The impact of the classical, lectin and alternative pathways of complement activation on protective immunity against *Streptococcus pneumoniae* infection following vaccination with established pneumococcal vaccines

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By

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Statement of originality

This thesis entitled: “The impact of the classical, lectin and alternative pathways of complement activation on protective immunity against *Streptococcus pneumoniae* infection following vaccination with established pneumococcal vaccines” has been submitted for the degree of PhD in Immunology. The reported results have been generated by the author, a externally funded full-time PhD student at the University of Leicester in the period between October 2012 and September 2016. All the work reported in this thesis is original unless otherwise acknowledged in the text or by references. Nothing of work has been submitted for another degree in this or any other University.

Abstract

Streptococcus pneumoniae (*S. pneumoniae*) is a pathogen that can cause infectious disease, such as meningitis, septicaemia and pneumonia. As part of innate immunity, the complement system plays a major role in the immune response to *S. pneumoniae*. Given this, the aim was to determine the impact of selective deficiencies for classical, lectin and alternative pathways of complement activation on vaccination response using pneumococcal polysaccharide vaccines. All results were achieved using CRM_{197} (a non-toxic mutant of diphtheria toxin), which elicited a different immune response in comparison to pneumococcal polysaccharide vaccines (PneumovaxII and Prevenar13). WT mice, $C1q^{-/-}$, MASP-$2^{-/-}$ and $fB^{-/-}$ mice were immunised subcutaneously or intraperitoneally (s.c./i.p.) with either 20µg of CRM_{197}, 1µg of PneumovaxII (pneumococcal polysaccharide vaccine) or 1µg of Prevenar13 (pneumococcal polysaccharide conjugate vaccine). Mice received a single or three spaced dose of CRM_{197}, PneumovaxII or Prevenar13 and then a final booster one week before the end of the experiment. Blood samples were taken at different time points and all mice responded with high antibody titres to CRM_{197}, PneumovaxII and Prevenar13; showing, an increase in antibody response to CRM_{197} immediately after the third immunisation and after the first immunisation for PneumovaxII and Prevenar13. This suggested that the antibody response to CRM_{197} was independent from the presence or absence of C1q (classical pathway) and MASP-2 (lectin pathway), but was increased in C1q deficient mice for PneumovaxII and was completely independent for Prevenar13. Imperative to the effective treatment of pneumococcal infections, this project significantly aided in the understanding of the complement system and has highlighted the importance of activation pathways in protective responses. Overall, PneumovaxII showed that the classical complement pathway might play a negative regulatory role, whilst the lectin and the alternative pathways are involved in the positive regulation and minor positive regulation of immune responses.

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List of abbreviation

AOM	pneumococcal acute otitis media
AP	Alternative Pathway
BBS	Barbital Buffer Saline
BHI	Brain Heart Infusion
bp	base pair
BSA	Bovine Serum Albumin
$C1q^{-/-}$	C1q knockout mice
C1r	Complement serine protease C1r
C1s	complement serine protease C 1s
CFU	Colony Forming Unit
CL-11	Collectin-11
CP	Classical Pathway
CR	Complement Receptor
CRD	Carbohydrate Recognition Domain
CRP	C-reactive protein
DAF	Decay Accelerating Factor
dATP	Deoxyadenosine Triphosphate
ddH_2O	nano- distilled water
dH_2O – D.W	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol-bis-N,N,Ń,Ń-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
fB	Factor B
$fB^{-/-}$	fB knockout mice
Fc	Fragment crystallizable
fD	Factor D
FH	Factor H
FI	Factor I
HIV	Human Immunodeficiency Virus
HSA	Human Serum Albumin
i.p.	Intraperitoneal
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K/O	Knockout
kDa	Kilodalton
LEA	Lectin pathway Effector Arm
LP	Lectin pathway
MAC	Membrane Attack Complex
MAp	MBL-Associated protein
MASP	MBL-Associated Serine Protease
$MASP\text{-}2^{-/-}$	MASP-2 knockout mice
MBL	Mannan Binding Lectin
mL	Milliliter
mM	Millimole
ng	Nanogram
NHS	Normal Human Serum

OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPS	Pneumococcal polysaccharides
PspA	Pneumococcal Surface Protein A
PspC	Pneumococcal Surface Protein C
RNA	Ribonucleic acid
RNAse	Ribonuclease
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
sMAP	small MBL-Associated Protein
SP	Serine Protease
TAE	Tris-base/Acetic acid/EDTA
TBS	Tris Buffered Saline
v/v	volume/volume
w/v	weight/volume
WT	Wild Type mice
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µL	Microliter

General Introduction

1 General Introduction

The immune system has two effector arms; the adaptive immune system (which has a high degree of specificity) and the innate immune system, which is largely less specific (Figure 1). The complement system (which is nominally a part of the innate immune system) plays a major role in defence against many infectious organisms as part of both the innate and antibody-mediated acquired immune responses. Named for some of the earliest observations of its activity- a heat sensitive material in serum that "complemented" the ability of antibody to kill bacteria- it is now known that complement comprises more then thirty circulating and membrane-expressed proteins which are found both on the surface of cells and in serum. Complement components are mainly synthesized in the liver by cells involved in the inflammatory response (*Yuste et al. 2010*). The components of the complement system are part of the innate immune system, which provides instant protection against invading pathogens. Nevertheless, complement activation can also be triggered by effector components of the adaptive immune system (*Abbas, Lichtman and Pillai 2010*).

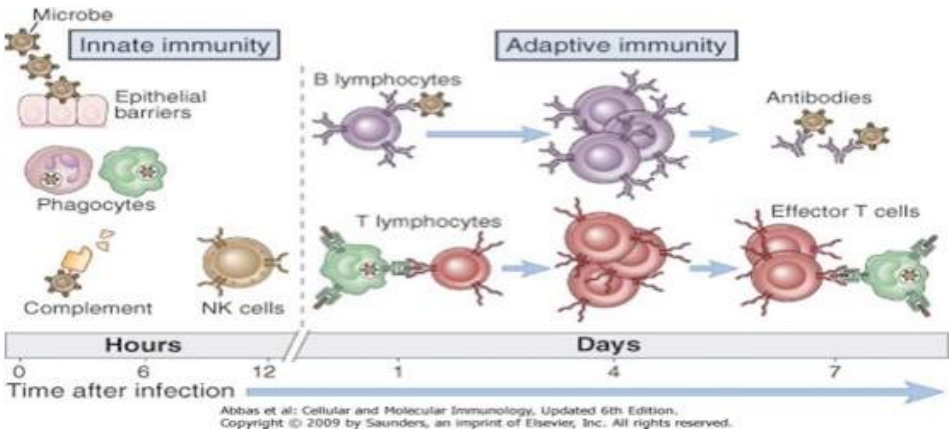

Figure 1: This figure shows the differences between adaptive and innate immunity. Adapted from Abbas et al. (2010).

Complement can be activated by three different activation pathways; the alternative pathway, the classical pathway and the lectin pathway (*Schwaeble et al. 2002*). The complement system plays a major a role in fighting against infectious diseases (*Trouw, Daha 2011*). The lectin and alternative pathways are activated directly on the surface of bacteria cells, while the classical pathway is activated by antigen and antibody complexes formed by the binding of antibodies to bacterial antigens. Moreover, all three pathways lead to the formation of C3- convertase, a protease complex that cleaves the abundant complement component C3 (*Brown et al. 2002*). The classical, the lectin and the alternative pathways are important components of the immune response against *Streptococcus pneumoniae* infections Both the classical and the lectin pathway lead to the cleavage of the abundant central complement component C3 by the formation the C3 convertase complex C4bC2a (*Yuste et al. 2010*).

1.1 Activation of the complement system

Activation of the complement system is very important within the immune response and this is achieved through different pathways such as the classical, the lectin and the alternative pathways (*Comerford 2014*). The most critical and essential function of the complement system is to recognise pathogens and their toxins and facilitate their elimination from the host. There are three independent complement activation pathways for enhancing the elimination of foreign bodies from the host is enhanced (*Acton 2012*). The classical pathway is initiated by antibodies and provides an efficient way to activate the complement system depends on the ability of the presence of either natural antibodies (mainly IgM) or specific antibodies (IgG) provided by adaptive immune response. The lectin pathway bypasses the absolute requirement for antibodies by its ability to be activated through direct binding of its recognition subcomponents to microbial pattern and drive efficient complement

activation on pathogens via the lectin pathway specific serine proteases MASP-1, MASP-2 and MASP-3. The alternative pathway is also efficient in providing an antibody- independent mechanism of complement activation and provides an amplification loop to enhance complement activation on pathogen surfaces (*Vinuesaa 2001*). As common feature, all three complement activation pathways (Figure 2), channel into the formation of a C3 convertase that converts the abundant component C3 into C3b and C3a. Accumulation of C3b in close proximity of either the lectin and classical pathway C3 convertase C4bC2a and or the alternative pathway C3 convertase C3bBb switches the substrate specificity of these C3 convertases from C3 to C5, hence forming the C5 convertase complexes C4b2a(C3b)n or C3bBb(C3b)n respectively which cleave C5 into C5b and C5a. C5b then initiate the terminal activation cascade by the subsequent binding of the complement components C6, C7 and C8 to form a C5b-8 complex that initiates the formation of membrane attack complexes by binding to and initiating the polymerisation of C9 to a cylindrical structure that leaves a patch or a hole in its centre which inserts into the lipid bilayer of the bacterial cell membrane as a membrane attack complex that can initiate to cell lysis through osmolytic processes (*Podack, Tschoop and Muller-Eberhard 1982*).

