 2mM EDTA, pH 7.4) .

2.2.5.3.2   mCL-11 purification on His-Trap column

The supernatant was collected and centrifuged at 3000xg for 5 minutes to remove the cell debris , then diluted with an equal volume of phosphate buffer pH 7.4, containing 100mM NaCl and 5mM imidazole and the mixture was applied on to a HisGravi Trap column (GE Healthcare). The column was washed with 20 ml of phosphate buffer pH 7.4, containing 100 mM NaCl and 25 mM imidazole, to remove weakly bound contaminants .After that bound protein was eluted using elution buffer (phosphate buffer pH 7.4, containing 100 mM NaCl and 500 mM imidazole).

2.2.5.4   SDS-PAGE

In order to   characterize the recombinant CL-11 sodium dodecyl sulphate polyacrylamide gel electrophoresis was used under reducing and non-reducing conditions. In the reducing conditions, 25µl of the recombinant protein were heated for 5 minutes at 95˚C with the loading dye containing SDS and 2-mercaptoethanol while, in the non-reducing condition used the SDS-loading dye without heating and without 2 mercaptoethanol. Then, the samples were loaded into 12% acrylamide SDS gel and the gels were run in 1X SDS running buffer at 150V for 1h. The gel was stained with Coomassie Brilliant Blue R250 (0.2g Coomassie brilliant blue R250, 10ml Glacial acetic acid, 40ml Methanol, dH$_2$O up to 100ml) for 30 minutes at room temperature with gentle shaking, followed by de -staining with de-staining solution (40ml Methanol, 10ml Glacial acetic acid, dH$_2$O up to 100ml) until the protein bands appeared.

2.2.5.5   Western blot

Western blot was used to confirm the identity and size of the protein by electroblotting the SDS gel onto nitrocellulose membrane and blocking with 5% skim milk in PBS for 1h to block the residual protein binding sites in the membranes. A rabbit anti mouse collectin 11 diluted 1:5000 in blocking buffer was added after washing three times with PBS 0.05%

Tween 20, and incubated for 1h with shaking. After that, the membrane was washed and anti-rabbit IgG HRP conjugate, diluted 1:2000 in blocking buffer was added and incubated 1h with shaking. Then, Luminata Crescendo Western HRP Substrate was added on the nitrocellulose membrane after washing it three times. The intensity of the signal was detected after exposing to Fuji film. In other Western blots, monoclonal mouse anti polyhistidine antibodies HRP conjugated (Sigma-Aldrich) diluted 1:5000 in blocking buffer were used to detect the recombinant mCL-11 with his tag.

Chapter Three: Results

Production of recombinant mouse CL-11 and examination of its ligand binding activity

3 Chapter Three: Results

3.1 Production of recombinant CL-11

3.1.1 Amplification of full length murine CL-11 cDNA

Murine CL-11 is encoded by mRNA with an open reading frame (ORF) of 816 base pairs in length. Linear cDNA from mouse liver mRNA was prepared (see 2.2.3.2.3) and used as a template to amplify the ORF of murine CL-11 by using mCL-11 –F Hind III primer and mCL-11 –R Xho1 primer by PCR (Table 2-6). (Fig. 3.1)
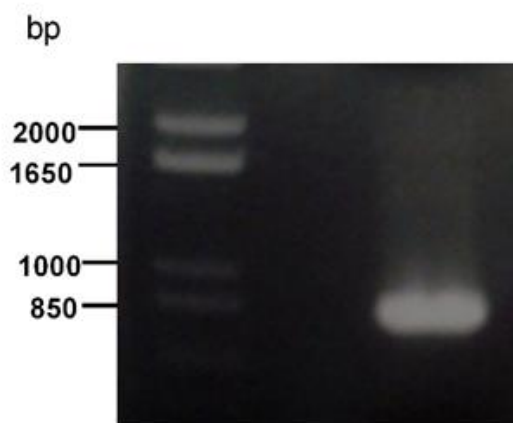
Figure 3-1 Amplification of murine CL- 11 ORF
The 816 pb coding sequence for mouse CL-11 was amplified from mouse liver cDNA using high fidelity Taq polymerase (see Materials and Methods)

3.1.2 Cloning into expression vector pSecTag 2/ Hygro B and pcDNA™3.1/Hygro expression vector

The full length ORF of murine CL- 11 was cloned into the pGEM-T Easy vector, plasmid mini-preps were prepared and the correct insertion of the ORF of murine CL-11 cDNA identified by restriction digest using HindIII and Xho1. The insert cDNA band has a calculated length of 816 bp while the pGEM-T Easy subcloning vector runs at approximately

3kb (Fig.3.2 A). The insert was then sub-cloned into the mammalian expression vector pSegTag2/Hygro (5.2 kb) and the sub-cloning was confirmed by restriction enzyme digestion (Fig.3.2 B) and DNA sequencing. Before the transfection of the eukaryotic CHO-K1 cell line, all of the constructs were sequenced to confirm that all clones were inserted in frame and to ensure that no other mutations were introduced during PCR amplification of the ORF sequence.
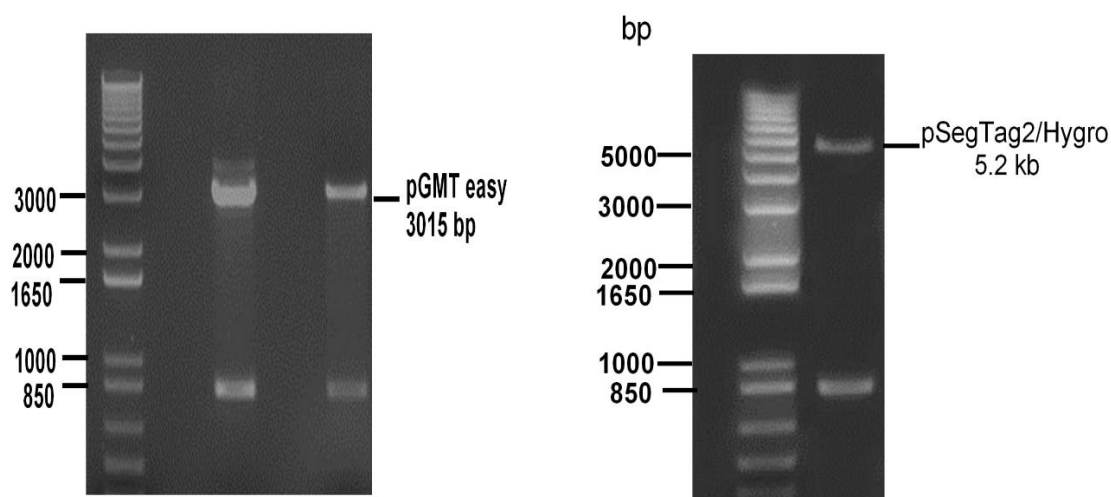
Figure 3-2 Agarose gels electrophoresis showing cloning of the murine CL-11 ORF cDNA sequence into the pGEM-T easy vector and subsequent subcloning into the pSegTag2/HygroB expression vector
(A) The full length ORF for CL-11 was cloned into the 3kb vector pGEM-T easy vector and digested with HindIII and XhoI. (B) Inserts were excised with HindIII and XhoI and sub-cloned into pSecTag2/Hygro 5.2kb pSegTag2/Hygro vector

The first transfection experiment failed to obtain a CL-11 producing cell line following transfection of the pSectag2/hygroB expression vector containing the murine collectin-11 ORF as analysed by SDS gel electrophoresis and DOT blot analysis (2.2.5.1 and 2.2.5.4), I therefore used another eukaryotic expression vector, i.e. pcDNA™3. 1/Hygro (+). The correct insertion of the ORF sequence was confirmed by restriction digestion and sequencing

to ensure the in frame cloning and the absence of mutations in the open reading frame within the expression plasmid pcDNA™3. 1/Hygro (+) vector. (Fig 3.3).
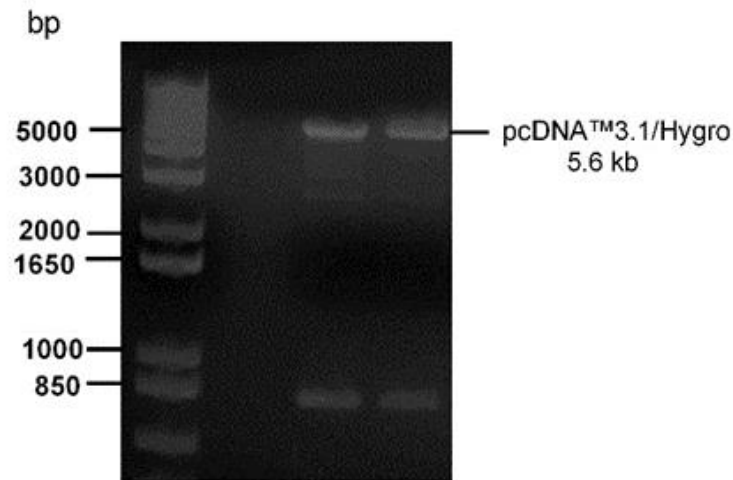
Figure 3-3 Agarose gel electrophoresis shows sub-cloning of murine CL-11 into pcDNA™3.1/Hygro expression vector
The full length coding sequence for murine collectin-11 (816 bp) was subcloned into the 5.6 kb pcDNA™3.1/Hygro (+) vector and excised with HindIII and Xho1

This second construct was transfected into the in HEK293 cell line. Using this procedure CL-11 expressing HEK293 clones were isolated after the cell line was exposed to 180μg/ml of Hygromycin-B in the cell culture medium to select for transfectants containing the Hygromycin-B resistance gene encoded by the expression vector. Single colonies of Hygromycin-B resistant clones were isolated and cultivated separately and their supernatants tested for CL-11 release using a commercially available polyclonal rabbit anti mouse collectin 11 antibody labeled with HRP (Fig. 3.4).
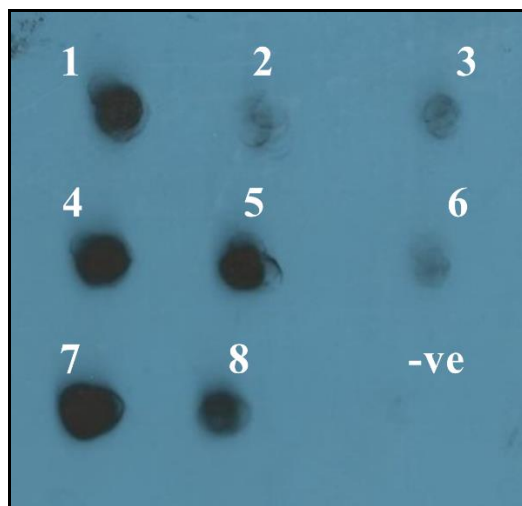
Figure 3-4 Dot blot assay of the supernatant of different HEK293 clones for expression of murine Collectin-11
Screening of several selected clones for expression of collectin-11 using A rabbit anti mouse collectin HRP. Supernatant from non-transfected HEK293 cells was used as a negative control.

A Mannose–Sepharose column was used to affinity purify recombinant murine collectin-11 (2.2.5.3.1). Elution fractions were collected and analysed by western blot analysis using the polyclonal rabbit anti-mouse CL-11 antibody described in (2.2.5.5). Purified murine collection-11 can be seen as a single stained band running at approximately at 36 kDa under reducing conditions while under non-reducing conditions the westen blot analysis detects CL-11 polymers forming a band at approximately 100kDa (representing the homotrimer), and occasionally larger bands at 200+ kDa, thought to be non-reduced higher order oligomers (2.2.5.4). (Fig. 3.5).
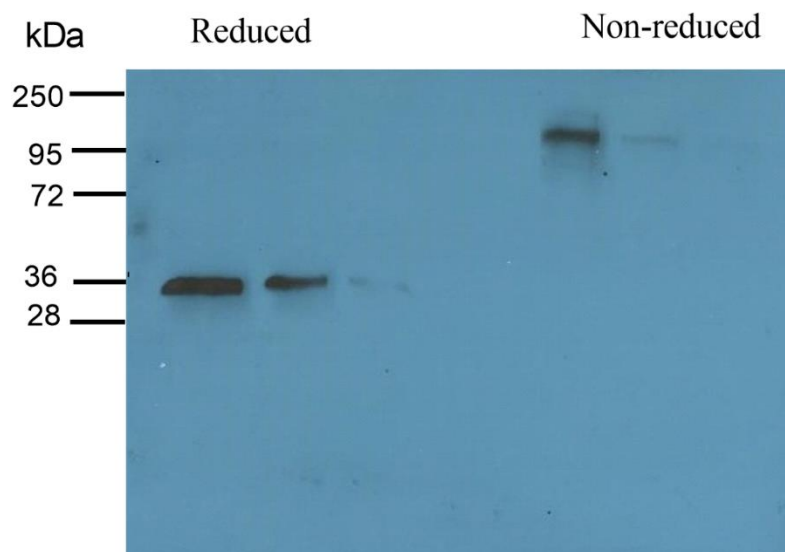
Figure 3-5 Western blot analysis CL-11 expression using anti CL-11 antibodies
Lane 1,2,3: different elution fractions under reducing conditions, lane 4,5,6 different elution fractions under non-reducing conditions

3.1.3   Cloning into expression vector pED4 vector

Although, the expression of murine CL-11 was successful when using pcDNA 3.1 as an expression vector in HEK293 cell line, the amount of the protein that was harvested after the purification using the manose-Sepharose column was relatively low at 50-100 µg / litre of supernatant. In order to achieve a higher yield of recombinant CL-11 I tried an alternative expression system using the pED4 vector, which contains the dihydrofolate reductase (DHFR) gene in DHFR deficient CHO cells, a sub-cell line called DXB11. Methotrexate MTX was added to the media to increase protein expression levels. MTX upregulates the expression of DHFR gene in the pED4 vector leading to increased recombinant protein expression. (Kaufman *et al.*, 1991). To establish expression constructs to express recombinant murine CL-11(mCL-11) with and without a 6xHis tag (for affinity purification of the recombinant protein), murine cDNA was used as a template to amplify the ORF encoding recombinant CL-11. The Histidine tag was used to facilitate purification of recombinant CL-11 using Nickel affinity purification columns. (Figure 3.6 and 3.7).

A

Signal peptide

10	20	30	40	50
MMMRDLALAG	MLISLAFLSL	LPSGCPQQTT	EDACSVQILV	PGLKGDAGEK
60	70	80	90	100
GDKGAPGRPG	RVGPTGEKGD	MGDKGQKGTV	GRHGKIGPIG	AKGEKGDSGD
110	120	130	140	150
IGPPGPSGEP	GIPCECSQLR	KAIGEMDNQV	TQLTTELKFI	KNAVAGLRET
160	170	180	190	200
ESKIYLLVKE	EKRYADAQLS	CQARGGTLSM	PKDEAANGLM	ASYLAQAGLA
210	220	230	240	250
RVFIGINDLE	KEGAFVYSDR	SPMQTFNKWR	SGEPNNAYDE	EDCVEMVASG
260	270			
GWNDVACHIT	MYFMCEFDKE	NL6HIS		

C TERMINAL 6 HIS TAG

B

mCL-11 *Pst* F   mCL-11 C-HIS R

CTGCAG GCCACC ATG... Signal peptide   6His TGA GAATTC

Figure 3-6 Schematic of the design of C terminal his tagged recombinant CL-11
(A) amino acid sequence including the C-terminal poly Histidine tag of recombinant murine CL-11 (B) Schematic of design the C-terminal His tag added to recombinant CL-11. Sequences are the restriction sites (blue), Kozak sequence (yellow)

A

Signal peptide   N terminal 6 HIS TAG

10	20	30	40	50
MMMRDLALAG	MLISLAFLSL	LPSGCP6HISQQTT	EDACSVQILV	PGLKGDAGEK
60	70	80	90	100
GDKGAPGRPG	RVGPTGEKGD	MGDKGQKGTV	GRHGKIGPIG	AKGEKGDSGD
110	120	130	140	150
IGPPGPSGEP	GIPCECSQLR	KAIGEMDNQV	TQLTTELKFI	KNAVAGLRET
160	170	180	190	200
ESKIYLLVKE	EKRYADAQLS	CQARGGTLSM	PKDEAANGLM	ASYLAQAGLA
210	220	230	240	250
RVFIGINDLE	KEGAFVYSDR	SPMQTFNKWR	SGEPNNAYDE	EDCVEMVASG
260	270			
GWNDVACHIT	MYFMCEFDKE	NL		

B

Figure 3-7 Schematic of design to add an N-terminal His tag to recombinant CL-11
(A) Amino acid sequence of C terminal his tag recombinant CL-11 (B) Schematic of design N terminal his tag recombinant CL-11. Sequences are the restriction sites (blue), Kozak sequence (yellow)

Full length recombinant CL-11 with either a C-terminal His tag or an N-terminal His tag was produced as described in Materials and Methods. The establishment of the expression constructs is documented in (Figure.3.8).
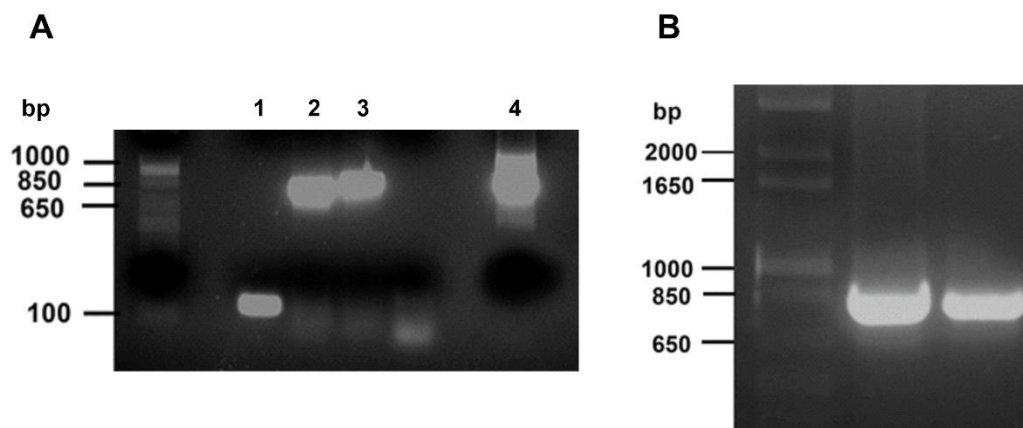
Figure 3-8 PCR amplification of murine CL- 11
(A) with histidine tag N-terminal and C- terminal (1): Fragment of the first PCR to generate N-terminal his tag. (2): Fragment of the second PCR to generate N-terminal his tag. (3) The full length coding sequence for murine CL- 11 with histidine tag N-terminal (4) The full length coding sequence of murine CL- 11 with the Histidine tag at the C-terminus (B) The full length coding sequence of murine CL- 11 without the Histidine tag coding sequence.

The PCR products were cloned into the pGEM-T easy vector (which facilitates subcloning of PCR products due to T-tailing of the blunt-cut cloning site). The primers used to amplify the PCR fragments were engineered to contain endonuclease restriction sites for *EcoR1* and Pst1 (Figure 3. 9).

A

B
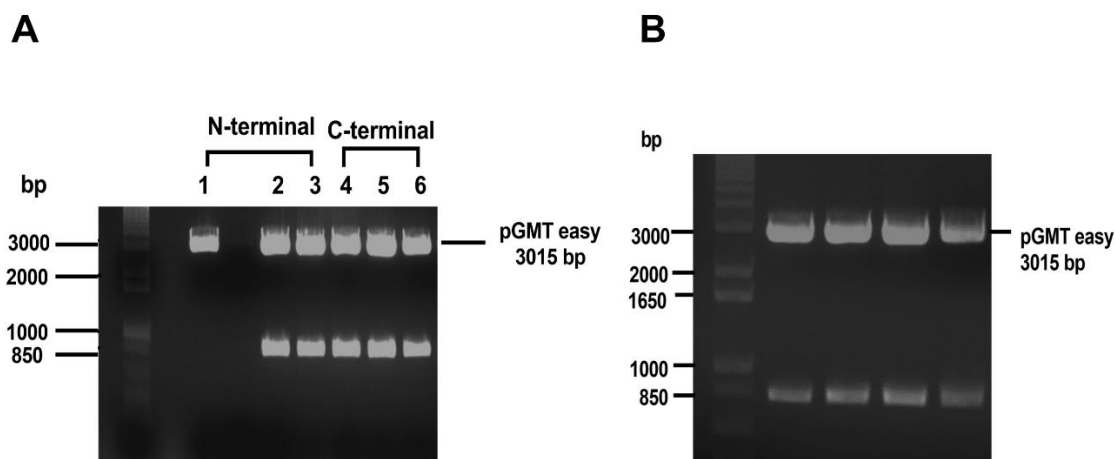
Figure 3-9 The full length coding sequence for mCL-11 cloned into pGEMT-easy
(**A**) CL-11 cDNA with an N-terminal his tag: (1,2,3) or C-terminal his tag (4,5,6) was cloned into the 3kb vector pGEM-T easy and digested with EcoR1 and Pst1. (**B**) CL-11 cDNA without addition of a His tag coding sequence.

The mCL-11 constructs were excised from pGEM-T easy vector by EcoR1 and Pst1 restriction digestion and subsequently subcloned into the expression vector pED4. (See Figure 3.10).

A

B
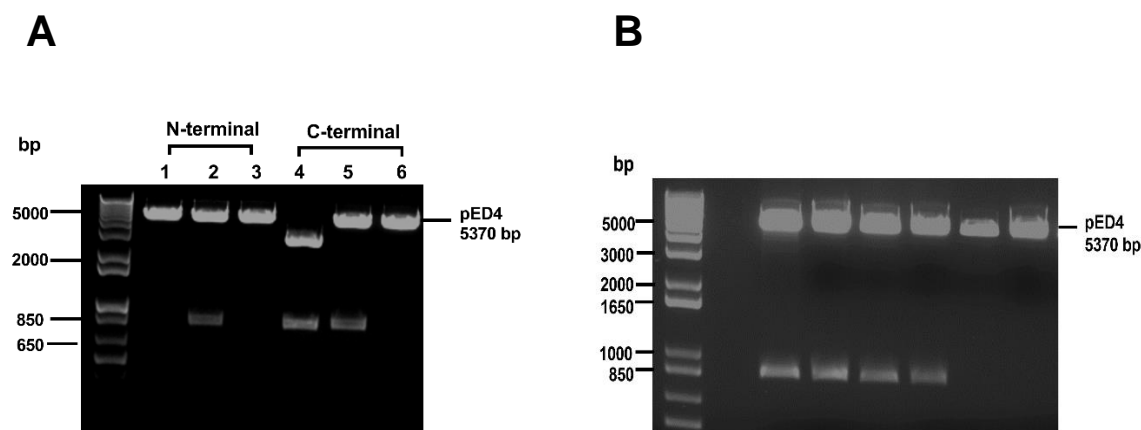
Figure 3-10 The full length coding sequence for CL-11 in pED4
(**A**) N-terminal His tagged cDNA (1,2,3) and C-terminal tagged cDNA (4,5,6,7) was cloned into the 5.3 kb pED4 vector and digested with EcoR1 and Pst1. (*B*) cDNA without addition of a His tag coding sequence.

The constructs to encode mCL-11 were sequenced by PNACL at the University of Leicester to confirm that all clones were inserted in the frame and to ensure that the PCR did not generate any random mutations.

CL-11 constructs were transfected into a mammalian cell line derived from Chinese hamster ovary (CHO- DXB11) cells (2.2.4.3). Following transfection, the expression of CL-11 in supernatants of different selected clones was assessed by dot blot analysis using an HRP-labeled anti His tag antibody or an HRP labeled polyclonal rabbit anti mouse CL-11 antibody. Unfortunately, the construct with a C-terminal His tag failed to generate any CL-11 producing clones, while clones with an N-terminal His tag and non-His tag encoding plasmids succeeded in expressing murine CL-11 (See Fig. 3.11).
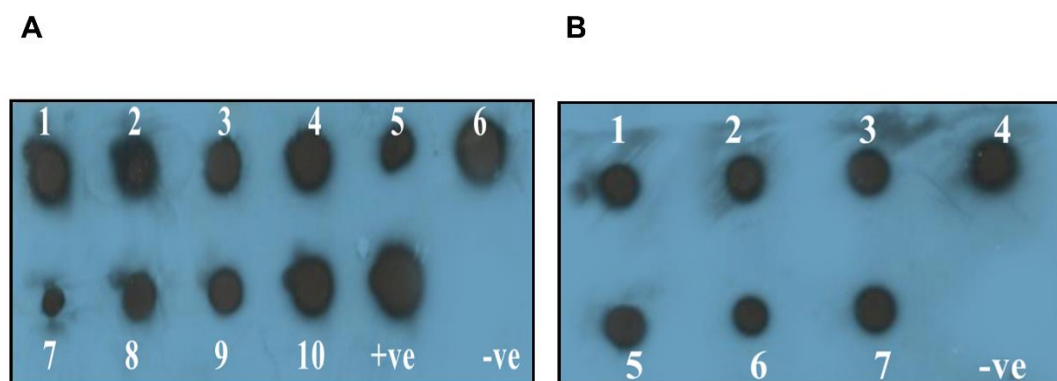
Figure 3-11 Screening of Supernatants of several clones encoding murine CL-11
(A) N-terminal his tag mCL-11 using a monoclonal antibody against the 6 histidine tag. Recombinant properdin with a histidine tag was used as a positive control. Supernatant from non-transfected cells was used as a negative control. (B) non his tag mCL-11 using rabbit anti CL-11, supernatant from non-transfected cells was used as a negative control.

Recombinant mCL-11 with an N-terminal His tag was purified from cell culture supernatant using a Nickel column affinity purification while recombinant m CL-11 without His tag was purified using Mannose–Sepharose column affinity purification (i.e. CL-11 binds to Mannose). The highly concentrated (1-2 mg/ml) of His tagged mCL-11 and (0.2 mg/ml) of mCL-11 without His tag were analyzed by SDS-PAGE and by western blotting. The mCL-11 formed a band at approximately around 100 -200 kDa when run under non reducing conditions and around 36 kDa when run under reducing conditions on SDS-PAGE (See Figure 3.12 and Figure 3.13). The protein band was sent for sequencing by mass spectrometry analysis and the sequence was confirmed to be identical with the deduced amino acid sequence encoded by murine CL-11 cDNA.
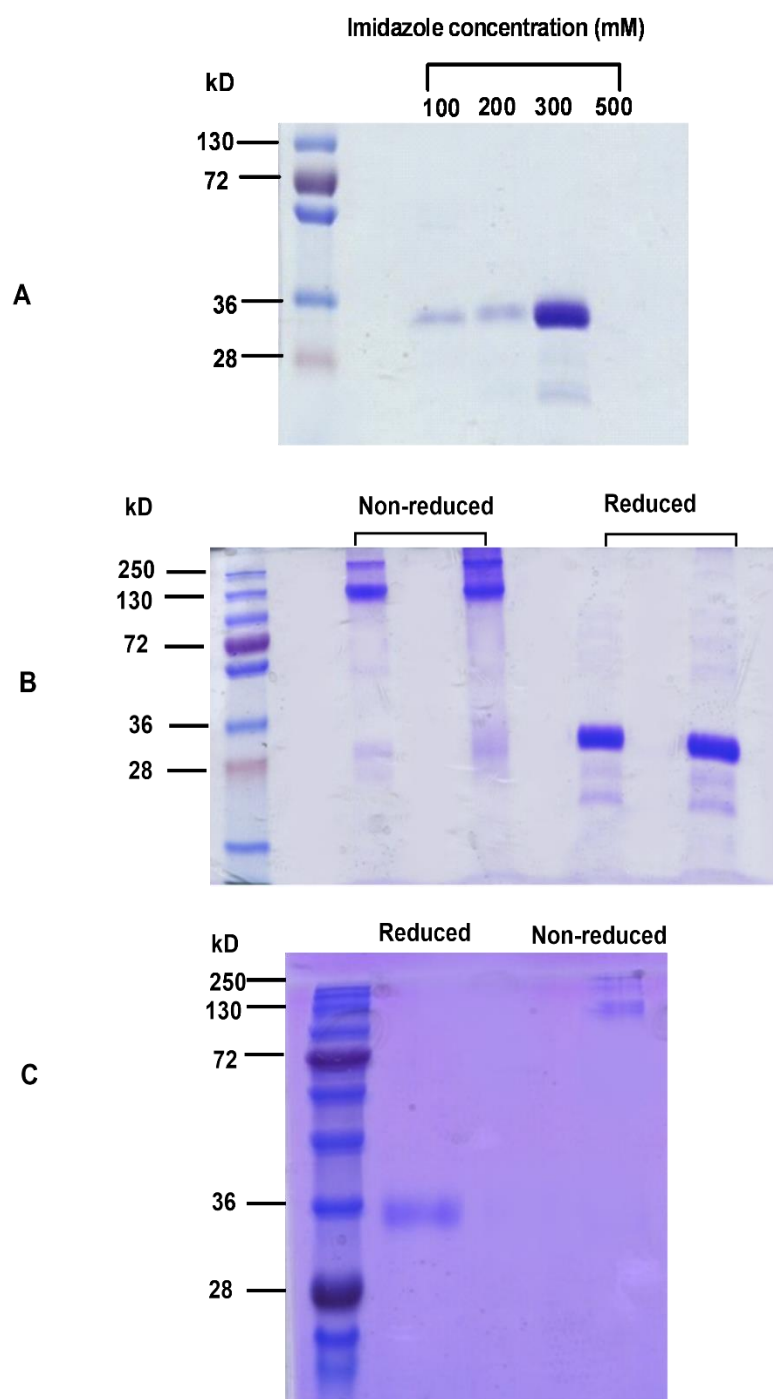
Figure 3-12 SDS-PAGE analysis of purified CL-11
(A) N-terminal his tag mCL-11 was purified by using His GraviTrap columns eluted with different concentrations of imidazole (100 mM, 200 mM, 300 Mm and 500mM). (B) The 300mM imidazole elution fractions separated under reducing and non- reducing conditions. (C) Non his tag mCL-11 was purified by Mannose–Sepharose column and run under reducing and non- reducing conditions.

A   **B**
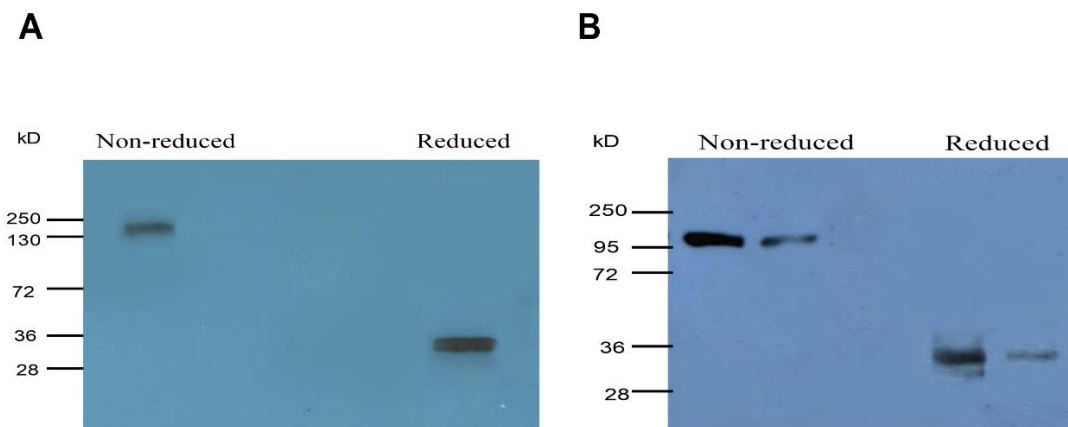
Figure 3-13 Western blot analysis of purified mCL-11 expression
(A) N-terminal his tag mCL-11 using anti his tag antibodies under reducing and non-reducing conditions, (B) non his tag mCL-11 using rabbit anti CL-11under reducing and non-reducing conditions.