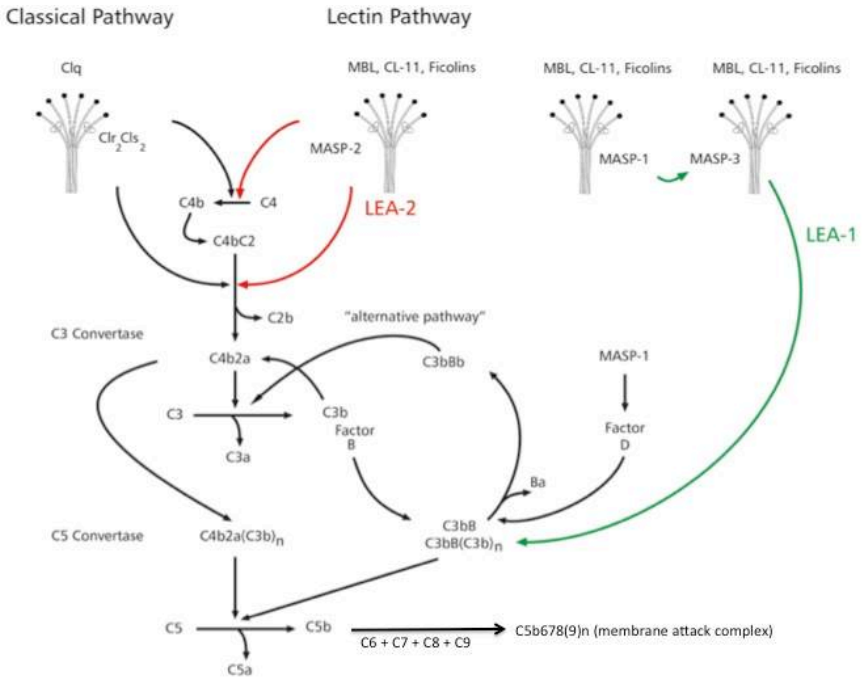

Figure 2: This figure kindly provided by Professor Wilhelm Schwaeble.

1.1.1 The Classical Pathway of Complement Activation

It was the first complement pathway that was described and therefore named classical pathway. Antigen-antibody complexes are the predominant activators of the classical pathway. C-reactive protein (CRP) binds to the polysaccharide phosphocholine expressed on the surface of many bacteria (e.g. *S. pneumoniae*) and activates the classical pathway of complement. In the classical pathway the sequence of activation of theearly components is C1, C4, C2, and C3 (*Schlapbach et al. 2010*). The C1 complex is made up of subunits, C1q, C1r, and C1s (Figure 3). There are three components that form the classical pathway initiation of activation complex C1. i.e. the multimolecular classical pathway recognition subcomponent C1q and the two classical pathway specific serine proteases C1r and C1s. C1q is composed of six identical subunits, each formed of a heterotetramer of each of the C1q chains C1q-

A chain, C1q- B chain and C1q.C chain. Each single chain of C1q is composed of a collagen-like tail portion (CLR), a flexible arm region and ends in a globular head unit. C1r and C1s are single chain proenzyme that form a heterotetrameric complex. Once the CIq recognition subcomponent binds with at least two or more globular heads to IgG or IgM immune complexes, it undergoes a conformational changes that convert the C1r components within the C1r/C1s heterotetramer into its enzymatically active form to cleave their only substrate C1s which in turn converts C1s into its enzymatically active form to convert C4 into C4a and C4b and subsequently C4b-bound C2 to form the classical pathway C3 convertase complex C4bC2a. Unlike IgG, which requires 2 or more antibody molecules to form an immune complex that cam activate C1, one antigen-bound IgM molecule is sufficient to activate C1 and start the classical complement activation pathway (*Faro et al. 2008*). Here, C1q can bind to IgM across the joining region of the pentamer. C4b can form covalent bonds with molecules that are nearby when it is when it is formed. This allows it to bind to many kinds of surfaces, including the immune complexes or antigens, or any other nearby molecules. C4b also participates in the formation of the classical pathway C3 convertase, C4bC2a (*Windbichler 2005*). C2b is the smaller fragment of C2, and it may contain the sequence responsible for the C2-kinin activity associated with hereditary angioedema. C2a is the larger fragment, and it contains a serine esterase active site. In order for C2a to act, it must bind to C4b, to form the C4bC2a complex which is the C3 convertase of the classical pathway.

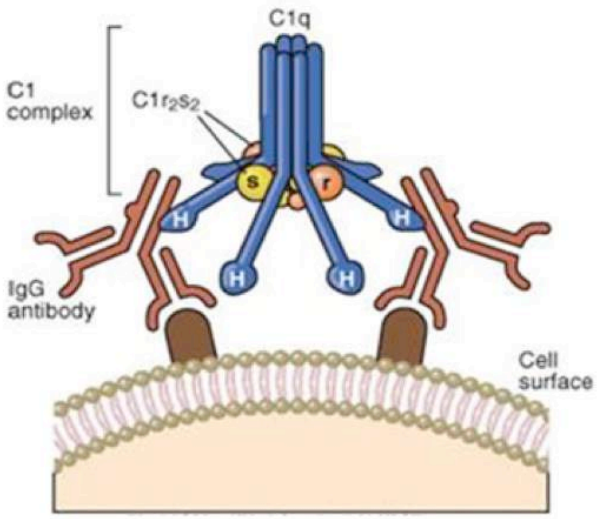

Figure 3: C1q, which is the key component of the classical complement pathway and it is activated by antibody–antigen complexes. C1q consists of six subunits, which is made up from collagen-like fibrous portion and a globular head that is involved in binding to diverse targets (*Francis et al. 2003*).

1.1.1.1 C1q Protein in mice

C1q is a flexible recognition protein that binds to various immune as well as non-immune ligands and is the first subcomponent of the C1 complex that sets off the activation of the classical pathway of complement (*Gaboriaud et al. 2003*). C1q has a molecular weight of 460 kDa and is comprised of 18 polypeptide chains, which are all of similar length as well as amino acid composition (*Gal, Ambrus 2001*). The polypeptide chains are composed of 6A chains (223 residues), 6B chains (226 residues) and 6C chains (217 residues) which are all of similar length. The three different forms of chains form trimers to produce collagenous triple helices at the N-terminal portion of the molecule with the globular heads at its C-terminal (*Gal, Ambrus 2001*) . According to the authors, one trimer of the three chains is held together by non-covalent bonds and

also by an interchain disulphide bridge existing between the A and B chains. Another similar interchain bridge also exists at the N-terminus between two C chains. One C-C and two A-B units form the structural unit with two triple A-B-C helices and two globular heads. Three of these structural units interact to give rise to the hexameric C1q structure presented in (Figure 4) below.

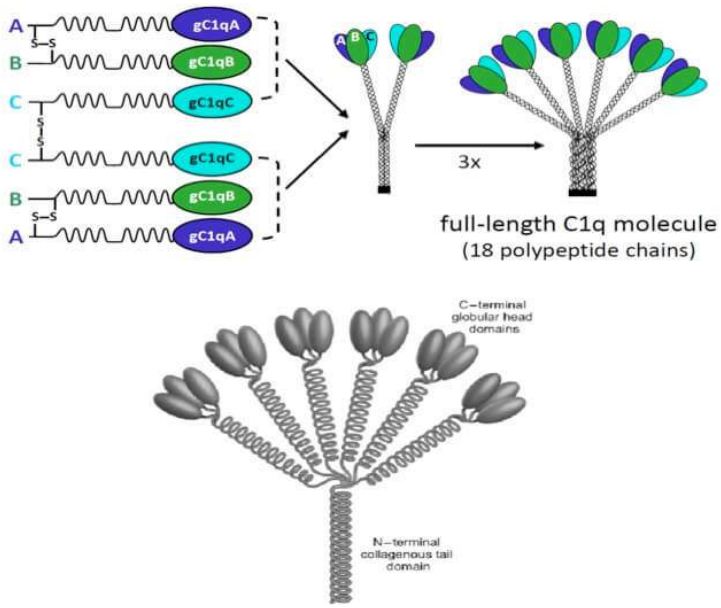

Figure 4: Schematic Presentation of the C1q Protein Structure, which is Interaction of the A, B and C chains and how they form the C1q molecule.

1.1.2 Alternative Pathway of Complement Activation

The alternative pathway (AP) of complement is triggered by a wide array of factors, including lipopolysaccharide from the cell wall of gram-negative bacteria, the cell wall of some yeasts, and cobra venom factor. Certain viruses, aggregated Ig, and necrotic cells; may also activate the alternative pathway (in addition to classical pathway). The alternative pathway requires three factors analogous to the classical pathway Factor D is the equivalent of C1s, factor B is equivalent of C2 and C3b takes the place of C4b. Properdin unique to the alternative pathway, is required for efficient convertase activity, it stabilises the AP convertase complexes by

preventing their decay though C3b binding competitors like factor H, which also serves as a cofactor of the factor-I mediated conversion of C3b to iC3b.. The first initiation step of the AP is the binding of factor B binds to C3b. Factor D cleaves C3b-bound factor B and converts the zymogen C3bB complex into its enzymatically active form C3bBb. The Ba fragment is released to the fluid phase and Bb stays bound to C3 to made up C3bBb which is the alternative pathway C3 convertase with the enzymatic activity being located on the Bb fragment. Properdin stabilizes the alternative pathway C3-convertase by binding to C3bBb. Factor H binds to C3bBb and displaces Bb. Factor H acts as a co-factor for Factor I, which cleaves C3b once to produce iC3b or twice to produce Cc and C3dg. CR1 and MCP can also act as co-factors for Factor I (*Schlapbach et al. 2010*).

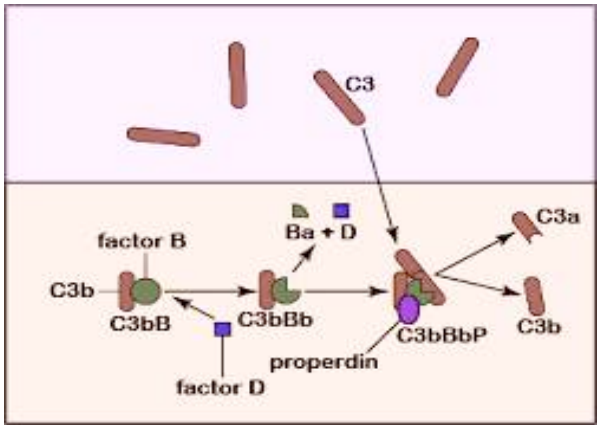

Figure 5: Alternative Pathway of Complement Activation diagram.

1.1.2.1 Factor B Protein in Mice (fB)

Factor B is one of the essential components that forms the C5 and C3 alternative pathway convertase complex (*Pekna et al. 1998*). It is a 93-kDa serine protease existing in serum at a concentration of between 170mg/L and 260mg/L. The amino acid chain of the factor B

polypeptides is comprised of 175 residues and share 90 percent sequence identity (*Belogrudov 2009*). Each polypeptide has 6 cysteine residues with the Cys94 and Cys92 being juxtaposed in the linear amino acid sequence. An analysis of the structure of the Factor B protein demonstrates that the C-terminal domain is comprised of 4 leucin-rich repeats and lacks the coupling activity of the full-length polypeptide. It has also been established that factor B mutants that have truncated N-terminal amino acids have diminished coupling activity. In this study, it is revealed that factor B has a crystal structure with an oblong, oval shaped molecule consisting of two domains; the C-terminal domain that consists of four leucin-rich repeats and a unique N-terminal globular domain (*Belogrudov 2009*). The first ten N-terminal residues fold as a short α-helix which projects outwards from the molecule and is considered to be what anchors factor B to the matrix end of the inner membrane of the mitochondria hence orienting the entire polypeptide molecule in a lateral direction alongside the surface of the membrane (*Belogrudov 2009*).

1.1.3 Lectin Pathway of Complement Activation

The lectin pathway, as the name suggests, is triggered by lectins. Lectins are the proteins that recognize and bind to specific carbohydrate targets. The mannose-binding lectin (MBL) is one such protein that takes part in the lectin pathway of complement activation. MBL is a large serum protein that binds to non-reduced mannose, fructose and glucosamine on bacterial and other surfaces of cells with Mannan (mannose-comprising polysaccharides) (Figure 6).

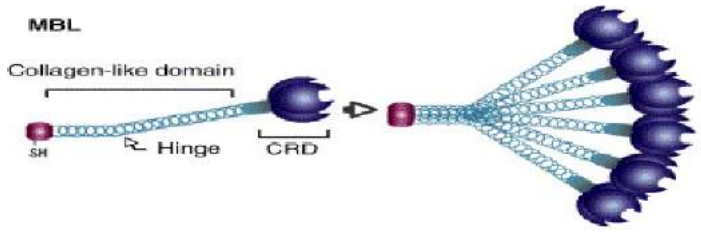

Figure 6: Domain and oligomeric structure of MBL (*Endo et al. 2006*).

The binding of MBL to a pathogen results in the secretion of two MBL-associated serine proteases: MASP- 1 and MASP-2. The MASP-1 and MASP-2 are similar to C1r and C1s respectively, and MBL is similar to C1q. Formation of the MBL/MASP-1/MASP-2 trimolecular complex results in the activation of MASPs and subsequent cleavage of C4 into C4a and C4b. The C4b fragment binds to the membrane and the C4a fragment is released into the microenvironment. Activated MASPs also cleave C1 into C2a and C2b. C2a binds to the membrane in association with C4b and C2b is released into the microenvironment. The resulting C4bC2a complex is a C3 convertase, which cleaves C3 into C3a and C3b. C3b, binds to the membrane in association with C4b and C2a and C3a is released into the microenvironment. The resulting C4bC2aC3b is a C5 convertase. The generation of C5 convertase is the end product of the lectin pathway (*Schlapbach et al. 2010*). It proceeds to produce the MAC in a similar way to the classical and alternative pathways.

1.1.3.1 MASP-2 in Mice

MASP-2 is a serum protease that contributes significantly to the activation of the complement system through mannose binding lectin (MBL). Once activation is complete by auto-catalytic cleavage, MASP-2 cleaves C2 and C4 triggering their activation and formation of C3 convertase. MASP-2 structure is similar to that of C1r or C1s but there are differences in oligomeric forms since MASPs form homodimers and while C1r and C1s form heterotetramers, MASP-2 is composed of 227

amino acids (*Gal, Ambrus* 2001).

1.2 Complement deficiency

The complement system plays a major role in the innate immune system which has an important role in fighting microbial infections. Deficiency of complement components is related to autoimmune disorders and recurrent infection. In addition, depletion of complement components and excessive complement activation can be related to deficiency of complement regulators (*Todd, Spickett 2010*).

1.2.1 Deficiencies of the classical pathway components

The complement system forms part of the immune system which is concerned with innate immunity. The deficiencies of the classical pathway are attributed to autoimmune disease. The common classical pathway component deficiencies include C1, C4 and C2 (*Kavanagh & Wright 2010*). Deficiencies in classical pathway components results in the failure of the complement system to clear immune complexes and apoptotic cells. For instance, C2 deficiency is often found in young children with recurrent infections. C1q deficiency leads to autoimmunity and this complicates the process of clearing apoptotic cells. Deficiency of one or more for the components of the classical pathway C1q, C1s and C4 tends to increase the risk of immune complex autoimmune disease (*Amano et al. 2008*). This observation has led to a better understanding of the major role that C1q plays in the clearance of immune complexes, Also C2 deficiency leads to severe infections such as streptococcal pneumonia disease (*Jonsson et al. 2005*).

1.2.1.1 C1q Deficiency in Mice ($C1q^{-/-}$)

Several studies have been conducted using C1q deficient mice. C1qa - deficient mice (C57BL/6J) are defined as lacking the C1q protein and therefore classical complement activity (*Stephan et al. 2013*) . C1q deficient mice have been found to develop a syndrome that is indicative of systematic lupus erythematous in humans with proliferative glomerulonephritis an antinuclear Abs (*Petry et al. 2001*) This study which was conducted on hybrid mice (129 x C57BL/6J) revealed that background genes play a significant role in the full expression of the autoimmune disease. They demonstrated C1q protein levels were significantly increased with age in the mouse as well as the human brain by up to 300 times (*Stephan et al. 2013*) . In this study the C1q deficient mice in demonstrated significantly less cognitive ability as well as memory decline and specific tests on hippocampus-dependent behaviour supported this. In these and other studies on C1q deficiency in mice, the mice were generated using a homologous recombination targeting exon 1 of the C1q gene which was backcrossed for seven to ten generations to C57BL/6J (*Mitchell et al. 2002*, *Stephan et al. 2013*, *Petry et al. 2001*).

1.2.2 Deficiencies of the lectin pathway

Lectin pathway deficiencies include MBL, MASPs, H-ficolin, M-ficolin, CL-11 and L-ficolin (*Ram, et al. 2010*). MASP-2 deficiency results in autoimmune manifestations, chronic inflammatory disease and severe infections and has been shown to be caused by specific mutations of the MASP-2 genes (*Ram, et al. 2010*). The common form of lectin pathway is MBL (*Harboe, et al. 2006*). The immunity of infants depends on the capacity of lectin pathway to fight bacterial infections. MBL deficiency is associated with an increase in the risk of autoimmune skin disease although many individuals with MBL deficiencies remain healthy (*Dahl et al. 2004*). However, MBL deficiency can also be found in cancer patients, immunocompromised (HIV) patients and transplant patients and

leads to an increase in the risk of recurrent severe infections and an increase in the colonization of bacteria in the lungs (*Cedzynski et al. 2009*).

1.2.2.1 MASP-2 Deficiency in mice (MASP-2$^{-/-}$)

MASP-2 deficiency is caused by defects in MASP-2 expression and gives rise to disorders that lead to autoimmune manifestations, chronic inflammatory disease and recurrent severe infections. Various studies seeking to establish the effects of MASP-2 deficiency have used MASP-2 deficiency mice by generating mutant mice through disruption of the eleventh and part of the twelfth exons "coding for the C-terminal half of the CCPII domain and encoding the serine protease domain; respectively" of the MASP-2 gene by replacing them with neo^{r}-gene as explained by: (*Schwaeble et al., 2011*) Given that this region was expected that MASP-2 would be intact in the knockout mice. However, their serum showed no presence of MASP-2 which is how the mice were determined to be MASP-2 deficient (MASP-2$^{-/-}$). Most of the studies on MASP-2 deficiency in mice generated these mice using the procedure developed by (*Schwaeble et al., 2011*). The gene disruption decreases expression levels of MASP-2 resulting in MASP-2$^{-/-}$ mice. Gene-targeted mice that lacked MASP-2$^{-/-}$ were generated by constructing a targeting vector to replace the specific exons (exon 11 and half of exon 12) for MASP-2 with a gene cassette that is resistant to neomycin (*Schwaeble et al., 2011*) .