3.2   In vitro studies

3.2.1   Binding of native CL-11 to different ligands

CL-11 binding was checked on different ligands, including zymosan, mannan, N-acetyl BSA, N-acetyl cysteine and DNA using wild type and CL-11 deficient mouse sera. CL-11 showed strong binding to zymosan and DNA and a weaker binding with N-acetyl BSA, mannan and N-acetyl cysteine (Figure 3.14).
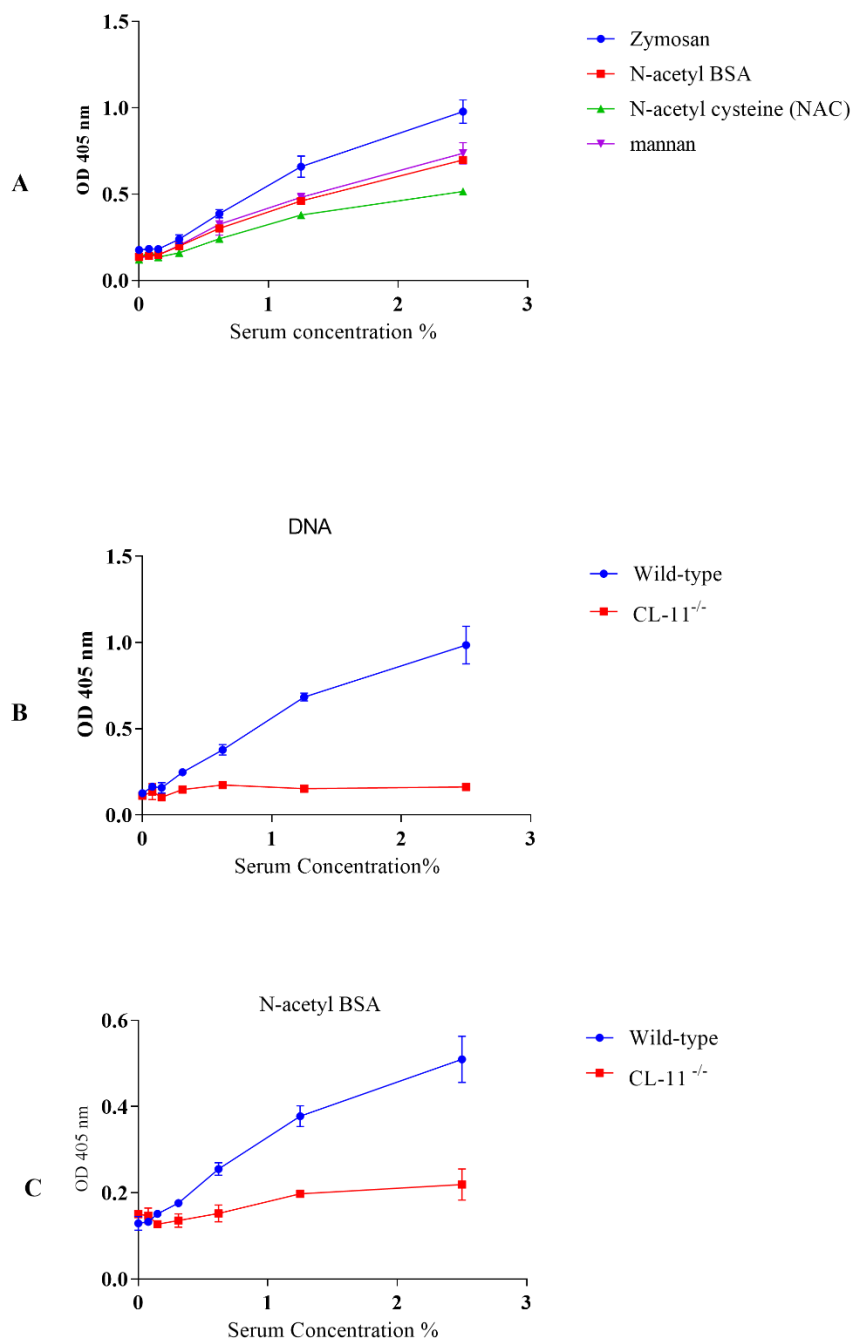
Figure 3-14 CL-11 binding assay on Zymosan, N-acetyl BSA , N-acetyl cysteine (NAC), Mannan and   DNA.
(A) Zymosan, N-acetyl BSA , N-acetyl cysteine (NAC) and Mannan incubation with WT serum   by ELISA using a specific rat anti-mouse CL-11 (B) DNA incubation with WT serum and CL-11 deficient serum (C) N-acetyl BSA incubation with WT serum and CL-11 deficient serum. Results are mean (±SEM) of duplicates and are representative of three independent experiment

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3.2.2   C3 deposition assay on different ligands

CL-11 shows binding to different ligands; zymosan, mannan, N-acetyl BSA, N-acetyl cysteine and DNA, so C3 deposition was assayed on these ligands using wild-type and CL-11 [-/-] sera. C3 deposition was higher on mannan in wild-type but was not affected by absence of CL-11 (in CL-11 [-/-] mice serum). However, reduced levels of C3 deposition were detected on zymosan, DNA and N-acetyl BSA using CL-11 [-/-] serum. (Figure 3.15), indicating that CL-11 plays a significant role in C3 turnover on these three ligands.
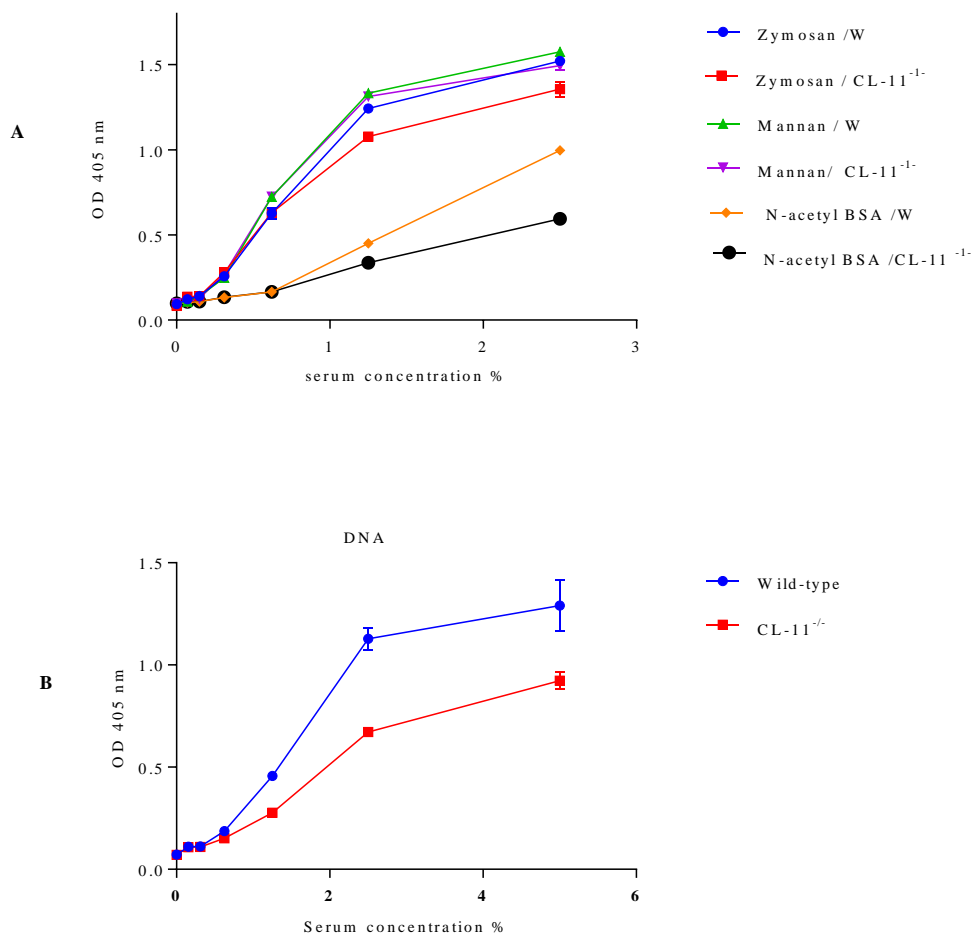
Figure 3-15 C3 deposition assay on mannan, zymosan, N-acetyl BSA and DNA using CL-11[-1-] mouse serum

C3 deposition assay on (A) mannan, zymosan, N-acetyl BSA and (B) DNA using CL-11[-1-] mouse serum Following coating of microtitre plates with mannan, zymosan and BSA, incubation with serum, C3b deposition was detected using specific rabbit anti-human C3c (Dako).

C3 cleavage assay was performed using sera from MBL A/C [-/-], Ficolin [-/-] mice and compared with wild-type and CL-11 [-/-] mice to assess the relative contribution of different lectin pathway recognition molecules towards activation of the lectin pathway on zymosan. The main contributor to C3 activation on zymosan appears to be MBL, with CL-11 and ficolin a approximately equal, more minor roles (figure. 3.16).
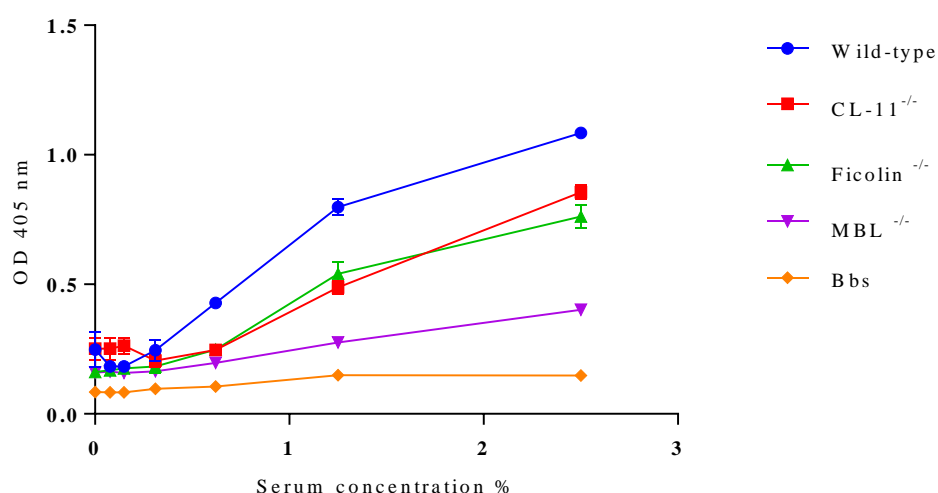
Figure 3-16 C3 deposition assay
C3 deposition assay on Zymosan using sera from gene targeted mice deficient in various carbohydrate recognition molecules: Following coating of microtiter plates with Zymosan incubation with sera, C3 deposition was detected using specific rabbit anti-human C3c (Dako). Results are mean (±SEM) of duplicates and are representative of three independent experiments BBS (barbital buffered saline in place of serum was the negative control).

3.2.3   Binding of recombinant CL-11 to different ligands

Recombinant CL-11 shows a strong binding to zymosan and DNA when compared to N-acetyl BSA. (Figure 3.17, Figure 3.18, Figure 3.19).
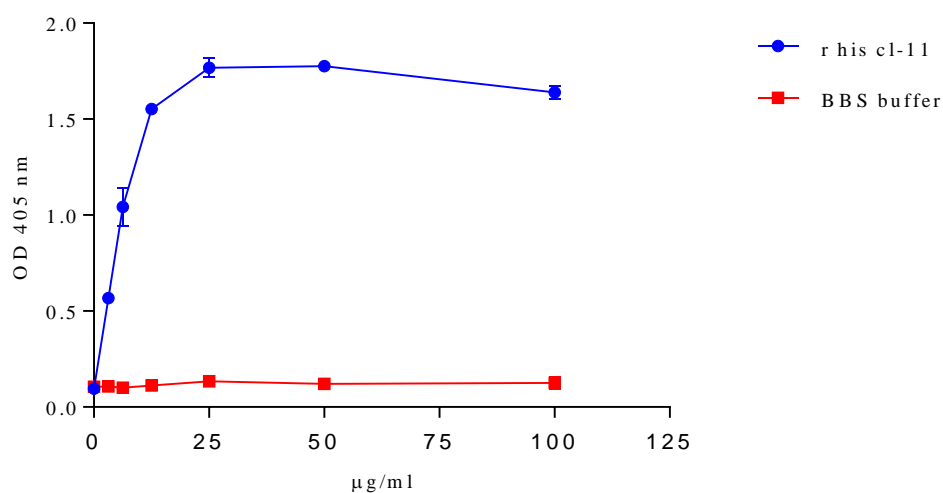
Figure 3-17 CL-11 binding assay on Zymosan by ELISA
Following coating of microtiter plates with zymosan , incubation with recombinant his mCL-11, CL-11 binding was assessed using a specific rat anti-mouse CL-11
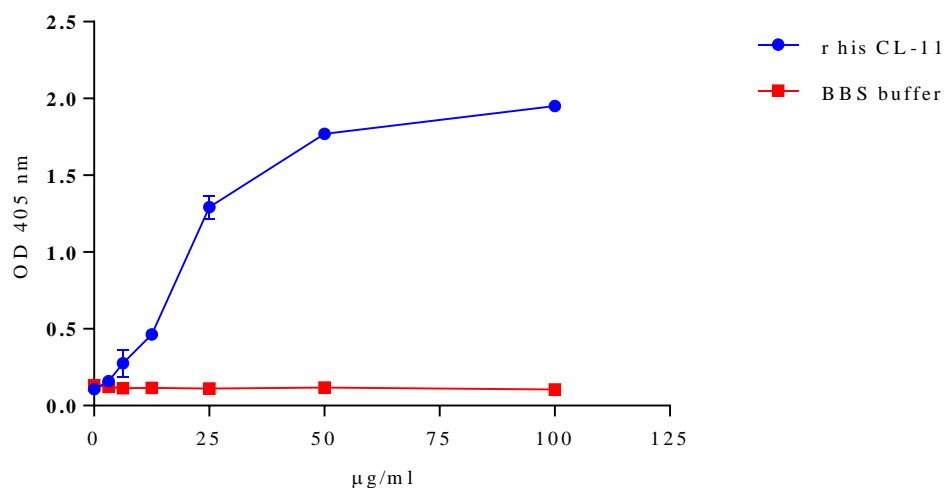
Figure 3-18 CL-11 binding assay on DNA by ELISA
Following coating of microtiter plates with DNA , incubation with his mCL-11 , CL-11 binding
was assessed using a specific rat anti-mouse CL-11
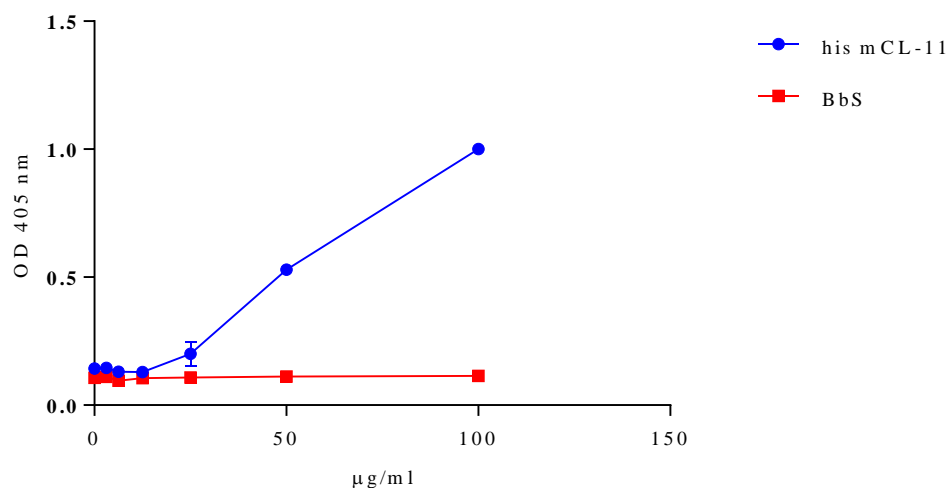
Figure 3-19 CL-11 binding assay on N-acetyl BSA by ELISA
Following coating of microtiter plates with N-acetyl BSA , incubation with his mCL-11 , CL-
11 binding was assessed using a specific rat anti-mouse CL-11