1.2.3 Deficiency of the alternative pathway

Deficiency of the alternative pathway can arise by loss of factor D, factor B or Properdin and affected people are prone to pneumococcal and meningococcal infections (*Frank 2000*). Properdin deficiency exposes individuals to a greater risk of Neisseria infection, while Factor D

deficiency leads to a reduced activity of bactericidal activity and is associated with severe meningitis disease that is associated with *S. pneumoniae* infections (*Todd, Spickett 2010*). Interestingly, factor B deficiency in humans has not been reported so far which suggests it plays a major role in survival.

1.2.3.1 Factor B Deficiency in Mice ($fB^{-/-}$)

Animals that have a deficiency of factor B lack all the functions for which the alternative pathway of complement activation is required but maintain functional classical as well as lectin pathways of complement activation (*Pekna et al. 1998*) . Factor B-deficient mice were generated using gene targeting in embryonic stem (ES) cells. The genomic factor B gene was mutated such that the gene became non-functional. A targeting construct (where the 1.8 kb fragment comprising a part of the exon 3 as well as exons 4-9 was removed and a neo cassette introduced) was used to transfect the ES cells. This resulted in deletion of the DNA sequence that encoded the entire Ba fragment as well as a considerable part of the first serine protease (*Pekna et al. 1998*) . This homologous recombination procedure using the described targeting construct to mutate the factor B gene in mice was found to lead to a null allele at the factor B locus. Factor B deficient mice were derived and bred in the normal way but their serum lacked alternative pathway-dependent haemolytic activity. This established that Factor B was critical for a protective immune response to hyper responsiveness and airway inflammation as factor B-deficiency in mice was found to increase airway hyper responsiveness (*Taube et al. 2006*). Factor B-deficient mice (using the targeting construct described by (*Pekna et al. 1998*) showed reduced the immunity against *Pseudomonas aeruginosa* (*Mueller-Ortiz, Drouin and Wetsel 2004*) .

1.3 The interaction of *S.pneumoniae* with complement system

Complement activation is an integral part of the protection against pneumococcal infection, as mice deficient in different components of the complement system present with increased susceptibility and severity of pneumococcal disease (*Alper et al. 1970, Ross and Densen 1984*). Indeed, infection with pneumococci has been shown to elicit a defined complement activation profile in-vitro and many in-vivo experiments have shown that complement activation is critical for an adequate host defense (*Ali et al. 2012, Bruyn et al. 1992, Winkelstein 1981*). Mechanistically, glycoproteins derived from pneumococci can activate the classical pathway through the interaction with the C1q molecule (*Stephens et al. 1977*). Pneumococcal capsule can hinder opsonophagocytosis by inhibiting both the classical and alternative complement pathways (*Hyams et al. 2010*). Several mechanisms involved in the evasion of the complement system have been described, including the PspA inhibition of opsonisation through the reduction of C3 deposition (*Poolman 2004*). Similarly, the activity of the alternative pathway could be affected by PspC pneumococcal surface protein C, which binds to factor H to inhibit crucial proteolytic process (*Poolman 2004*). Similarly, carbohydrates on the structural proteins of pneumococci bind to MBL to activate the lectin pathway (*Bouwman et al. 2006*). Moreover, pneumolysin has cytolytic toxin that also activates complement system which is play role in protective and against complement mediated clearance which indicating the important of pneumolysin in the pathogenesis of *S. pneumoniae* infection (*Yuste et al., 2005*).

1.4 *S. pneumoniae*

Streptococcus pneumoniae is a Gram-positive bacterium which causes diseases such as septicaemia, pneumonia and meningitis (*Hyams et al. 2011*) (Figure 7). *S. pneumoniae* is a normal resident of the tract flora of the upper respiratory system, but under the right conditions it can become pathogenic (*Tuomanen 2004*).

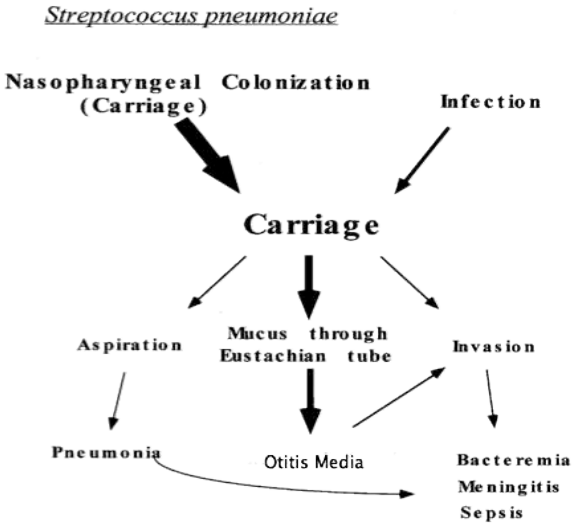

Figure 7: Shows the different infections caused by *S. pneumoniae* Figure modified from *Briles et al (2000)*.

In many developing countries, the highest number of deaths recorded in children under 5 years old was caused by pneumonia and every year over 1 million of infants die from pneumonia (*Kadioglu et al. 2008*). In addition, people aged between 65 and 85 years have an increased risk of infection of *S. pneumoniae* with a mortality rate of about 20% and 40% respectively (*Butler, Schuchat 1999*). More than 90 serotypes of *S. pneumoniae* have been classified, which can be divided into 46 serogroups based on differences in capsular polysaccharides of *S.*

pneumoniae (*Henrichsen 1995*); (*Park et al. 2007*) and (*Calix, Nahm 2010*). 10 serogroups have been recorded in England and Wales, and another 10 different serogroups have been recorded in Scotland (Table 1).

Table 1: The most common serogroups/types of pneumococci causing invasive disease in Great Britain (*Clarke 2006*)

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

1.4.1 Pneumococcal virulence factors and disease

The virulence factors caused the pathogenicity of pneumococci and there are many virulence factors of *S. pneumoniae* which are consist of capsule, pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumolusin (Ply), hyaluronate lyase (Hyl), enolase (Eno), autolysin A (LytA), hydrogen peroxide (H_2O_2), pneumococcal iron acquisition A (PiaA), pneumococcal adhesion and virulence A (ParA), the metal binding proteins of pneumococcal surface antigen A (PsaA) and pneumococcal iron uptake A (PiuA) (Kadioglu et al. 2008)

Table 2: Pneumococcal virulence factors and their main role in colonization and disease, Adapted from *Kadioglu et al. (2008)*.

Pneumococcal virulence factors and disease	Main role in colonization
Upper-airway colonization	
Capsule	Prevents entrapment in the nasal mucus, thereby allowing access to epithelial surfaces. Also inhibits effective opsonophagocytosis
Phosphorylcholine (ChoP)	Binds to rPAF on the epithelial surface of the human nasopharynx.

Choline-binding protein A (CbpA or PspC)	Binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium (process of reverse transcytosis).
β-Galactosidase (BgaA) β-N-Acetylglucosaminidase (StrH)	Act sequentially to cleave terminal sugars from human glycoconjugates, which might reveal receptors for adherence.
Hyaluronidase (Hyl)	Breaks down hyaluronan-containing extracellular matrix components.
Enolase (Eno)	Binds to plasminogen.
Pneumococcal adhesion and virulence A (PavA)	Binds to fibronectin.
Respiratory-tract infection and pneumonia	
Pneumolysin (Ply)	Cytolytic toxin that also activates complement. An important determinant of virulence in vivo models of disease. Wide range of effects on host immune components at sub-lytic concentrations.
Pneumococcal surface protein A (PspA)	Prevents binding of C3 onto pneumococcal surface. Also binds lactoferrin.
Autolysin (LytA)	Digests the cell wall, which results in the release of Ply.
Pneumococcal surface antigen A (PsaA)	Component of the ABC transport system, which is involved in resistance to oxidative stress.
Pneumococcal iron acquisition A (PiaA) Pneumococcal iron uptake A (PiuA)	Component of the ABC transport system.
Neuraminidases (NanA and NanB)	Aid colonisation by revealing receptors for adherence, modifying the surfaces of competing bacteria that are within the same niche and/or modifying the function of host clearance glycoproteins.
IgA1 protease (IgAP)	Cleaves human IgA1.
Superoxide dismutase A (SodA)	Detoxifies superoxide anion.

1.4.2 Capsular Polysaccharides

There are more than 90 serotypes of *S. pneumoniae* which can be divided into 46 serogroups depend on the differences in capsular polysaccharides of *S. pneumoniae* (*Henrichsen 1995), (Park et al. 2007) and (Calix,*

Nahm 2010). The virulence factor of *pneumococcus* is the polysaccharide capsule (*Catterall 1999*). One of the factors that aid the amplification of multi-resistance in other serotypes is in the exchange of capsule components as has been seen in highly resistant strains such as 6A, 6B, 9V, 14, 19F and 23F (*Tomasz 1999*) and (*Wuorimaa, Kayhty 2002*). The result of the presence of the negative charge of the capsule the immunogenicity of the surface nature among the polysaccharides located in the pneumococci capsule is poor. This hinders phagocytosis but can be overcome by the antibody and complement mediated opsonisation (*Lee et al. 2001*). In children less than two years old, there is not much development of the MZ is not fully developed and the polysaccharide antigens need to be conjugated to proteins to trigger a T cell dependent pathways (*Klein Klouwenberg, Bont 2008*).