3.2.4   Recombinant CL-11 reconstitution

In order to confirm the ability of the recombinant CL-11 to activate the lectin pathway, C3 deposition assay was performed on different ligands using recombinant CL-11 to reconstitute CL-11$^{-/-}$ serum. C3 deposition was higher on zymosan and DNA in comparison to N-acetyl BSA,   and N-acetyl cysteine. (Figure 3. 20).
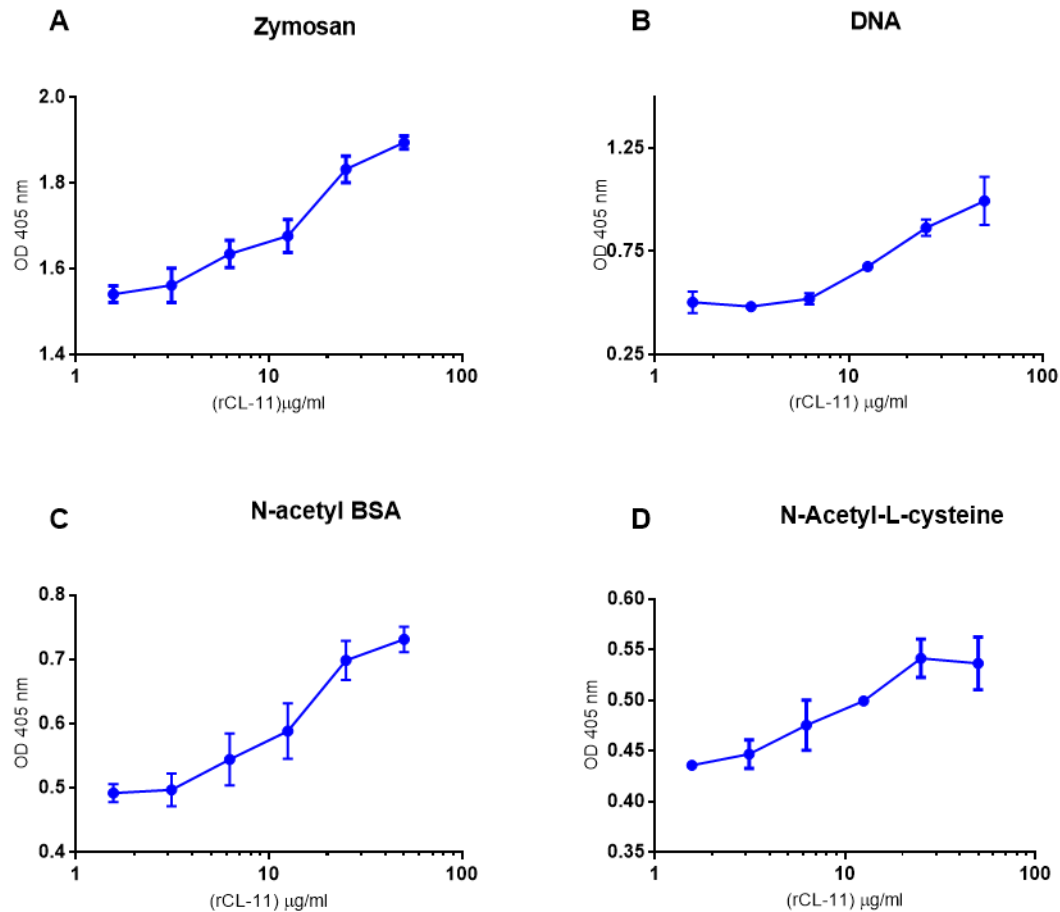
Figure 3-20 Recombinant CL-11 reconstitutes C3 deposition in CL-11$^{-/-}$ serum
MaxiSorb microtiter plates were coated with 1 µg/ml A) Zymosan. B) DNA.C) N acetyl BSA. D) N-Acetyl-L-cysteine. Serial dilution of r CL-11 were added to the plates, starting at 50 µg/ml and incubated for 1 hr. CL-11 $^{-1-}$ serum (2.5%) was added as a source of other complement components and incubated for 30 min at 37C°.C3 deposition was detected using anti-human C3c (Dako).followed by α rabbit IgG AP and pNPP substrate

3.2.5   Binding of CL-11 and C3 deposition assay   on *Streptococcus pneumoniae* D39

To define the potential role of CL-11 in activating lectin pathway, their binding affinity was tested on *Streptococcus pneumoniae* D39. This binding assays showed that CL-11 has strong binding to *S. pneumoniae* D39 (Fig 3.21)
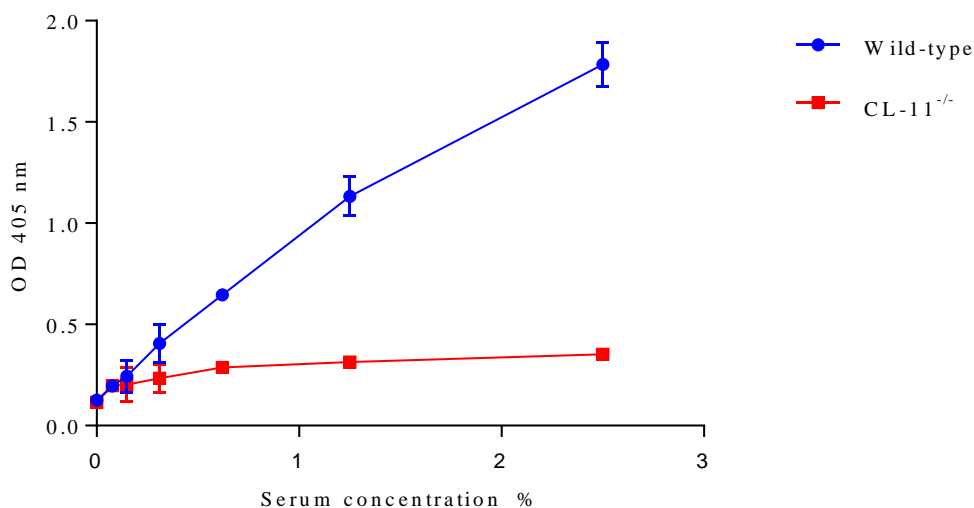
Figure 3-21 Binding assay on *S. pneumoniae* using sera from WT and CL-11$^{-/-}$
Following coating of microtitre plates with *S. pneumoniae* incubated with wildtype (blue) or CL-11 KO (red) mouse sera, binding (or absence of binding) of CL-11 was detected using a rat anti mCL-11 polyclonal antibody.

Using sera deficient in CL-11, MASP-2, MBL, Ficolin A and MBL A/C/Ficolin A. No C3 deposition was observed in sera from MASP-2$^{-/-}$ mice. In contrast, a high level of C3 deposition was observed on the surface of *Streptococcus pneumoniae* D39 in sera from MBL A/C $^{-/-}$ (See Fig. 3.22). Sera deficient in CL-11 or Ficolin A (Fcna) showed reduced C3 deposition. In sera triply deficient in MBL-A, MBL-C and Fcna, the C3 deposition was the same as seen for the Fcna $^{-/-}$ serum, providing further evidence that MBL plays no role in C3 activation on *S. pneumoniae*. No C4 deposition was detected on the surface of *Streptococcus pneumoniae* D39 using the same deficient sera.
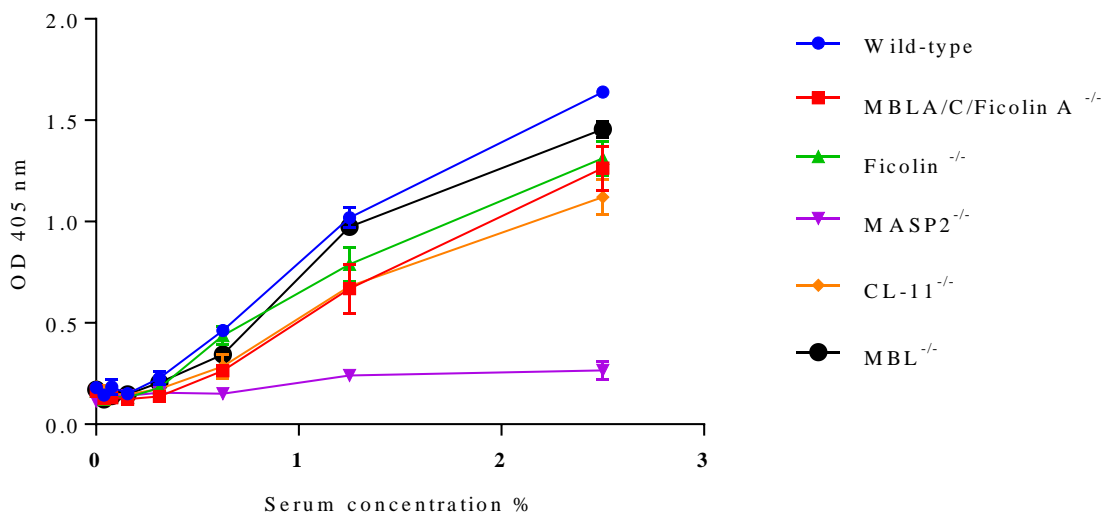
Figure 3-22 C3 deposition assay on *Streptococcus pneumoniae* D39 using sera from gene targeted mice deficient in various carbohydrate recognition molecules
Following coat of microtiter plates with formalin fixed bacteria, incubation with sera, C3 deposition was detected using specific rabbit anti-human C3c (Dako).

Also Recombinant mCL-11 showed very strong binding to *S. pneumoniae* D39 (see Figure 3.23).
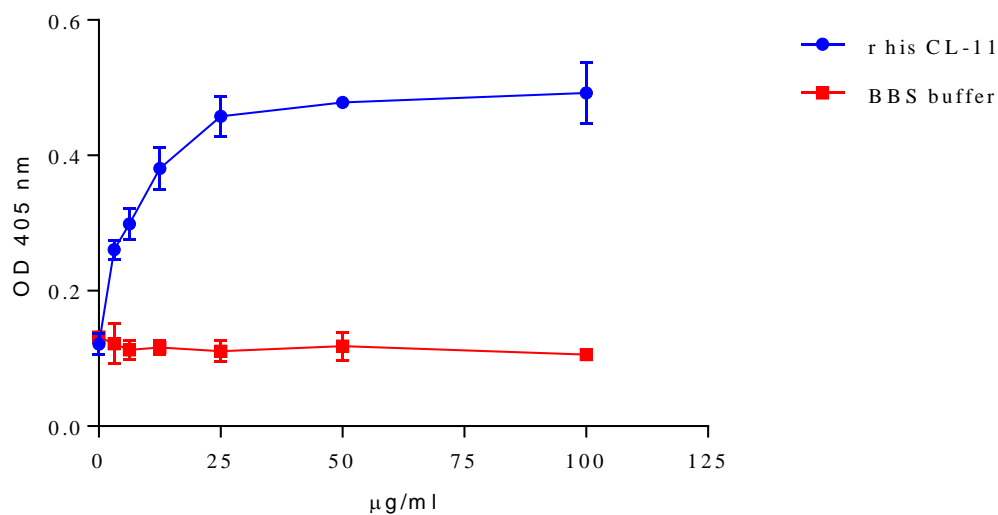
Figure 3-23 CL-11 binding assay on *Streptococcus pneumoniae* by ELISA
 Following coating of microliter plates with *Streptococcus pneumoniae*, incubation with recombinant his mCL-11, CL-11 binding was assessed using a specific rat anti-mouse CL-11.

3.3   Discussion

My study aimed to characterise the role of murine CL-11 in activating the lectin pathway against *S. pneumoniae* using a gene-targeted mouse line (on C57BL/6 background) with total CL-11 deficiency. I analyzed the binding of both serum derived and recombinant murine CL-11 to *S. pneumoniae* D39. CL-11 was shown to strongly bind to *S. pneumoniae*. A pSectag2/HygroB eukaryotic expression vector was used to express CL-11 in CHO-K1 (derived from a Chinese hamster ovary cell line). Since my first approach to express CL-11 in this expression system failed (I could not detect any recombinant CL-11 in transfected cell clones), I changed the vector and used pcDNA3.1 Hygro instead of pSectag2/HygroB in HEK cells (see Materials and Methods), an approach that achieved expression of recombinant CL-11, albeit in low quantities (the amount of recombinant CL-11 recovered from transfected cell lines was only in a range between 50-100 µg/L). In order to increase the expression yield I sub cloned the ORF encoding mouse CL-11 (encoding with either no added sequence encoding a poly Histidine-Tag or an N-terminal His-Tag into the pED4 expression vector containing a dihydrofolate reductase (DHFR) gene as a selective marker to express the construct in the in DHFR deficient CHO sub-cell line DXB11. Methotrexate MTX was added to the media to increase protein expression levels by substrate induction. MTX upregulates the expression of DHFR gene encoded by the pED4 vector leading to an increase in the expression of the vector encoded recombinant protein (Kaufman *et al.*, 1991) .His-Tagged recombinant mouse CL-11 was purified by nickel column affinity chromatography using the histidine Tag added to the N-terminus achieving a good yield of recombinant murine CL-11 in the range of 2 mg/L of cell culture supernatant.

As shown in (Figure 3.12) the recombinant murine CL-11 that I generated runs on SDS gels under reducing conditions as a single chain at approximately 36 kDa, while under non-reducing conditions (and regardless whether or not my recombinant CL-11 contains a poly-Histidine-Tag) two larger protein bands are detected in the confluence zone ranging from 100 to 200 kDa after Coomassie staining. This indicates that my recombinant CL-11 forms polymers of homotrimers, a feature well described for MBL. These results were confirmed by Western blotting using either an anti-His-Tag monoclonal antibody from the preparation

furnished with a His-Tag or a polyclonal rabbit antibody directed against murine CL-11 (see Figure 3.13).

In order to identify if my recombinant CL-11 behaves similarly to native serum resident CL-11, I have assessed the binding interactions of native mouse serum CL-11 to various potential ligands and found that native CL-11 binds strongly to genomic DNA and zymosan, and shows weaker binding to mannan, N-acetyl-Cysteine and N-acetyl BSA (see Figure 3.14.). This is well in line with the ligand profile of CL-11 described in previous publication (Keshi *et al.*, 2006; Hansen *et al.*, 2010; Ali *et al.*, 2012). This analysis was repeated using the recombinant CL-11 that I have produced. As shown in (Figures 3.17, 3.18, 3.19) the recombinant mouse CL-11 that I generated shows strong binding to genomic DNA, Zymosan and weaker binding to N-acetyl BSA, indicating that the recombinant CL-11 that I produced behaves similarly to than serum derived CL-11.