1.5 *S. pneumoniae* vaccines

S. pneumoniae vaccines play a major role in protecting individuals against infection and many children have been saved from the fatal course of *S. pneumoniae* infection through the use of vaccines (*Madhi et al. 2008*). There are two major types of pneumococcal vaccines (Table 3). The first one is pneumococcal polysaccharide vaccine (Pneumovax23) which is contain 23 different serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F). Pneumococcal polysaccharide vaccine is safe and inexpensive vaccine (*Artz, Ershler and Longo 2003*). There are 23 different polysaccharide serotypes in the Pneumovax23 vaccine which are represents approximately 90% of *S. pneumoniae* infections covered by Pneumovax23 (*George et al. 1997*) and (*Kyaw et al. 2000*). The second vaccine type is the pneumococcal conjugate vaccine (7-Prevenar) which is often used for young children because it works on seven different serotypes (4, 6B, 9V, 14, 18C, 19F and 23F). This vaccine protects over 90% of children under 2 years old from *S. pneumoniae* infection (*Clark et al. 2006*). Moreover, pneumococcal conjugate vaccine (Prevenar13),

which is licensed to used in 2011 and contain 13 different serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F).

Table 3: Serotypes of *S. pneumoniae* covered by Pneumococcal polysaccharide and Pneumococcal polysaccharide vaccines

Vaccine	Serotype covered
Pneumococcal polysaccharide vaccine	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A,12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F
Pneumococcal polysaccharide conjugate vaccine (7-valent)	4, 6B, 9V, 14, 18C, 19F and 23F
Pneumococcal polysaccharide conjugate vaccine (Prevnar13)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F

1.5.1 Pneumococcal Polysaccharide Vaccine

There are 90 known serotypes of pneumococcus but only a few cause diseases in humans. The virulence factor of pneumococcus is the polysaccharide capsule. The host response needs to form anti-capsular IgG2 antibodies to clear the infection caused by pneumcoccus (*Catterall 1999*). Vaccine preparation has to take into account the need for the production of high antibody titres and antibody affinity. The capsule of the serotypes, which trigger disease, is the main ingredient of the present vaccines against pneumococci. The level of antibody titre that is needed to provide defence against pneumococcus has not yet been confirmed. In 1977, the United States gave the first license to a pneumococcus vaccine which was made up of a purified polysaccharide preparation from 14 serotypes. It was later replaced in 1983 by a 23-valent polysaccharide vaccine (Pneumovax-II, Pnu-Immune-23) which contained purified polysaccharide antigens from 23 serotypes obtained from a number of pneumococcus strains obtained from a number of developing countries ( ). It can only provide protection against the serotypes, which are present in the vaccine, so vaccines appear to be serotype specific. Indications for

receiving the vaccine involve those who are at risk of pneumococcal infection. It is also recommended for older patients as it is found to be very cost effective (*Ament et al. 2000*).

The parts of the population which respond poorly to this polysaccharide vaccine involves children under the age of 2, 20% of old patients and the HIV positive and immune suppressed patients. These age groups have the greatest incidence of invasive and non-invasive infections and are at high risk from pneumococcal infections (*Wuorimaa, Kayhty 2002*). In infants the immune system not fully developed which would allow the polysaccharide to induce an immune response via the induction of interferon-gamma [IFN–γ] which can enhance antibody production by B cells and induce class switching. Also some elderly patients have a weak IgG response because of which they react inefficiently to polysaccharide antigen (*Wuorimaa, Kayhty 2002*). Unfortunately the polysaccharide vaccine cannot give protection against the carriage of pathogens and it cannot prevent the spread of invasive or resistant strains (*Wright et al. 1981*) and (*Obaro, Adegbola 2002*).

1.5.2 Pneumococcal Conjugate Vaccine

Conjugate vaccines were invented to increase the shortcomings of polysaccharide vaccine in infants. The immune response can be geared towards a thymus-independent process (not requiring T cell help) to a thymus dependent one using this vaccine preparation which covalently links the polysaccharide antigen to a carrier protein (Figure 8). The subsequent T cell dependent response leads to the stimulation and development of T cell memory and thus promotes the maturation of B cells and the development of a protective secondary immune response. In several countries, a conjugate vaccine was utilised against *Haemophilus influenza* type B (Hib) which became a huge hit and it is now regularly used (*Scheifele 2001*). Many conjugate vaccines for example 7-, 9- and 11-valent preparation have been produced and they are at different stages

of testing. The polysaccharide of a 7-valent conjugate vaccine contains serotypes 4, 6B, 9V, 14, 18C, 19F and 23F and is covalently linked to a non-toxic CRM_{197} diphtheria carrier protein. In February 2000 the United States licensed a 7-valent conjugate (Prevnar™ *7*-Prevenar®, Wyeth Lederle Vaccines) since then 17 million doses were given. Subsequently the Pneumococcal polysaccharide conjugate vaccine was licensed in 2006 for use in the UK.

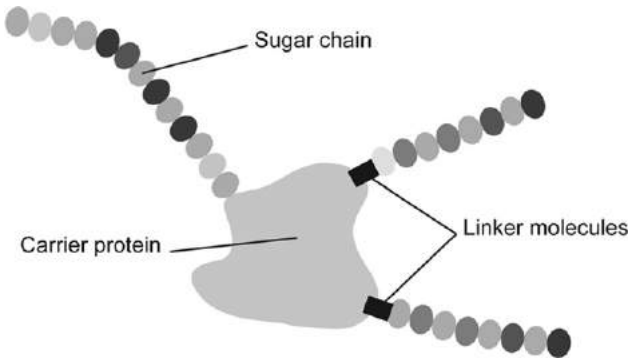

Figure 8: The structure of conjugate vaccine, taken from (*Broker et al. 2011*).

It has been shown that Prevenar™ is protective against otitis media, pneumonia and invasive disease which have been found to be causes by vaccine serotypes (*Blacketat., 2000; Eskolaetat.,2001*). It has also been shown that it induces serotype cross protection and mucosal IgA responses in infants under the age of 2 and other high risk groups (*Choo et al. 2000*).

The 7-valent conjugate vaccine has been considered successful but it still has many negative aspects for instance, it is more expensive to produce than the 23-valent polysaccharide. Perhaps the most important negative aspect to this vaccine is that all serotypes cannot be included in its

preparation so it will be unable to provide defence for all those who require it. During the launch of the 7-serotype conjugate vaccine at the start of the 20th century in the United States, it was shown to be effective against only 15% and 35% in adults and children respectively. However, now with the passage of time it has been shown to give protection against more than 80% of pneumococcal diseases (*Feikin, Klugman 2002*). The colonization of pneumococcal serotypes can be decreased through the power of the vaccine itself which can lead to replacement of common serotypes. *S. pneumoniae* produces hydrogen peroxide which hinders the growth of *H. influenzae* a common respiratory tract pathogen (*Pericone et al. 2000*) and it can result in desialylation of the lipopolysaccharide found in *H. influenzae* and *Neisseria meningitidis* (*Shakhnovich, King and Weiser 2002*). In Asia, Africa and Oceania this vaccine does not provide sufficient protection to *S. pneumoniae* because of the presence of a range of different serotypes found in this region and pneumococcal infection remains a serious threat to health. However, it does largely provide protective immunity in North America and some European countries (*Wuorimaa, Kayhty 2002*).

Research conducted in Finland showed that cases of pneumococcal acute otitis media (AOM) were reduced by 57% with the use of this vaccine (*Eskola et al. 2001*) and the same vaccine was shown to reduce AOM incidence in the US by 84% (*Joloba et al. 2001*). The one drawback to the use of this conjugate vaccine is that it increases the chances of infection with other organisms and resistant strains of *S. pneumoniae*. This was highlighted by a study which showed enhanced chances of acquiring AOM through exposure to non-vaccine serotypes (*Eskola et al. 2001*).

1.5.3 CRM_{197} protein

CRM_{197} is a non-toxic mutant of diphtheria toxin with a single amino acid substitution of glutamic acid for glycine (*Rappuoli, 2012*). Protein

CRM_{197} is the carrier for polysaccharides and greatly enhances their immunogenicity and CRM_{197} is used as a carrier protein for various vaccines including meningitis and pneumococcal bacterial infections. Both the structure and function of CRM_{197} is similar to the native diphtheria toxin produced by *Corynebacterium*. CRM_{197} itself is a single polypeptide chain of 535 amino acids with two subunits which are linked by disulfide bridges which allows the stable part of the protein to penetrate the host cell (Acton 2012). In the 52 kDa CRM_{197} protein (Figure 10) glutamic acid replaces the glycine in position 197 and prevents ADP ribosylation activity - hence it is not toxic to cells. CRM_{197} is a protein which is now well defined as a carrier protein for polysaccharides to increase their immunogenicity. CRM_{197} has been used as a carrier protein for many polysaccharide vaccines such as the pneumococcal polysaccharides vaccine. The effectiveness of using CRM_{197} as a carrier protein for conjugate vaccines has been shown by its ability to promote a protective immune response in very young infants in whom the immune system is only at the early stages of maturation and can promote both antibody and cell mediated immune responses (*Harboe, et al.2006*) (*Leonard, et al. 2003*).