Subsequently, I established a C3 cleavage assay to measure the degree of CL-11 mediated LP functional activity on different ligands. This was achieved by comparing the amount of C3b/iC3b deposition between CL-11 sufficient and CL-11 deficient mouse sera on various ligands in parallel. When using mannan-coated ELISA plates, there was as good as no difference between sera of WT and CL-11$^{-/-}$ mice, since on mannan-coated plates, the majority of LP activation complexes that bind and drive LP activation are MBL / MASPs complexes. When the plates were coated with DNA, however, a very clear discrepancy between the degree of C3 deposition in CL-11 sufficient and CL-11 deficient serum was detected (see Figure 3.15). Likewise, a significantly reduced C3 deposition was also detected when using N-Acetyl-BSA, a ligand that I had shown to bind CL-11, but not MBL. When using zymosan-coated plates, it became apparent that CL-11 deficiency also affects the relative degree of C3 deposition in my assay (see Figure 3.16).

Recent studies demonstrated that C3b deposition on *S. pneumoniae* is dramatically reduced in MASP-2 deficient mouse serum. The major lectin pathway recognition molecules that mediate MASP-2 dependent C3 deposition on the surface of *S. pneumoniae* were identified

to be ficolin A and CL-11(Ali *et al.*, 2012) . I repeated these assays and confirmed independently that CL-11 binds to *S. pneumoniae* (see Fig, 3.21). I then progressed to assess the impact of CL-11 deficiency on the degree of C3b/iC3b/C3dg opsonisation of *S. pneumoniae*. In order to compare the relative contribution of CL-11 to the deposition of C3b and iC3b and C3dg on ELISA plates coated with *S. pneumoniae*, I incubated sera from WT mice, CL-11 deficient mice, MBL A and C double deficient mice, ficolin A deficient mice, and ficolin-A and MBL A and C triple deficient mice. As shown in (Figure 3.22), MBL deficient serum appears to deposit C3 on *S. pneumoniae* with nearly similar efficiency than WT serum, while the degree of C3 deposition is markedly reduced in either ficolin-A or CL-11 deficient serum. This indicates that MBL appears to play a rather minor role in driving the LP on the surface of *S. pneumoniae*. The degree in which ficolin-A and MBL A and C triple deficient mouse serum deposits C3 on the surface of *S. pneumoniae* indicates that CL-11 on its own can still mediate a visible degree of residual LP functional activity on the surface of *S. pneumoniae*. Again, no C3 deposition on S. pneumoniae was detected in sera from MASP-$2^{-/-}$ mice suggesting that MASP-2 is essential to deposit C3 on the surface of *S. pneumoniae*. The absence of binding of MBL-A and a weak binding of MBL-C to *S. pneumoniae* has been described previously (Krarup *et al.*, 2005) and strongly supports my own findings.

In order to assess the ability of the recombinant mouse CL-11 that I generated to bind to *S. pneumoniae*, I incubated my recombinant CL-11 in increasing concentrations in *S. pneumoniae* coated ELISA wells and developed the plate using a polyclonal rat-anti mouse CL-11 serum. As shown in (Figure 3.23), my CL-11 binds in a concentration dependent manner to the *S. pneumoniae* coated surface of the ELISA plate and binding reaches a saturation point at a concentration of 25 microgram of CL-11 per milliliter.

Next, I used my recombinant CL-11 to reconstitute LP functional activity in CL-11 $^{-/-}$ mouse serum. I measured LP functional activity using a C3 deposition assay in ELISA plates coated with either Zymosan or DNA or N-Acetyl BSA or N-Acetyl cysteine. As shown in Figure 3.20, by adding increasing concentrations of recombinant CL-11 to CL-11 deficient serum

achieved a marked increase in the amount of C3 deposition throughout, indicating that the addition of CL-11 to CL-11 deficient serum forms new CL-11 / MASPs complexes that bind to each of the tested ligands and visibly increase the amount of LP mediated C3 deposition on these activating surfaces. Taken together these results add strong evidence for a significant role of the LP recognition molecule CL-11 in the innate immune defense against *S. pneumoniae.*

Chapter Four: Results

CL-11 deficiency worsens the outcome of *S. pneumoniae* infection *in vivo*

4   Chapter Four: Results

4.1   Genotyping of CL-11 $^{-/-}$ mice

Collectin 11 deficient mice were maintained in the University of Leicester Biomedical services facility and backcrossed for 11 generations with C57BL/6 mice. Genotyping of Collectin-11 $^{-/-}$ mice was performed by using a multiplex PCR analysis of genomic DNA prepared from mouse ear snips. Three primers were used to identify wild-type and the targeted (i.e. disrupted) allele. The primer scr_F1 and wto-R1 amplify the wild-type allele fragment of 470bp, while scr_F1 and Neo3a amplify the targeted allele producing a 600bp fragment. DNA from heterozygous mice gave rise to two PCR amplification products of 470bp and 600bp (Fig. 4.1)
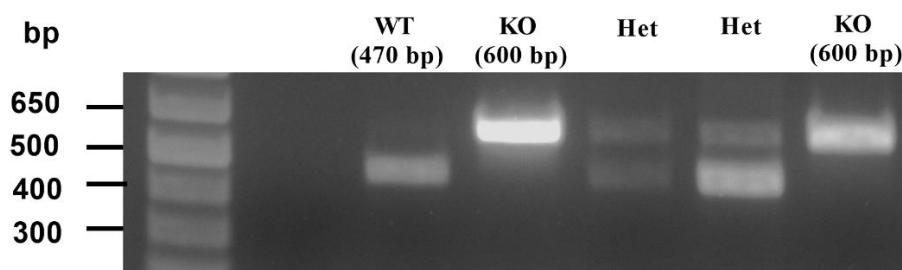
Figure 4-1 Genotyping of CL-11 -/- mice.
The 1 Kb DNA ladder size standard was loaded in the left hand lane. DNA of wild type mice or homozygous KO mice gives rise to PCR products of 470 bp (see WT) or 600 bp (see KO), respectively, while DNA of heterozygous mice gives rise to both amplification products (see Het)

4.2   Survival of wild type mice and CL-11 deficient mice following intranasal infection with *Streptococcus pneumoniae* D39

To assess the role of CL-11 in the innate immune response against *Streptococcus pneumoniae*, an infection survival experiment was carried out. Ten wild type mice (CL -11 sufficient) and 9 CL-11 deficient mice were challenged with 1×10^6 CFU of *S. pneumoniae* D39 by intranasal infection. Infected mice were monitored for up to 7 days post infection.

Five of the 9 CL-11^{-/-} mice (55.5%) failed to clear symptoms of progressing infectious disease and had to be euthanized before progressing to a severe stage of disease at day 2. At day 3, an additional 2 mice of the CL-11^{-/-} group had to be euthanized, while none of the 10 WT control mice had to be culled at day 2 and two of the WT control mice (20%) had to be culled at day 3. Over the observation period of 7 days, the overall survival throughout the experiment was therefore 22.22% for the CL-11^{-/-} group and 80% for the WT control group. The significance of these differences in survival was determined in a Mantel-Cox log-rank test and revealed a P-value of 0.0058 which underlines that the absence of CL-11 significantly increases mortality in models _S. pneumoniae_ D39 infection. (Fig 4.2).
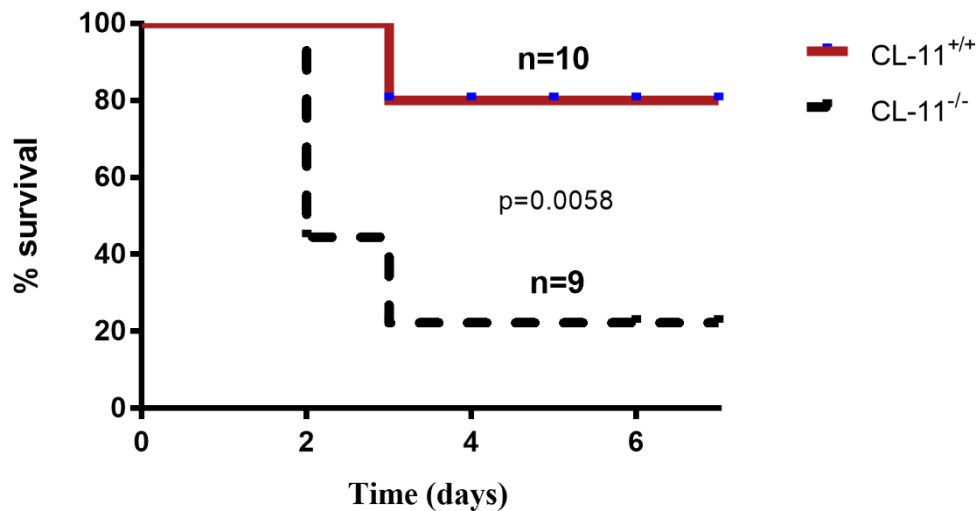
Figure 4-2 Survival of CL-11 -/- and CL-11+/+ mice (WT) after intranasal infection with 1x106 CFU Streptococcus pneumoniae D39
CL-11 [-/-] mice show significantly higher mortality as compared to wild-type mice p < 0.01 (Kaplan Meier survival curve and log rank analysis).
.

4.2.1   Detection of with *S. pneumonia* D39 in lungs and blood of infected CL-11 $^{-/-}$ and CL-11$^{+/+}$ mice following intranasal infection over different observation time points

Two groups of female age-matched CL-11 deficient and CL-11 sufficient mice were challenged with 1×10^6 CFU *Streptococcus pneumoniae* D39 via the intranasal infection route. 3 mice were culled at time points 0, 12, 24, 36 hours post-infection and 5 mice at 48 hours post-infection to assess the viable bacterial load in blood and lungs. The bacterial counts in lungs of CL-11 deficient mice increased continuously post infection until the end of the observation period. In contrast, the bacterial counts decreased in lung tissue of WT mice at 24 hrs after infection, then slightly increased at 36 and 48 hrs post infection (Figure 4.3).
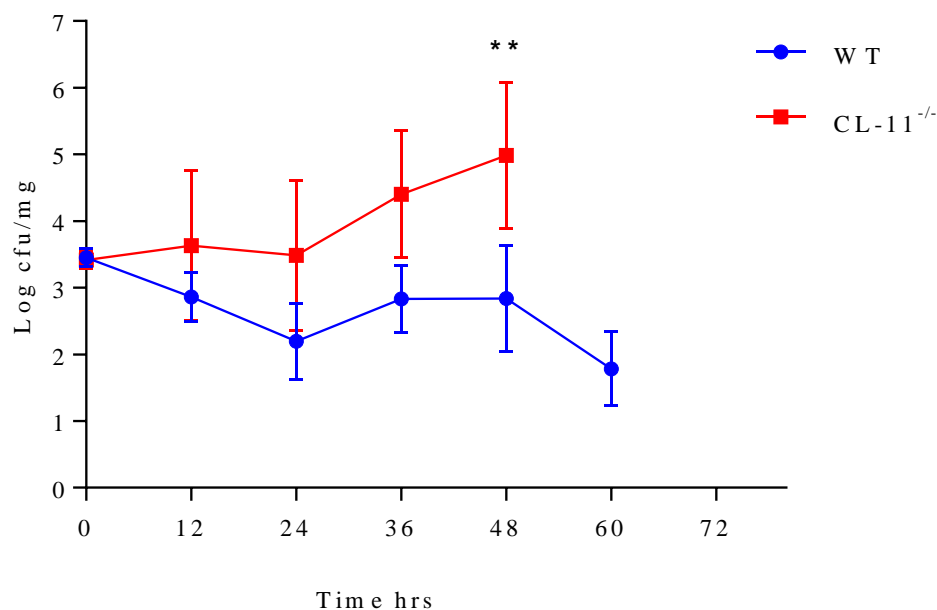
Figure 4-3 Viable counts of *Streptococcus pneumoniae* D39 in lung
Viable counts of *Streptococcus pneumoniae* D39 in lung homogenates after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Bacterial loads were copared between both groups by Tow-way ANOVA.*P<0.05 ** P<0.01

The bacterial counts were higher in blood of CL-11 deficient mice compared to CL-11 sufficient controls at all time points after infection (Fig. 4.4).
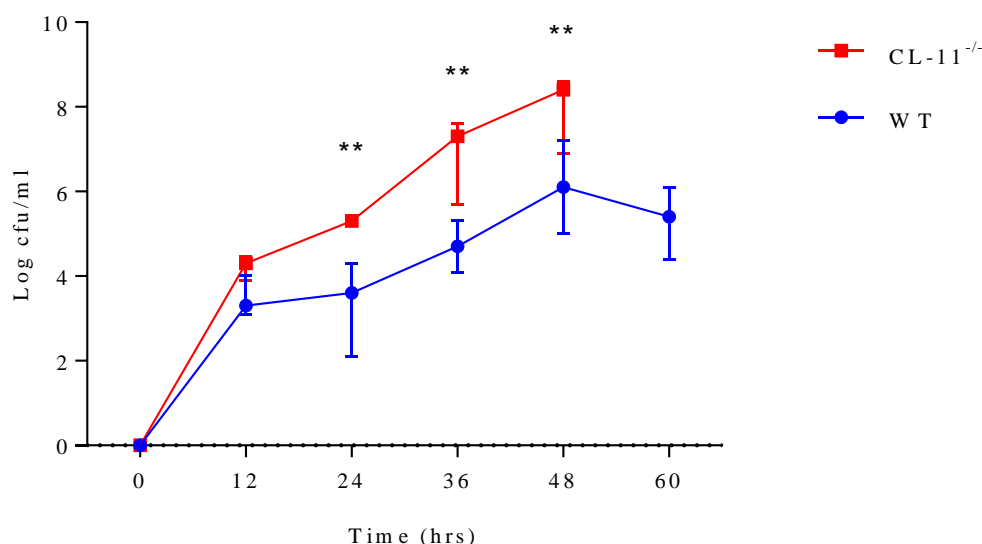
Figure 4-4 Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood
Viable counts of *Streptococcus pneumoniae* D39 in peripheral blood after intranasal infection with 10^6 CFU *S. pneumoniae* D39 at different time points post infection. Bacterial loads were compared between both groups by Two -way ANOVA.*P<0.05 ** P<0.01

4.2.2 mRNA expression profiles of inflammatory cytokines in mouse lung tissues post *S. pneumoniae* infection

The mRNA expression of different inflammatory cytokines in lung tissues were determined in CL-11 $^{-/-}$ and CL-11$^{+/+}$ mice at time points 0, 12 ,24 ,48 hours after intranasal infection with 10^6 CFU *S. pneumoniae* D39 using qRT-PCR technique. All mice showed the expected inflammatory response (Figure 4.5). By 48 hours, the WT mice showed signs of reduced inflammation, with the TNF-α expression dropping significantly, while it remained high in the CL-11 deficient mice. A similar trend was seen for the other proinflammatory cytokines (IL-1 ß and INF-γ, but the results did not reach significance (2 way ANOVA, with post hoc t-test).
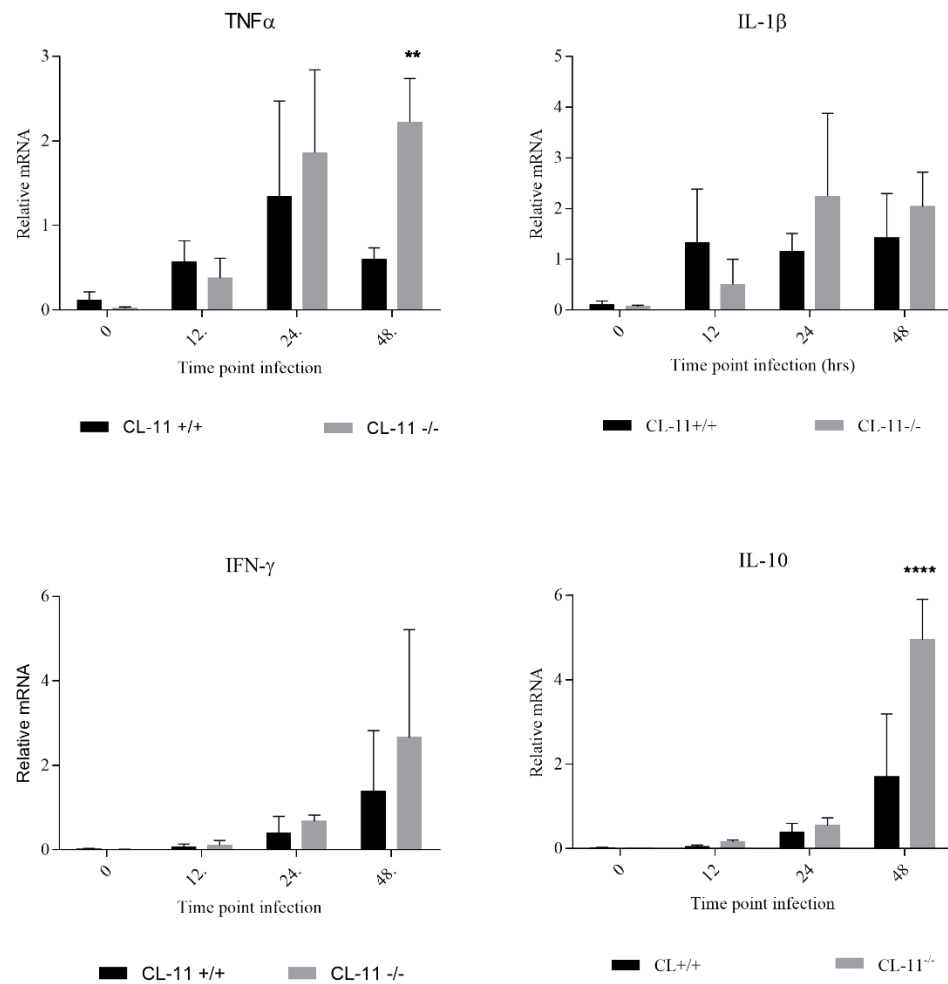
Figure 4-5 Quantitative RT-PCR analysis of mRNA expression of TNF- α, IFN- γ, IL-1β, IL-10
Quantitative RT-PCR analysis of mRNA expression of TNF-α, IFN-γ, IL-1β, IL-10, in the lung following intra nasal infection with *Streptococcus pneumoniae* D39. Lungs were removed 0, 12, 24 and 48 hours after infection.

4.3   Discussion

Since my work added strong evidence for a significant role of the LP recognition molecule CL-11 in the innate immune defense against *S. pneumoniae* I tested this hypothesis in a mouse model of *S. pneumoniae* infection. For this purpose, I bred up female CL-11$^{-/-}$ mice on C57/BL6 background and purchased strain, sex and age matched C57/BL6 WT control mice. At the age of 9 weeks, 9 CL-11$^{-/-}$ mice and 10 WT control mice were infected with 10^6 CFU of *S. pneumoniae* D39 and the course of infectious disease was carefully monitored over an observation period of 7 days. As shown in (Figure 4.2) 5 of the 9 CL-11$^{-/-}$ mice (55.5%) progressed to clear symptoms of progressing infectious disease and had to be euthanized before progressing to a severe stage of disease at day 2. At day 3, an additional 2 mice of the CL-11$^{-/-}$ group had to be euthanized while none of the 10 WT control mice had to be culled at day 2 and two of the WT control mice (20%) had to be culled at day 3. Over the observation period of 7 days, the overall survival throughout the experiment was therefore 22.22% for the CL-11$^{-/-}$ group and 80% for the WT control group. The significance of these differences in survival was determined in a Log-rank test and revealed a P-value of 0.0058 which underlines that the absence of CL-11 significantly increases mortality in models *S. pneumoniae* D39 infection. This is the first report that identifies CL-11 to be a key component of innate immunity against pneumococcal pneumonia.

In a second set of experiments, I determined the likely differences between CL-11 deficient mice and their WT littermate controls in the bacterial burden found in lungs. For this purpose seventeen female 9 week old CL-11$^{-/-}$ mice and seventeen strain, age and sex matched WT controls were infected and of each group 3 mice were killed at time point 0, 3 mice at time point 12 hours, 3 mice at time point 24 hours, 3 mice at time point 36 hours and 5 mice at time point 48 hours post infection. After culling the mice were exsanguinated by cardiac puncture and the lungs of these mice were taken out and homogenized and the homogenate used to determine the number of recoverable CFUs as described in Materials and Methods. As shown in (figure 4.3), the bacterial counts were increased in lungs of CL-11 deficient mice in comparison to the numbers of recoverable CFUs found in the lungs CL-11 sufficient WT control mice throughout each and every time point after infection.

When comparing the degree of bacteremia in both test mouse groups, we also see a significant increase in recoverable CFUs in the CL-11$^{-/-}$ group, indicating that the absence of CL-11 favours the progression of infection. A previous study of our laboratory comparing ficolin A deficient mice (sufficient in CL-11) with WT control mice revealed a similar degree of increased susceptibility to Streptococcus pneumoniae D39 infection in ficolin A deficient mice, showing high mortality at 72 hours, post infection (Ali *et al.*, 2012). The best way to understand the phenomenon that taking out one of several other recognition subcomponent that may bind equally well to the surface of a pathogen like *Streptococcus pneumoniae* has such a great impact on the susceptibility to infection is to recapitulate the physiological events that drive lectin pathway activation. Although the organization of lectin pathway activation complexes may look very similar to that of classical pathway complexes, there is a remarkable difference in the activation mode of both pathways. The classical pathway activation complex, i.e. C1, formed by the 18 chain macromolecule C1q (a hexamer of heterotrimers formed out of C1q A-chain, B-chain and C-chain) as the recognition subcomponent and a heterotetramer of the C1q associated serine proteases C1s-C1r-C1r-C1s form an independently operating functional unit, since the heterotetramer of C1r and C1s sits within the calyx of C1q (Wallis *et al.*, 2010b; Girija *et al.*, 2015).

Conformational changes within the C1q recognition subcomponent (when two or more globular head domains bind to the Fc region of immune complexes of the immunoglobulin classes IgG1, IgG2, IgG3 or IgM) catalyze the autoactivation of C1r which subsequently cleaves and activates its only substrate C1s and allows C1s to be converted into its active form cleaving complement components C4 and C4b-bound C2 to form the classical pathway C3 convertase C4bC2a. All activation steps can occur within a single C1 complex and the juxtaposition of other classical pathway activation complexes is not a requirement since each C1 complex works as a single operational unit. In contrast, lectin pathway activation complexes are differently organized: Here, the recognition subcomponents are polymers of homotrimers of single chains which can form dimers or trimers or tetramers or higher polymers of homotrimers. The lectin pathway specific serine proteases MASP-1, MASP-2 or MASP-3 bind as homodimers to dimers, trimers or tetramers of their respective recognition subcomponents MBL, or CL-11 (complexed with itself or complexed with CL-10), or Ficolin A. Most lectin pathway activation complexes are only loaded with one MASP

homodimer and the antiparallel orientation of the homodimers results in the serine protease domains being pointed into the opposite direction and since the flexibility within the homodimers is limited to such an extent that one enzymatically active serine protease domain cannot reach and cleave the serine proteases domain of its direct binding partner within a single LP activation complex. What is required for that to happen is that two lectin pathway complexes have to bind in close proximity to each other that the activated enzyme domain sticking out from one lectin pathway complex can cleave the zymogen MASP of the other complex to initiate a chain reaction of activation events that drive lectin pathway activation. This chain reaction is perpetuated by the observation that enzymatically activated MASP-1 can cleave zymogen MASP-1 or zymogen MASP-2, like activated MASP-2 can cleave zymogen MASP-2 or zymogen MASP-1 in juxta-positioned complexes. These juxtapositioned complexes can have different recognition subcomponents binding to their ligands on activating surfaces and I hypothesise that the explanation why the deficiencies of either ficolin A or CL-11 present with similar effects in terms of increased susceptibility to *S. pneumoniae* infection or reduction of C3 opsonisation on the pathogen surface is that both recognition subcomponent bind to ligands on the pathogen surface that form a complex network of activation events driven by the density in which these different lectin pathway activation complexes form a network of activation and drive complement activation on this surface (Degn *et al.*, 2014)

As the pro-inflammatory responses initiated by complement activation play an important role in the antimicrobial immune defense of the lung to control the infection (Kerr *et al.*, 2002), the levels of different cytokines –as read-outs of inflammatory responses were measured at different time points post infection (Figure 4.5).

Both TNF-alpha and IL-1 beta levels responded early after pneumococcal invasion. These pro-inflammatory cytokines induce the nuclear translocation of the transcription factor NF-kB and switches on many different inflammatory genes. Complement activation also leads to the recruitment of neutrophils from the blood stream to lung tissues to help clear the

bacteria (Jones *et al.*, 2005; van der Sluijs *et al.*, 2006; Barichello *et al.*, 2009) . The most potent mechanism by which complement activation recruits neutrophils is via the release of the complement anaphylatoxin C5a, a small but biologically highly active cleavage product of the cleavage of the fifth complement component. Neutrophils are loaded with high numbers of C5a receptors (belonging to the 7TM rhodopsin like receptor family). CL-11 sufficient WT mice show higher mRNA expression levels of both TNF-alpha and IL1-beta at time point 12 hours as compared to CL-11 deficient mice. Then the TNF-α expression is increased in CL-11 deficient mice at time points 24 and 48 hours. This is in line with previous reports that show that TNF-α mRNA expression is increased during the infection with *Streptococcus pneumoniae* and TNF levels may become fatally high at the late stage of disease (Kerr *et al.*, 2002). It has also been shown that blocking TNF-α using an anti-TNF-α monoclonal antibody results in a reduction of neutrophil recruitment and a decreased clearance of *Streptococcus pneumoniae* (van der Poll *et al.*, 1997). Another study analyzing human subjects showed that the treatment with monoclonal anti-TNF-α antibodies causes an increased to invasive pneumonia and other opportunistic infections (Calbo & Garau, 2010). Moreover, it has been indicated that the TNF-α deficient mice are highly susceptible to *S. pneumoniae* D39 infection in comparison to WT mice (Jeong *et al.*, 2011).

My own results indicate that IL-1 ß mRNA expression levels of wild type mice were higher than those seen in CL-11 deficient mice at time point 12 hours, because the CL-11 deficient mice showed a lower pro-inflammatory response at the early stage of infection because of a reduced degree of lectin pathway activation. The importance of complement in surviving *S. pneumoniae D39* infection has been demonstrated in previous studies carried out in C3 deficient mice, which all succumbed to infection and showed remarkably low expression of IL-1-ß in a model of pneumococcal meningitis (Rupprecht *et al.*, 2007). The increase in   IL-1 ß expression levels in CL-11 deficient mice at time points 24 and 48 hours is most likely caused by the higher severity of infection in these partially lectin pathway deficient mice and most likely not caused by any direct effect of CL-11 deficiency, but rather by the higher degree of bacteremia in CL-11 mice. IL-10 is a downregulator of pro-inflammatory events and aims to protect the body for overshooting inflammation as an anti-inflammatory

cytokine. The expression of IL-10 is not enough to control the proinflammatory response in collectin 11 deficient mice and remains high at 48 hours and leads to septic shock. It is therefore not at all surprising that IL-10 deficient mice shows higher mortality from septic shock (Latifi *et al.*, 2002). The IFN-γ expression in collectin 11 sufficient and deficient mice increases with no significant difference between them during the observation time of the experiment.

Chapter Five: Results

Study of human CL-11 core promoter elements by gene reporter assays

5   Chapter Five: Results

5.1   Study of human CL-11 core prompter elements by gene reporter assays

The promoter is an essential region of the regulatory machinery of a gene. This region is located mainly upstream the transcription start site (TSS). A promoter is composed of two regions; the core and the proximal regions. The core region is located within -40 to +40 nucleotides of transcription start site (TSS) and contains several motifs such as the TATA box, BRE (TFIIB-recognition element), DPE (downstream promoter element), DCE (downstream core element), Initiator element (Inr) and MTE (motif ten element). These elements have binding sites to bind specific proteins named transcription factors to start transcription. The proximal region is located several hundred bp upstream the core-promoter.(Zeng *et al.*, 2009; Juven-Gershon *et al.*, 2008).

These core promoters are not all the same, some core promoter lack many of known promoter elements and some of them lack all of these elements. A TATA-box is a motif (8mer TATAWAAR (R denotes A/G and W denotes A/T) located 30 bases upstream of the transcription start site (TSS), which is a site of TATA binding protein. It has been shown that some promoters lacks a TATA-box and it may be that the promotor is controlled by other elements.(Juven-Gershon *et al.*, 2008; Zeng *et al.*, 2009). It has been found that TATA box is present in about 5–7% of eukaryotic promoters and, promoters that lack a TATA box may contain the Inr element (a 7-mer YYANWYY (N denotes A/G/C/T and Y denotes C/T). Some promoters have TATA and Inr elements together. (Roy & Singer, 2015).

The function of the TATA box and the initiator (Inr) is to provide a site for binding TATA-binding protein (TBP), which is a component of the TFIID (Transcription initiation factor) complex. Once the TFIID binds to a TATA box, other transcription factors TFIIA, B, E, and F also bind and initiate transcription by RNA polymerase II.(Berg *et al.*, 2002). Downstream promoter elements (DPE) are located +28 to +32 relative to nucleotide at +1 (consensus sequence A/GGA/TCGTG).The gene transcription by RNA polymerase is initiated by recognition of the DPE by the transcription factor II D (TFIID) subunits and it has been shown that this occurs cooperatively with Inr.(Juven-Gershon *et al.*, 2008; Butler & Kadonaga, 2002). There is a promoter element located downstream (BREd) (sequence

G/ATT/AT/GT/GT/GT/G) or upstream (BREu) (sequence G/CG/CG/ACGCC) of some TATA boxes which is named the TFIIB recognition element (BRE).This element has consensus G/C-G/CG/A-C-G-C-C which is recognized by TFIIB. It has a role in transcription not only as activator but also as repressor. Also DCE is another downstream element located at +9, +18, +32   (sequence CTTC, CTGT, AGC) and recognized by TFIID (Roy & Singer, 2015).

The proximal promoter regions is localized a few hundred base pairs upstream of the core promoter and is characterized by the presence of CpG islands, which are long sequences of DNA (500bp to 2kb) with a high GC nucleotide content (higher than 55%) and a high frequency (higher than 0.65) of CpG dinucleotides. It has been found that 60% of human genes have CpG islands and that promoters with TATA boxes usually lacks a CpG island. (Maston *et al.*, 2006).

Promoters of some complement human genes have been studied and the studies showed that the promotors of C2, C4 and factor H all lacked a TATA box (Williams & Vik, 1997). Also it has been reported that the MASP2 gene and C1s gene lack TATA box but have GC box-like sequences. The MASP1/3 core promoter gene has a TATA-like sequence. (Endo *et al.*, 2002; Stover *et al.*, 2001).

Genetic variation in eukaryotic promoters can have significant effects on gene expression; in the case of human L-ficolin, serum concentrations vary substantially with the promoter haplotype and there is a substantial body of research showing that disease susceptibility is affected by variations in the promotor (Munthe- Fog *et al.*, 2007).