Table 4: Vaccines based on CRM_{197} (*Lee, Blake 2012*)

Vaccine	CRM197 content (µg)
Meningococcal C	15
7-Valent pneumococcal	20
9-Valent pneumococcal	22
H. influenzae type b	25
13-Valent pneumococcal	32

1.6 Future Vaccines

As already mentioned above the short comings of conjugate vaccines include high expense, low serotype coverage and poor performance in infants - thus there has been a demand for the development of a

pneumococcal vaccine which can be manufactured at low cost, have enhanced serotypes coverage and be effective in infants. The use of so called 'deep epidemiological exploration' has been used to identify the spectrum of serotypes which cause disease and their geographical dominance. The idea of producing tailored vaccines for individual environments is interesting but this could pose a heavy financial burden on poorer countries so a vaccine preparation that would be effective across geographical boundaries is being sought. For a vaccine to be effective it should be able to stimulate the production of IgA and IgG antibodies, they should have high affinity and be cross protective against a large number of serotypes.

Currently a number of studies are underway to examine the use of pneumococcal proteins for use as vaccines (for example pneumolysin and pneumococcal surface protein A (PspA)) (*Alexander et al. 1994*) and (*Paton, Giammarinaro 2001*). These would provide the great advantage of not having to consider geographical factors such as in the great variability of polysaccharide epitopes. The search for acceptable candidates for an *S. pneumoniae* vaccine involves signature tagged mutagenesis (STM), differential fluorescence induction (DFI) and motif searches for surface expressed molecules. For their detection several techniques have been put into action (*Pearce, Yin and Masure 1993*), (*Polissi et al. 1998*), (*Lau et al. 2001*) , (*Wizemann et al. 2001*), (*Hava, Camilli 2002*) and (*Marra et al. 2002*) and an enormous number of genes have been discovered through such genomic searches. However, there is now a need for thorough evaluation of their appropriateness through comprehensive examination.

1.7 Antibiotics and Resistance

In 1928 Alexander Fleming discovered, accidently, that blue and green mould colonies (*Penicillium notatum*) which had infected his staphylococci seeded agar plates produced a substance which inhibited

the growth of the bacteria. This observation eventually led to the purification of the soluble product which in 1938 Howard Florey and Ernst Chain at Oxford University with the help of colleagues in America was purified and used to treat patients suffering from severe infections Antibiotics are a product, which inhibits the growth of/or kills bacteria. Antibiotics are thus chemical agents that have revolutionised the treatment of infectious disease (*Mossialos 2010*).

The first antibiotic resistance was reported in 1940 when Penicillin resistant *Staphylococcus aureus* strains were descried. This has led to a challenge for the development of new antibiotic to stopped the rabidly grown of infection (*Pray 2008*). Antibiotic resistance is a result of the changes in bacterial characteristics that stops the effectiveness of drugs which previously had been used to kill them (*Elizabeth, Bancroft 2007*). The resistance to antibiotics has increased in the pool of human pathogens. The mechanisms of antibiotic resistance include: resistance due to making a drug target alteration in the cell of the bacteria, or by making a limitation and modification of enzymes aimed at stopping active drug efflux (*Poole 2002*). The enzymatic modification or destruction of resistance is due to mutations in microbial genes which lead directly to resistance or to the transfer of resistance to the next generation of microbes. The diagnosis of a disease is very important in order for hospital staff to decide which antibiotic is most suitable for the patient. This is because laboratories might take a day or more to isolate the specific pathogen or because of stretched laboratory resources (*Russell 2002*). There is close relationship between the treatment of bacterial infection and antibiotic resistance especially when patients develop multidrug resistant infections for specific diseases such as Tuberculosis (*Mayer 2007*).

In many developing countries, patients can easily buy antibiotics without the need for a prescription even when symptoms are not related to bacterial infection and this can lead to an increase the occurrence of antibiotic resistant organisms in these countries. In developed countries,

such as the UK and the United States, it is more difficult to obtain antibiotic prescriptions because the governments of developed countries have introduced rules with regard to prescribing antibiotics. It is therefore likely that antibiotic resistant organisms are less likely to arise in developed countries than in developing countries.

1.7.1 Antibiotic and Antibiotic Resistance to *S. pneumoniae*

Pneumococcal infection was once known as 'captain of the men of death' by Dr. William Osler who was a prominent physician at the start of the 20th century. In the late 1930's sulphonamides were introduced as a means of treatment which was then followed by penicillin in 1945. The discovery and use of antibiotics decreased the dominance and high death rate which was associated with pneumococcal infection. The development of a suitable vaccine was on hold for several years due to the triumph of antibiotics.

In 1969, a strain of *S. pneumoniae* found in Papua, New Guinea was shown to be resistant to penicillin (*Tomasz 1999*). It was found that the multiplication of resistant strain of S. *pneumoniae* was exceptionally distant. This was assess and explained by an Editorial and was found to be close to fact. In Spain, it has been reported that there has been an increase in the incidence of infections caused by penicillin non-susceptible strains of *S.pneumoniae*. Internationally, this has then led to a proliferation in the number of resistant strains and has been reported in the US as well as in some developing countries In fact, it has been said that almost 80% of all bacterial isolates may contain penicillin resistant bacteria(*Low 2000*) and (*Henriques Normark et al. 2001*). From 1992 to 1999 studies carried out in Scotland established that 61% of *S. pneumoniae* strains were resistant to penicillin and erythromycin was also found to be ineffective against 14% of isolates in the same study (*Kyaw et al. 2002*). Lactam antibiotics are the third generation of new antibiotics and work by inhibiting cell wall biosynthesis and include

cephalosporin, ceftotaxime and ceftriaxone but over time bacteria have become able to change the constituents of their cell wall proteins and highly resistant isolates of *S. pneumoniae* which are resistant to penicillin have arisen (*Linares et al. 1992*). It has also been reported that *S.pneumoniae* has also been found to be tolerant to against vincomycin (*Novak et al. 1999*) and (*Henriques Normark et al. 2001*). It has been suggested that the development of resistance to antibiotics is the over prescribing of antibiotics. Some *S. pneumoniae* serotypes such as 6A, 6B, 9V, 14, 19F and 23F are more commonly linked with antimicrobial resistance as compare to other serotypes and these are included in the Prevenar 7-valent conjugate vaccine (*Wuorimaa, Kayhty 2002*).

One of the factors that aid the amplification of multi-resistance in other serotypes is in the exchange of capsule components as has been seen in highly resistant strains (*Tomasz 1999*). It is believed that horizontal gene transfer and homologous recombination is mainly responsible for the cross transmission between strains of bacteria and the ready transformability of pneumococcus helps in the rapid development of resistance (*Dowson et al. 1997*).

1.8 Antibody Structure

Antibody are generally Y-shaped and the structure of an antibody consists of two heavy and light chains linked by disulphide bonds (Figure 9). There is a constant variable region in every chain. The function of the variable region is to identify and link various molecules. The constant region, on the other hand, conducts effector processes which includes recognition by antibody fine specificity and phagocytes.

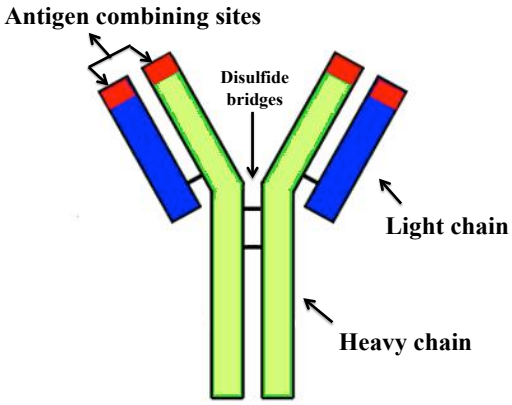

Figure 9: Structure of the typical IgG antibody molecule

The variable regions in an immunoglobulin produce two binding site which are responsible for identifying and binding the various antigen by epitopes. The rearrangement of genes causes difference in the variable region. Variable heavy gene families are of varying nature and are seven in total. They consist of 51 gene loci, 27 diversity segments and 6 joining segments. In addition to this, due to their two different types i.e., lambda (λ) and kappa (κ), there is greater variety in the variable light chain. The variable light chain repertoire comprises 71 variable genes and 9 joining segments in total (*Murphy, Janeway 2011*) .

The formation to create V, D and J segment, somatic rearrangement takes place in every gene segment. A complete and diverse variable region is created by this segment. A total of 3 million variable regions of different types were discovered due to the variety of potential gene segment combinations. In addition to this, the diversity is increased due to nucleotide insertions and deletions in the CDR3, variable heavy and light gene pairing and somatic hypermutation. The grouping of the complete B cell receptor and antigen stimulation is followed by somatic hypermutilation. Due to this process, there are alterations within the variable region which, in turn, result in higher affinity for an antigen in the variable region.

This general category however consists of five different immunoglobulin classes; IgG, IgM, IgA, IgE and IgD which can be differentiated by their C regions. This current study is however interested in the IgG antibody molecule.

1.8.1 IgG Antibody

IgG antibodies are generally large molecules that have a molecular weight of roughly 150 kDa and comprise of two different types of polypeptide chain (*Cook 2000*). One chain of roughly 50kDa is referred to as the heavy or H chain while the other of approximately 25 kDa is referred to as the light or L chain (Figure 9). Each IgG molecule comprises of to light and two heavy chains. The heavy chains are linked by disulfide bonds while each H chain is linked to an L chain also by a disulfide bond (*Liu & May, 2012*). The two H chains and the two L chains are usually identical in any given immunoglobulin molecule hence gives an antibody molecule two antigen-binding sites that are identical to each other hence the ability of this molecule to simultaneously bind to two identical structures (*Janeway et al., 2001*).

In Figure 9, heavy chains are represented by the green colour while the light chains are illustrated using the blue colour and as can be seen, the two pairs are joined by disulfide bonds such that each H chain is linked to an L chain and the two H chains are linked to each other.