5.1.1   The CL-11 promoter

Analysis of CL-11 gene 700bp upstream and 200bp downstream of exon 1 using the Eukaryotic Promotor Database (EPD) identified a putative transcription start site (TSS) with four TATA boxes upstream. No other regulatory consensus sequences were identified (GC box and CCAAT box). The putative TATA boxes are almost certainly too far 5' of the TSS to be functional, suggesting that the CL-11 promoter is unconventionally regulated (figure 5.1). The EPD also identified alternative promoters in intron 1, which might drive expression of the other, shorter transcript variants, but these were not analyzed.

Here, I defined the minimal promoter for CL-11 transcript variant a, using a set of nested deletions cloned into an *in vitro* reporter vector. The eventual aim was to study the effect of genetic variation in the promoter on the level of CL-11 expression, using the same system.

```
>FP002400 COLEC11_1 :+U EU:NC; range -700 to 200.
TTGTCTAAGTTGTCAAATTTATGTATAACTTCAATAATACGTTTTAAATAAAGAATCCAT      -640


TATTTTTCCTTAATTTTATTATGTGTGTGTGGAATTTGTGTTTTTACAGTTTTATTTTATGA    -580
                                 rs1864480(0.22)

                 F primer -557           TATA BOX
TTTCTCAGTCTAAATAAATATTCACCTTTGAACCTAGCTTCTTATGATTGTAATTTTATA     -520   -557


TGTATCTTTTTCTTAAAGAAGGTTCCGCTATCCCCCAAAACATGCAAGATTCGGGCTGCA     -460

                                         TATA BOX
TAGAAGCTGGATCTGTCCAGCCCCTGACTAACATTTGTTTCTGGCATTAAATGAAACTTG      -400
                                              rs4849953(0.25)

      TATA BOX
CCTTTAAATGAAACTTAAATGCGTAGAGTGAGTTTCCGGACTCACGTGTGGCCAGGTCTC     -340

                                     TATA BOX
CACAGGCCGTGGTCAAGCTCTTGCTGACTTTTGAAGCGAAATCCCATATGAACAAAGGAA     -280

                       F primer -251       TATA BOX
AGGAGATCAGTCAGCTGCCCTCGTTTCTGTCTGATTGGTGCCAGGTGATGAGCGTCCCTC     -220   -251
                                              rs34596301 (0.05)
TATA BOX
CTCTTTATGCTCTTTTGAATTTCCACAGTAGTCATGCTTATTTTTTCAAAAAACGAAAAG     -160
         rs3820897 (0.33)
                             F primer -125
AAAGAAGAGAAAGCCAAAAAAGAAACAAAATTGGGATGTTCGGGTTGGAGAGTTAGCAGG    -100   -125


GGCATCTCCAGGAGGGTCCTGCCCCCCACCCCAAAGCCGGCCTCAGGGAGTTCCAGGCGT     -40

                         +1  TSS
TCCCACACTCCCCAGGCAGGACGGTGGACGCAGCGCAGACAGGAAGCTCCCCGAGATAAC      20


GCTGCGGCCGGGCGGCCTGATTTGCTGGGCTGTCTGATGGCCCGGGCCGAGGCTTCTCCC      80


TGCGCCTGGGACTGCGGCCGCCTCTCTAAATAGCAGCCATGAGGCGCCTGGGGGCAGTGT     140

                         R primer
CCTCGCGGGCCAGCGACGGGCAGGACGCCCCGTTCGCCTAGCGCGTGCTCAGGTAGGAGA     200
```

Figure 5-1 Sequence of the CL-11 promotor

Showing exon 1 (blue text; known 5' end of NM_199235), the predicted transcription start site (TSS; blue highlight) and four predicted TATA boxes (pink highlight). The TSS and TATA box predictions are derived from the Eukaryotic Promotor database (http://epd.vital-it.ch/cgi-bin/get_doc?db=hgEpdNew&format=genome&entry=COLEC11_1). Common allelic variations (frequency of the rare allele >0.05) are highlighted in green (extracted from dbSNP via Ensembl).Positions of the PCR primers using in the in vitro promotor study are shown red text

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5.1.1.1   Results

Gene reporter assays are used to study the activity of promoters *in vitro*. The system consist of a plasmid encoding a reporter gene with a multiple cloning site localized upstream of the reporter gene. Putative promotor sequences are cloned into the multiple cloning site MCS and the constructs transfected into a eukaryotic cell line. If the promotor is active in the cell line, the reporter gene will be expressed and its activity can be assayed, usually by using a simple enzymatic or luminescence measurement.

In this study, I have used the Dual Luciferase Assay (Promega) to study the human CL-11 promoter. In this assay the firefly and Renilla luciferase activities are measured sequentially in the same sample. Firefly luciferase catalyzes conversion of luciferin to oxyluciferin and produces light at 560nm, while Renilla luciferase catalyzes the conversion of coelenterazine to coelenteramide and produces light at 480 nm. The cell lines used for these assays were the human embryonal kidney line HEK293 and a hepatic cell line HepG2, chosen because CL-11 should be produced in these lines and it is therefore likely that they produce all the transcription factors necessary to drive the promotor.

The accuracy of the reporter assay depends on transfection efficiency and the efficiency of plasmid in the host cells. These factors may affect the experiment, therefore an internal control plasmid pRL-SV40 (see map in the 2.2.3.1.13.1) is used in a Dual Luciferase Assay. This internal control plasmid (which encodes Renilla luciferase under the control of the SV40 promotor) is co-transfected to the host cell with the main reporter plasmids (which encode firefly luciferase), and the results from the control are used to normalize the results from the experimental plasmids. Constructs containing fragments from the 5' flanking region of the human CL-11 gene were generated by PCR and ligated upstream of the luciferase reporter gene. The primers used are listed in table (2.11) and their positions are indicated in figure (Figure 5.1), above. The forward primers were modified to incorporate a *XhoI* site and the reverse primer contained a *HindIII* site.The PCR fragments were 316bp (125bp upstream and 191 downstream of the TSS), 442bp (251bp upstream and 191 downstream of the TSS) and 748 bp (557bp upstream and 191 downstream TSS) (Figure 5.2).
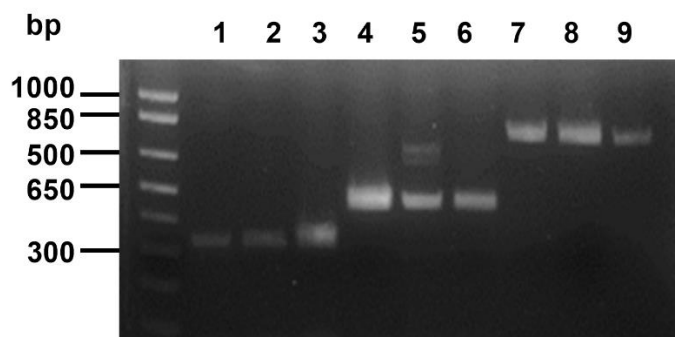
Figure 5-2 CL-11 promotor PCR products were generated using human genomic DNA: Lanes 1, 2 & 3 fragment to ntd -125; 4, 5 & 6 fragment to ntd – 251; 7, 8 & 9 fragment to ntd - 557.

The PCR products were cloned into pGEMT-easy then excised and ligated into the *HindIII* and *XhoI* sites of pGL3-basic and pGL3-enhancer (Figure 5.3). The pGL3-enhancer plasmid contains a downstream SV40 enhancer that gives greater luciferase expression, a feature that may be important when studying the activity of core promoter fragments that lack proximal promoter elements. pGL-3 basic lacks any plasmid-encoded enhancer or promotor elements.

A

B
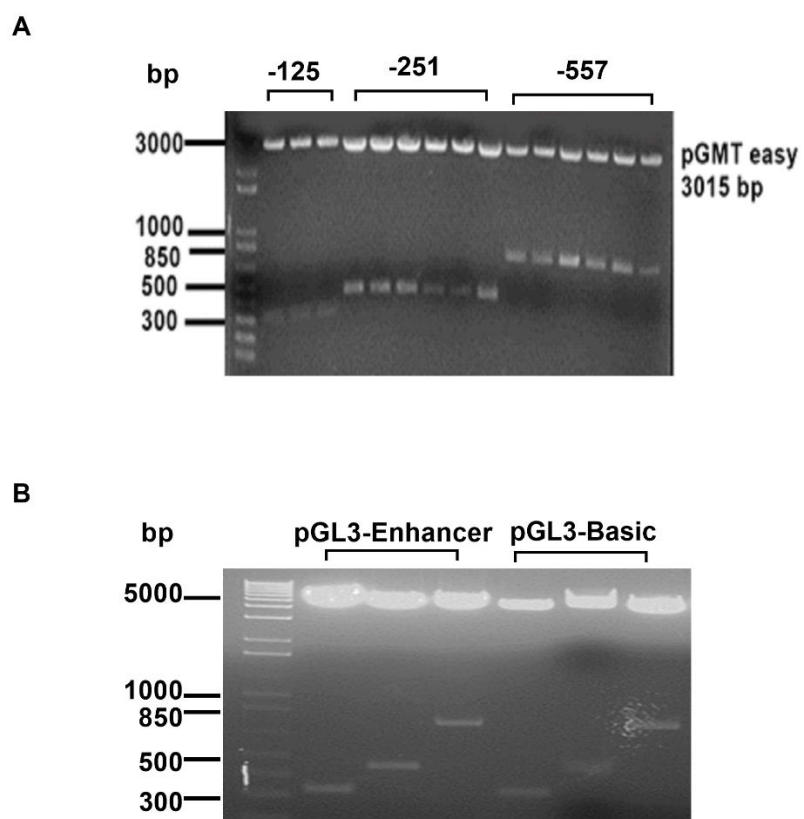
Figure 5-3 : Double digest using HindIII and XhoI for plasmid constructed of pGEMT-easy and Pgl3 vectors
 (A):   pGEMT easy-125, 2: pGEMT easy - 251and pGEMT easy -557   (B): pGL3 Enhancer fragment   -125, - 251 , -557. and pGL3 Basic fragment   -125, - 251 , -557.

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The constructs were transfected into HepG2 and HEK293 cell and analyzed for luciferase activity after 48hr as described in section 2.3.2.1.13.3. Cells transfected with pRL-control alone were added in the experiment as a negative control to read the background which shows no firefly luciferase expression. In both cell lines the shortest fragment (extending 125 upstream of the TSS) produced luciferase activity significantly above the background control, indicating that this region contains the minimal promoter. Luciferase activity increased with the length of the construct showing the presence of some distal control elements. The presence of the SV40 enhancer element in pGL3 enhancer gave the expected increase in luciferase expression, but results were otherwise essentially the same as those seen using pGL3-basic. The results do not exclude the possibility of other enhancer elements further upstream, since the luciferase activity was not seen to flatten off between the 2nd longest and longest fragments. These results demonstrate a viable system for studying the effect of natural genetic variation in the CL-11promoter.
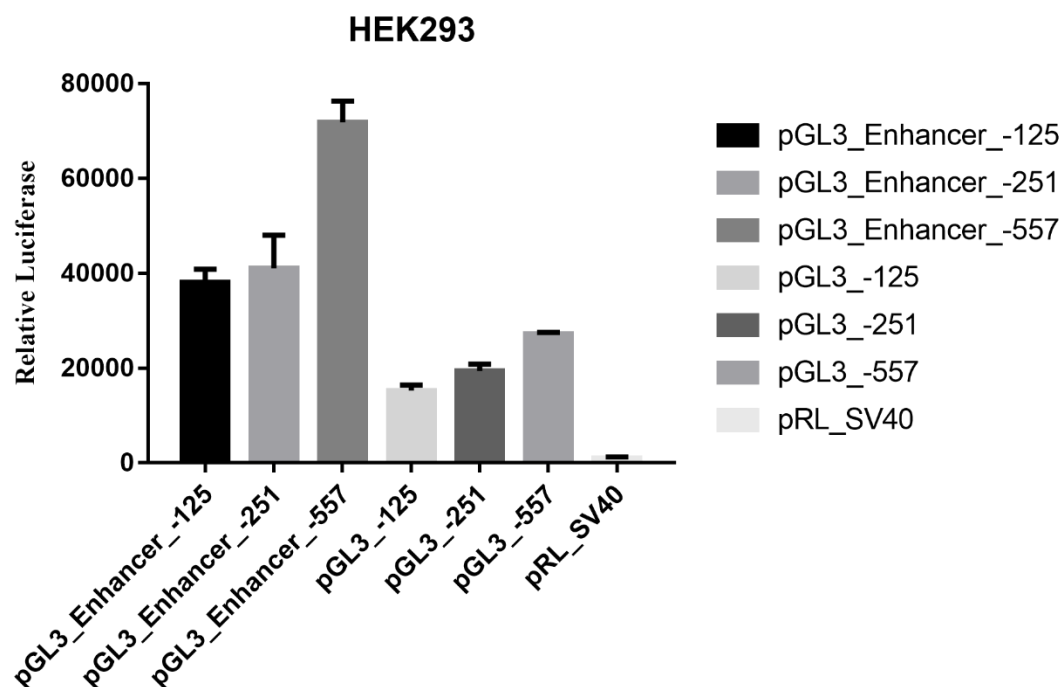
Figure 5-4 In vitro analysis of CL-11 promoter activity using HEK293 cells
Hek293 cells were transfected with pGL3 or pGL3-enhancer containing the CL-ll promoter fragments described above. Cells were co-transfected with a constant amount of pRL-control. After 48hrs, the cells were harvested in cell lysis buffer and firefly and renilla luciferase activity assayed using the Promega Dual Glo Luciferase assay reagents. Results were normalized to correct for differences in cell density and growth, and transfection efficiency, by dividing the values obtained for firefly luciferase (from the experimental pGL3 constructs) by those obtained from the renilla luciferase (the pRL-control values). Data are means ± SD of triplicates and are representative of three independent experiments.

Figure 5-5 In vitro analysis of CL-11 promoter activity using HepG2 cells
HepG2 cells were transfected with pGL3 or pGL3-enhancer containing the CL-ll promoter fragments described above. Cells were co-transfected with a constant amount of pRL-control. After 48hrs, the cells were harvested in cell lysis buffer and firefly and renilla luciferase activity assayed using the Promega Dual Glo Luciferase assay reagents. Results were normalized to correct for differences in cell density and growth, and transfection efficiency, by dividing the values obtained for firefly luciferase (from the experimental pGL3 constructs) by those obtained from the renilla luciferase (the pRL-control values). Data are means ± SD of triplicates and are representative of three independent experiments.

5.2   **The correlation of SNP (rs7567833) of the human CL-11 gene with a clinically established pre-disposition for frequent microbial infections**

The human genome comprises of approximately three billion nucleotide with a variation among individuals (Feuk *et al.*, 2006). This variety is created by gene polymorphisms which might be repeating in the sequence of nucleotides, deletion or insertion of nucleotides or substitution of one nucleotide by another nucleotide which are named single nucleotide polymorphisms (SNPs) (Haraksingh & Snyder, 2013).

Single nucleotide polymorphisms (SNPs) are the variations in a single nucleotide position in the genome. SNPs are common in the human population with the frequency of each base is approximately above 1%) (Vignal *et al.*, 2002; Li *et al.*, 2014). SNPs lead to genome evolution, diversity among individuals and traits of families such as curly hair. Also, SNPs have a role in variation of human phenotypes, such as susceptibility to disease, susceptibility to infection and responses to drugs and chemicals. SNPs occur in coding regions or in non-coding regions of genome and 50% of SNPs are occurred in non-coding regions which may be in promoters, enhancers and 3′ termini. In coding regions, SNPs may do not change the encoded amino acids which called synonymous SNPs or may change the encoded amino acids which are then called nonsynonymous SNPs. Nonsynonymous SNPs may lead to diseases and may be either missense (change in the amino acid sequence of a protein and its function which may lead to disease) or nonsense (point mutation that leads to change a sense codon to stop codon (Shastry, 2009; Haraksingh & Snyder, 2013).

Recent data indicate that there is a polymorphism in the promoter region of *COLEC11* gene 9570 C > T (rs3820897) which causes increased serum concentration levels of CL-11 (an increase of 10%, P = 0.044). However, CL-11 p.His219Arg (rs7567833) in the carbohydrate recognition domain (CRD) (minor allele frequency 0.033) has no effect on the level of CL-11 in serum among healthy Danish Caucasians (Bayarri-Olmos *et al.*, 2015). CL-11 p. His219Arg (rs7567833) have been associated with 40% decrease in CL-11 serum concentration; P = 0.03) in sera of among Nigerian individuals with that SNP (Antony *et al.*, 2015).

5.2.1   Allelic Discrimination Analysis

To determine whether the His219Arg (rs7567833) mutation effects susceptibility to infectious disease, either as a result of the amino acid substitution or due to the reported effect on Cl-11 serum levels , an association study was carried out using a cohort of pre-term neonates. A subset of the patients had suffered from at least one episode of bacterial infection requiring antibiotic treatment (Dzwonek *et al.*, 2008).

Quantitative Polymerase Chain Reaction was performed for genotype and allelic discrimination by using the Taqman SNP Assay to amplify and detect specific CL-11 SNP COLEC11 His219Arg (rs7567833) (Applied Biosystem) in purified genomic DNA samples of 155 premature neonates (97 Polish and 58 British) (Figure 5.6) .

Table 5.1 shows the genotypes obtained. In each of the sample groups, the distribution of the genotypes was in good agreement with that expected from the Hardy-Weinberg equilibrium. The minor allele frequency (MAF) in the Polish patient samples was 0.025, in close agreement with that reported for Danish Caucasians (Bayarri-Olmos *et al.*, 2015), suggesting that there was unlikely to be any association of the SNP with premature birth or disease. Unfortunately, complete clinical data for cohort could not be obtained at the time of publication, so it is impossible to divide the patients into those with and without infectious disease. In the case of the UK samples, the MAF was very much higher than expected (0.215 vs 0.022). Again no clinical data was available, so although this discrepancy might reflect a disease association, no solid conclusion can be drawn. It is equally possible that the high MAF in the UK patients reflects the ethnicity of the patients recruited in London.

Allelic Discrimination Plot
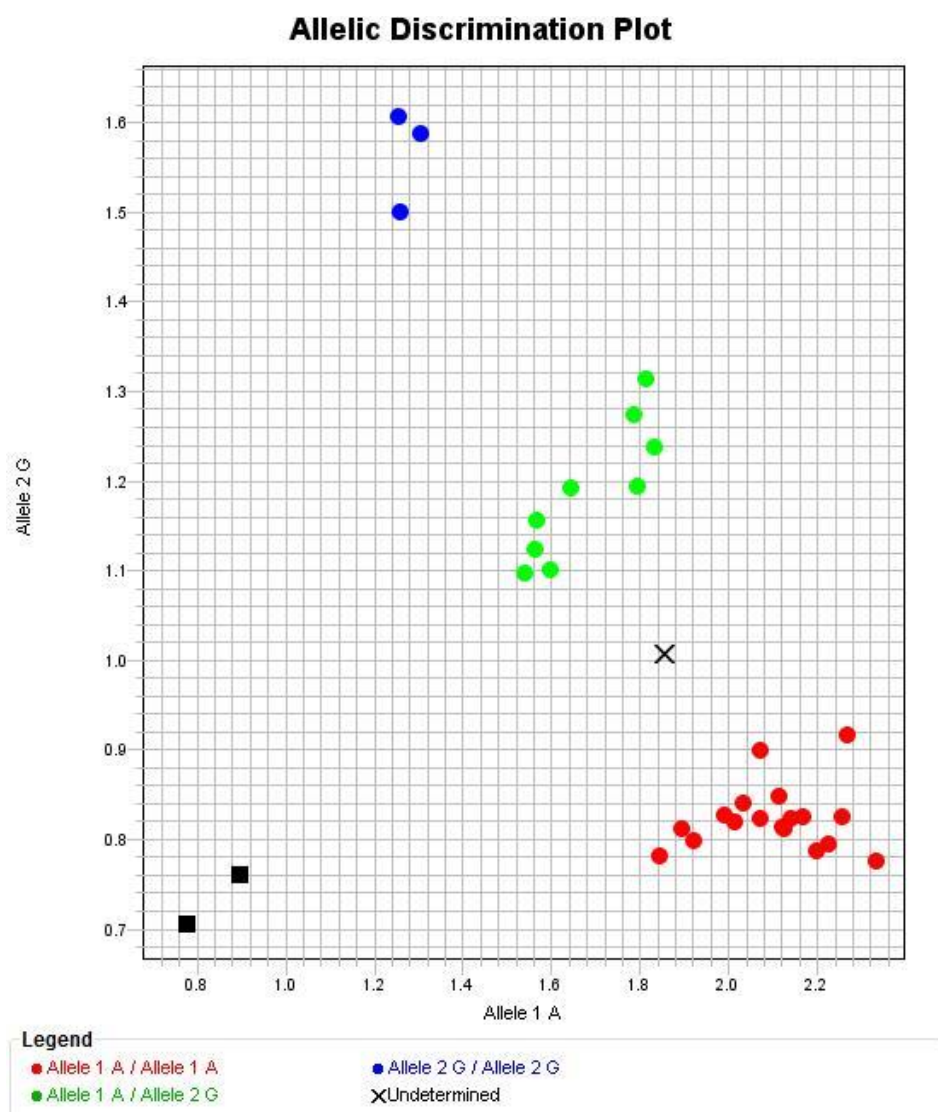
Figure 5-6 Allelic discrimination plot of TaqMan SNP genotyping assay for rs7567833.
Red cluster is homozygous   (AA), blue cluster is homozygous (GG) and the green one is heterozygous (AG). Black cluster shows the controls with no template and the failed amplifying samples.

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Table 5-1 TaqMan SNP genotyping assay for rs7567833

SNP rs7567833	AA	AG	GG	MAF
UK	39(67.2%)	13(22.4%)	6 (10.3%)	0.215
POLISH	92(94.8%)	5(5.1%)	0	0.025

5.3   Discussion

Natural genetic variation in eukaryotic gene promoters can have a massive effect on the transcription levels, protein levels, and hence phenotype. L-ficolin (FCN2) and MBL are well described examples of complement proteins whose serum concentration varies significantly with the promotor genotype. MBL deficiency is the commonest complement deficiency, affecting up to 1 in 10 individuals. The main determinants of MBL serum concentrations are 3 mutations in exon 1, which disrupt the Gly-Xaa-Yaa motifs of the collagen-like domain, and one of three common promoter mutations, which is located at ntd -221 and is given the symbol X/Y. Individuals who have two dysfunctional exon 1 alleles have undetectable serum MBL, as do those who have one dysfunctional exon 1 mutation on one chromosome and the X allele of the other. X/X individuals have serum levels about $1/10^{th}$ of that of a normal individual. MBL deficiency has been associated with various phenotypes, the best studied of which is recurrent infection in neonates and patients with cystic fibrosis (Wallis & Lynch, 2007). Three mutations in the promoter of *FCN2* have been shown to have a significant effect on L-ficolin serum concentrations.

In this study, the regulation of the CL-11 gene was studied by quantifying the gene expression using the dual luciferase system. In this assay, the regulatory elements in the CL-11 promoter were cloned upstream of the firefly luciferase gene and transfected to the mammalian cells (HEK293 and HepG2), with Renilla luciferase used as an internal control. The high luciferase activity which was obtained from SV40pGL3 positive control was as an indicator of a successful transfection. The success of a transfection depends on the right orientation of a promoter that is inserted upstream of the luciferase gene and also depends on the cell viability. Therefore, the normalization was done by using internal control SV40pRL to reduce the variation in cell viability and the efficiency of the transfection. The result showed the fragment -125 in both basic and enhancer promoter have a higher firefly luciferase level comparable to the internal control pRL and the activity level was increased with increasing the length of the fragments indicating the presence of some distal control elements. In addition, the luciferase activity in Hek293 cells (kidney cells) was higher than that in HepG2 cells (hepatic cells) under the same transfection conditions and this may be indicating the kidney specific expression of CL-11 gene.

In silico analysis of the CL-11 promoter using the Eukaryotic Promoter Database (EPD) analysis for regulatory element prediction showed there were many TATA boxes upstream (four boxes in range $^-557$ to $^+191$) and no GC boxes nor CCAAT boxes were identified. These TATA boxes were located too far 5' of the TSS to be functional, indicating that the CL-11 promoter is unconventionally regulated. In previous reports, it was shown that the promoter regions of complement regulatory proteins such as C1-inhibitor, complement factor I, complement factor H and C4-binding protein do not contain the typical TATA box and these promoters may have other initiator elements which were shown to be common in constitutive genes that are inducible by cytokines. (Volanakis, 1995; Sullivan *et al.*, 1994; Williams & Vik, 1997; Fraczek & Martin, 2010). Also, it has been described that IL-1β can act as an up regulator for the promoters of the *MASP1* and the *MASP2* gene, while IFN- α is a down-regulator for the *MASP1* promoter. In addition, the IL-6, IL-1β and IFN- α have an unregulating effect on the C1s promoter (Endo *et al.*, 2002).

During the course of this work, another group published results showing that a polymorphism in the promoter region of *COLEC11* 9570 C > T (rs3820897) causes an increase of approximately 10% of the CL-11 levels in the serum. There are conflicting reports on the effect of CL-11 His219Arg (rs7567833) in the carbohydrate recognition domain (CRD) on the level of CL-11 in serum. Among healthy Danish Caucasians this SNP had no effect (Bayarri-Olmos et al., 2015), whereas, the Nigerian population the same SNP caused a decrease of approximately 40% in serum CL-11 (Antony et al., 2015).

We aimed to test the effect of this mutation on the susceptibility of babies to bacterial infections in a well-characterized cohort. Although the study is complete, we have been unable to correlate the SNPs that I discovered with the susceptibility to infectious disease, because after I carried out my study and approached the clinician that collected this DNA cohort, I was told that I could not be provided with clinical data related to this DNA cohort, since the pediatrician that collected the cohort has left to a new job overseas without leaving the clinical data related to this DNA cohort with her supervisor, Prof. Nigel Klein, Institute for Child Health, Great Ormond Street, London.

The system developed during the course of this work was originally intended to be used to determine the effect of CL-11 promoter variation on gene expression. This question has been

partially addressed by another group who published results showing that a polymorphism in the promoter region of COLEC11 9570 C > T (rs3820897) causes an increase of 10% in the serum concentration of CL-11 levels during the course of this work. However, the system could equally well be used to determine whether the promoter responds to, for example, cytokines, and where the response elements are located.

Chapter Six: Conclusion and future directions

6   Conclusion and future directions

6.1   Conclusion

Previous studies have reported a critical role of complement activation via the classical activation pathway (CP) and the alternative activation pathway (AP) in the protection against *S. pneumoniae* infections. The key role of the lectin pathway (LP) subcomponents MASP-2, ficolin A in fighting pneumococcal infection has been studied using gene-targeted deficient mouse strains in a model of infection. It has been shown that the deficiency of either MASP-2 or ficolin A causes a significant predisposition to *S. pneumoniae* infections while MBL deficiency (combined deficiency of both mouse MBL genes, i.e. MBL A and MBL C) appeared to impair the immune defense against *S. pneumoniae* to a much lesser extent, implying that ficolin A plays a more prominent role in recognizing ligands on the surface of *S. pneumoniae.*   All the more important is the finding reported here which clearly demonstrates for the first time that in addition to ficolin A, CL-11 is a key recognition molecule driving LP activation of the surface of pneumococci.+

In vitro studies also showed that native CL-11 activates the LP on the surface of *S. pneumoniae.* This was confirmed by using recombinant murine CL-11, which I produced (with or without an N-terminal poly-Histidine-Tag).

In my *in vivo* studies, I compared the performance of CL-11 $^{-/-}$ mice with that of sex, age and strain matched WT control mice in an established mouse model of *S. pneumoniae* infection. CL-11$^{-/-}$ mice were significantly more susceptible to *S. pneumoniae* infections with a much more severe course of infectious disease presenting with significantly higher mortality (80% survival of the WT controls compared to only 22% in the CL-11$^{-/-}$ mice) and a significantly higher bacterial burden in the blood and the lungs of CL-11$^{-/-}$ mice following intranasal infection with *S. pneumoniae* D39.

 C3b deposition on *S. pneumoniae* was assessed by using sera from a selection of LP deficient mouse strains including mice deficient of MASP-2or with a combined deficiency of both MBL-A and MBL-C, ficolin A, and a mouse line deficient of 3 LP recognition molecules, i.e. with a combined MBL-A/C/ficolin A deficiency. There was no detectable C3 deposition

on *S. pneumoniae* in MASP-2 deficient serum. This clearly demonstrates that MASP-2 is essential as the key enzyme of the LP to drive the activation of C3 on the bacterial surface. The impact of a combined MBL-A and C deficiency on C3b deposition on the surface of *S. pneumoniae* was relatively small indicating that MBL-A and C have only a minor role in activation the lectin pathway on *S. pneumoniae.* CL-11 deficiency, however, showed a similarly high impact on C3b deposition as Ficolin A deficiency, indicating that ficolin A and CL-11 both are of similar importance as key recognition components for this pathogen. In contrast to MASP-2 deficient serum, triple deficient MBL-A/C and ficolin A$^{-/-}$ mouse serum showed a detectable levels of C3 deposition on *S. pneumonia*, indicating i) that MASP-2 functional activity is essential for C3 deposition on the surface of this pathogen and that ii) CL-11 on its own can drive a residual LP in absence of the other LP recognition subcomponents MBL and ficolin A.

I have also shown that the minimal promoter for the CL-11 gene is contained in a region between nucleotides -125 and +200 with respect to the TSS and that most of the activity is within the 557bp from the TSS. In common with other complement gene promoters, the CL-11 promoter appears to be atypical in that it does not contain known regulatory elements sufficiently close to the TSS to be functional.

6.2 Future plans:

When I started producing recombinant murine CL-11, I hoped to achieve a much higher yield of recovery of this recombinant LP recognition molecule to use to substitute and compensate for example ficolin A deficient animal with high levels of CL-11 and see, if the reduced resistance against *S. pneumoniae* in ficolin A deficient mice can be compensated. Unfortunately, the low yield of expression that I received in my initial clones expressing recombinant CL-11 was insufficient to do this. I therefore considered to use a transgene approach in vivo applying adenoviral expression systems to overexpress CL-11 *in vivo*. There were, however safety considerations to use adenovirus expression vectors in our animal house facility that blocked me from progressing towards this strategy. However, using another eukaryotic expression vector to produce recombinant murine CL-11 towards the end of my experimental work achieved yields of 1-2 milligram of recombinant mouse CL-11

from one liter of culture supernatant. At the progressed stage of my experimental work program it was, however, too late and the many other experiments I performed had drained the funds available to do this type of work that this very nice proof of principle study could not be performed. It is an unfortunate reality that the high cost of experimental animal work at times of restricted research funds limits what can be done significantly.

The primary purpose of my promoter study was to examine the possibility that variation in the Cl-11 promoter has a similar effect to that shown for variation in the MBL and L-ficolin promoters. The rationale for these experiments was largely lost when another group, using another approach, demonstrated that CL-11 promoter variations have only a minor effect on serum concentration in an experimental cohort (Bayarri-Olmos *et al.,* 2015). Nonetheless, the reporter system I have developed could be useful to examine the expression of CL-11 in vitro in different cell lines and under different stimuli, and to identify regulatory elements in the promoter.

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