$CD8^+$ T cells are necessary for IgG antibody production to pneumococcal polysaccharides. A study was conducted on mice which measured IgG Ab production following immunisation with pneumococcal polysaccharides in mice with disruptions in selected genes of the T cell pathway and it was found that CD1 molecules together with MHC class I-restricted CD8+ cells were essential for the production of an effective neutralising immune response (*Kobrynski et al. 2005*). Pneumococcal polysaccharide vaccines are more immunogenic in immunocompetent

than they are in imunocompomised individuals. Kobrynski et al. 2005 study sought to examine the efficacy of PPs vaccines in individuals who are immunocompromised and the host cellular subsets that are necessary for vaccine effectiveness against pneumococcal disease by vaccinating CD4-deficient, CD8-deficient and secretory immunoglobulin M-deficient mice and wild-type mice with a conjugate of Pneumococcal polysaccharide of tetanus toxoid and serotype 3 and investigating the antibody response as well as effectiveness of vaccination against systemic and pulmonary problems with serotype 3 pneumococcus in mice that have been immunised compared to control mice (*Tian et al. 2007*) . The findings showed that there was a difference in the response of the isotype and predominant IgG subclass between immunodeficient mouse strains and also between the immunodeficient and the wild type mice with CD8-deficient mice showing the most robust response. It was found that the vaccine protected the wild type, $CD4^{-/-}$, and $sIgM^{-/-}$ mice from death as a result of pulmonary challenge while those with CD8 deficiency were not protected. The findings of this study indicate that pneumococcal polysaccharide vaccine is effective in producing IgG antibodies in wild type and CD4-deficient mice but not in CD8 deficient mice.

It was established that $CD4^{+}$-T-cells are essential for IgG antibody production in mice were immunised with polysaccharide-protein conjugate vaccines. Polysaccharide conjugate vaccines in mice induce IgG antibodies but mainly require type 2 T-cell independent antigens for the IgG production to be induced (*Bartoloni et al. 1995*) . Immunisation of mice with polysaccharide conjugate vaccines has been shown to generate a protective immune response against various pneumococcal strains as the carrier protein stimulates T-helper cells which in turn stimulate B cells to produce the antibodies (*Käyhty, Eskola 1996*) . IgG production in mice requires a T-cell dependent response and for a pneumococcal conjugate vaccine to be effective in aged mice, with regard to producing IgG antibodies, it is necessary stimulated B cells to receive 'help' from $CD4^{+}$-T-cells thus allowing them to switch from IgM

to IgG antibody production (*Sen, Chen and Snapper 2006*) . Thus in older mice, where populations of $CD4^+$-T-cells are depleted, there is a reduced efficacy of the vaccine (*Sen, Chen and Snapper 2006*)

1.9 The role of the Spleen in the Immune Response to *S. pneumoniae*

The spleen has a primary role in acting as a 'blood filter' but is also a vital organ in allowing the clonal proliferation of immune cells reacting to infectious organisms such as *S. pneumoniae* It has been shown for instance that splenectomised rats have a much lower survival percentage after pneumococcal challenge than normal rats and it follows that splenectomy impacts on the survival and the risk of pneumococcal disease is higher in splenectomised patients (*Ram, Lewis & Rice 2010*). This is interlinked to the proposition that protection can be provided through preserving adequate splenic tissues (*Malangoni et al. 1985*).

It thus appears that splenic clearance is a vital feature in fighting pneumococcal infection and cannot be equipoised by the hepatic clearance (*Brown, Hosea & Frank 1981*). At the time of the induction of the innate immune response there is interaction among the complement system and the spleen, in which specialised cells in the marginal zone (MZ) of the spleen play an key role in directing the adaptive immune response. There are MZ macrophages B cells, dendritic cells and metallophilic macrophages (found in rodents but not in humans) in the MZ (*Steiniger, Timphus & Barth 2006*). There is a difference among marginal zone B cells and follicular B cells (of the germinal centres) and the former act as rapidly responding innate cells. The MZ B cells are intensified by the MZ macrophages in a T cell independent manner such that IgM can be produced (*Koppel et al. 2008*) which is essential for initial control of the disease and later stimulates the adaptive immune response afterwards by means of cell-cell interaction *(Salehen, Stover 2008*). The multivalent antigens (e.g. capular polysaccharide) (which are

able to cross-link their B cell receptor) can activate the B cells directly and encourage the secretion of antibodies along with macrophage activation (*Clutterbuck et al. 2012*).

As a result of the presence of the negative charge of the capsule the immunogenicity of the surface nature among the polysaccharides located in the pneumococci capsule is poor. This hinders phagocytosis but can be overcome by the antibody and complement mediated opsonisation *(Lee et al. 2001*). In children less than two years old, there is not much development of the MZ is not fully developed and the polysaccharide antigens need to be conjugated to proteins to trigger a T cell dependent pathways (*Klein Klouwenberg, Bont 2008*).

Hypothesis and Aims

2 Hypothesis and aims

2.1 Hypothesis

It is now accepted that the complement system is critical in many biological functions, including the maintenance of homeostasis. It serves as a first line of defence against infection, wherein it has roles in direct killing, or opsonisation and phagocytosis of pathogens. More specifically, the complement system is attributed to the development of adaptive immunity. Its role in the clearance of apoptotic bodies, and modulation of both T-lymphocytes polarisation and humoral responses through B-lymphocyte mediated mechanisms makes the complement system a critical part of the immune system. The crucial role of this system is underlined by the finding that deficiencies and uncontrolled activation of some of the components of the complement system is predisposes to severe infectious diseases. Irrespective of the array of information that is available on the mechanisms of the complement system, it is still unclear what role it plays in vaccine mediated immunity against infections. Combined with the aspects of vaccination discussed above, it is hypothesised that the complement systems a play role in the induction of the immunogenic response by PneumovaxII and Prevenar13, through differential modulation of the three complement activation pathways. To investigate this hypothesis, the study defined the contribution of the individual activation pathways to vaccine-induced immunity against *S. pneumoniae* using gene-targeted mutations that cause defects in the three complement pathways. Including the classical pathway in $C1q^{-/-}$, lectin pathway in MASP-$2^{-/-}$, and alternative pathway in $fB^{-/-}$ mice. Specifically, the production of antibody and infiltration of immune cells to the spleen was used to define the extent of the contribution of each pathway. Similarly, varying doses and different components of pneumococcal vaccines were investigated to determine potential differences in immunogenic response.

2.2 Aims

To determine whether any of the three complement pathways contributes to antibody response induced by

- Different vaccines (CRM_{197}, PneumovaxII and Prevenar13).
- Different doses regimes (initial dose and number of boosters).
- Different routes (s.c. and i.p.).
- To test the efficacy of antibody titre analysed. C3 deposition on CRM_{197}, PneumovaxII and Prevenar13 was assessed by ELISA and, in addition, sub- populations of lymphocyte subsets infiltrating the spleen were analysed by flowcytometry.

Materials and Methods

3 Materials and Methods

Table 5: Chemicals

Name	Supplier
1 kb plus DNA ladder	Invitrogen
1X TAE	Thermo scientific
2-Propanol	Sigma-Aldrich
6X DNA loading dye	Thermo scientific
Acrylamide bis	Sigma-Aldrich
Agarose, electrophoresis grade	Melford
Barbital	Sigma-Aldrich
Barbitone sodium 99+% for analysis	Fisher scientific
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Coomassie Brilliant Blue G-250 Dye	Thermo scientific
Deoxyadinosine triphosphate (dATP)	Thermo scientific
Deoxyribo nucleotide PCR grade (dNTPs)	Promega
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
EDTA- Ethylenediaminetetraacetic acid	Sigma-Aldrich
EGTA - Ethylene glycol-bis-N,N,Ń,Ń-tetraacetic acid	Sigma-Aldrich
Ethanol	Fisher
Ethidium bromide	Sigma-Aldrich
Foetal bovine serum	Invitrogen
Formaldehyde solution	Sigma-Aldrich
Glycerol	Fisher scientific
Hydrochloric acid solution	Sigma-Aldrich
Mannose	Sigma-Aldrich
OCT embedding cryoembedding Matrix	Fisher scientific
RNAse A solution	Promega
Sigmafast p-Nitrophenyl phosphate tablets	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium carbonate, anhydrous	Fisher scientific
Sodium chloride	Fisher scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium hydrogen carbonate	Fisher scientific
Tris base	Santa Cruz biotechnology
Tris-HCl	Sigma-Aldrich
Tween-20	Sigma-Aldrich and Fisher scientific
Zymosan	Sigma-Aldrich

Table 6: Commercial Antibodies and Antigen

Antibodies & Antigen	**Manufacture**
Anti-bovine whole serum developed in rabbit	Sigma-Aldrich
Anti-mouse polyclonal IgG antibody	Sigma
APC anti-mouse CD19	Cambridge bioscience
APC Rat IgG1, λ Isotype Ctrl	Cambridge bioscience
APC/Cy7 anti-mouse CD4	Cambridge bioscience
APC/Cy7 Rat IgG2b, κ Isotype Ctrl	Cambridge bioscience
Baby rabbit complement serum & cells	Fisher scientific
C1q Protein	Complement technology inl
C1q, Mouse: Mab, Clone 7H8	Cambridge bioscience
Chicken monoclonal anti-human C4c	Immunsystem AB
CRM_{197}	Reagent Proteins
CRM_{197} (as a gift)	GSK Vaccines S.r.l.(formerly Novartis Vaccines & Diagnostics) (Italy)
Factor B	Complement technology inl
Goat anti-mouse igg (whole molecule)alk	Sigma-Aldrich
goat anti-mouse igg (whole molecule)alk	Sigma-Aldrich
Mouse monoclonal anti-human MBL	Antibodyshop
PE anti-mouse CD3	Cambridge bioscience
PE/Cy7 anti-mouse CD25	Cambridge bioscience
PerCP/Cy5.5 anti-mouse CD8	Cambridge bioscience
Pneumococcal cell wall polysaccharide (CWPS multi)	Statens serum institut
Pneumococcal polysaccharide conjugate vaccine (Prevenar13)	Pfizer (movianto uk)
Pneumococcal polysaccharide vaccine (PneumovaxII)	Sanofi Pasteur MSD Limited
Purified anti-mouse CD16/32	Cambridge bioscience
Rabbit anti-rat igg (whole molecule)alka	Sigma-Aldrich
Rabbit polyclonal Anti-human C3c IgG	Dako
rat anti mouse CD19:FITC	Cambridge bioscience
rat anti-mouse CL11	Antibody shop
rat anti-mouse MBL-A	Hycult Biotech
rat anti-mouse MBL-C	Hycult Biotech

Table 7: Commercial Kits

Purchased Kits	**Manufacture**
QIAquick gel extraction Kit	Promega
Wizard genomic DNA purification kit	Promega
Wizard plus SV minipreps DNA purification kit	Promega
MyTaq red DNA polymerase	Bioline

Table 8: Media

Media Medium	Manufacture
ACK lysing buffer (erythrocyte lysis solution)	Scientific Lab and life technologies
Agar Base	Oxoid
ALUM	Thermo scientific
Brain Heart Infusion (BHI)	Oxoid
Hanks Buffered Salt Solution (HBSS) (1x)	PAA Laboratories and Fisher scientific
Horse blood defibrin	Oxoid, Fisher scientific and Scientific Lab
Penicillin -streptomycin solution	Sigma-Aldrich
RPMI-1640	Sigma-Aldrich

Table 9: Tips, Syringes and Pipets

Tips size	Manufacture
10 – 200 µL	Starlab and Sarsted
10 cc Syringe disposable sterile plastic	Fisher scientific and VWR
10 mL Pipette stripe tissue culture sterile	Fisher scientific
100 - 1000	Starlab and Sarsted
100 – 1250 µL	Starlab and Fisher
2 cc syringe disposable sterile plastic	Fisher scientific and VWR
20 µL	Sarsted
25 mL Pipette Stripette tissue culture sterile	Fisher scientific
5 mL Pipette Stripette tissue culture sterile	Fisher scientific and VWR
50 cc syringe disposable sterile plastic	Fisher scientific and VWR
Filter tips 0.5 – 10 µL	Started
Filter tips 10-200 µL	Starlab
Filter tips 100 – 1000 µL	Starlab and Sarsted

3.1 Mouse Strains

Wild type (strain C57BL/6J) mice were obtained from Charles River, UK and $C1q^{-/-}$, MASP-$2^{-/-}$ and factor $B^{-/-}$ (C57BL/6J –background) mice were obtained from CRF- University of Leicester. Mice were at 6 - 8 weeks old before use. Upon mice arrived into the CRF-Building (University of Leicester), they kept to acclimatise for at least one week. Then mice were kept in cage with good ventilated system and with allowed unlimited water and food. A Home Office personal license holder and training record carried out all animal procedures. Following experiments immunisation with CRM_{197}, pneumococcal polysaccharide vaccine (PneumovaxII) and pneumococcal polysaccharide conjugated vaccine (Prevenar13), mice will keep them in separate cage with specific ventilated system. All mice were used in this project issued by project license, which is under the Animal Scientific Procedures Act. 1986 UK.

3.2 Preparation of formalin fixed *Streptococcus pneumoniae*

Streptococcus pneumoniae D39 were added to 10 ml of BHI (Brain Heart Infusion), and incubated overnight with at 37^{o}C. In the next day spun down the bacterial at 300xg for 10 minutes. After that, the Supernatant was thrown and the coagulated was kept to wash it by PBS (phosphate buffer saline) and re-spun it again, this step should be repeated three times. Then 0.5% formalin in PBS was added to bacterial pellet, which was incubated for 2-3 hours at room temperature. After that bacteria were spun and washed again with PBS two times. Finally, re-suspended the bacteria in the coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and adjusting the OD into 550 nm to have 0.6 of suspension (*Lynch et al.* 2004) and (*Hyams et al.* 2010)**.**

3.3 Preparation of CRM_{197} for immunisation

Mice were injected by 20µg CRM_{197}/mouse/dose s.c. or i.p. Alum adjuvant (containing an aqueous solution of aluminium hydroxide. Magnesium hydroxide plus inactive stabilizers from Thermo) was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and CRM_{197} protein and kept for 30 minutes at room temperature. Then each mouse was immunised using 100µl of the final diluted solution.

3.3.1 SDS-polyacrylamide gel electrophoresis

The characterisation of the protein was assessed using SDS-PAGE. 20µl of CRM_{197} protein was incubated with 2-mercaptoethanol and SDS loading dye (2.5 mL glycerol, 5.8 mL Tris-HCl pH 6.8, 1 mg bromophenol Blue and 0.83 g SDS) at 95oC for 10 minutes. Then, the sample was loaded into 12% SDS-polyacrylamide gel which was run using running buffer (192 mM glycin, 25 mM trizma base and 0.1% SDS) for 1 hour at 150V. After that the gel was stained using Coomassie brilliant blue G-250 dye at room temperature for 30 minutes. Then, the gel was washed using de-staining solution (10% acetic acid glacial, 60% methanol and 30% ddH_20 water) to let the band of the protein to be coming visible. After that dH_2O was used to wash the gel (Figure 10).

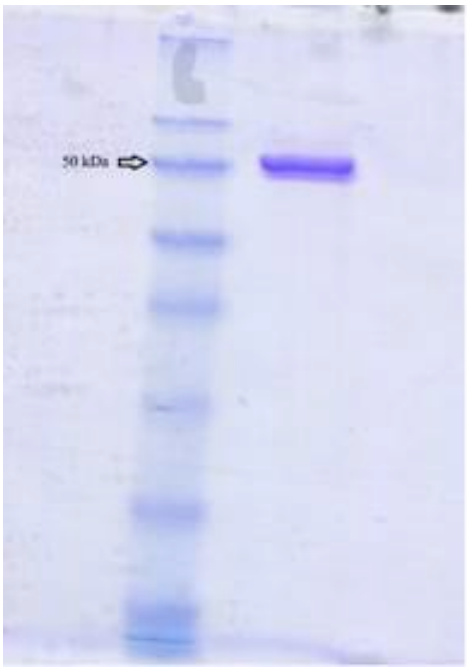

Figure 10: SDS-polyacrylamide gel electrophoresis of purified Diphtheria toxin mutant CRM_{197}

3.4 Preparation of PneumovaxII for immunisation

Mice were injected by 1µg PneumovaxII/mouse/dose s.c. or i.p routes and other mice were injected by 5µg PneumovaxII/mouse/dose i.p route. Alum adjuvant (containing an aqueous solution of aluminium hydroxide. Magnesium hydroxide plus inactive stabilizers from Thermo) was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and PneumovaxII vaccine and the immunisation suspensions was mixed and kept at room temperature for 30 minutes before immunisation. Then each mouse was immunised using 40µl of the final diluted solution (for 1µg PneumovaxII groups) and using 200µl of the final diluted solution (for 5µg PneumovaxII groups).

3.5 Preparation of Prevenar13 for immunisation

Mice were injected by Prevenar13 which was administered by (human dose/25) /mouse/dose i.p. Alum adjuvant (containing an aqueous solution of aluminium hydroxide. Magnesium hydroxide plus inactive stabilizers from Thermo) was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and Prevenar13 vaccine and the immunisation suspensions was mixed and kept at room temperature for 30 minutes before immunisation. Then each mouse was immunised using 40µl of the final diluted solution.

3.6 Preparation of sera

The blood of this project was collected from mice from saphenous vein bleed or by cardiac puncture (after using 2.5% isoflurane for deeply anaesthesia with oxygen 1.8 L O_2/min) and from human healthy volunteers by arm vein. Then transferred immediately and incubated for about 2 hours on ice to stop the complement activation. After that the spun down was used to separate the blood for 11 minutes at 11000rpm in 4^{o}C. Finally, the serum was collected in eppendorf (100µL each) then stored in -80^{o}C until used.

3.7 Genotyping of MASP-2$^{-/-}$, C1q$^{-/-}$ and fB$^{-/-}$

3.7.1 Genomic DNA extraction from ear snips

All mice were checked before starting any experiment to make sure that it were K/O or not. Genomic DNA was isolated from mouse ear snips using Promega Wizard Genomic DNA kit. Mice ear snips approximately (0.3 cm) were digested overnight at 55 ^{o}C in 300µl lysis buffer (250µl nuclei lysis solution + 60µl 0.5M EDTA) and 10 µl of 20 mg/ml proteinase K (Invitrogen) with over night gentle shaking. Next day, 1.5µl

of Rnase A solution (4 mg/ml) was added to each sample and mixed by inverting the tubes several times. The tubes were incubated at 37°C for 15 minutes and samples were allowed to cool to room temperature for 5 minutes before proceeding. After that, 100µl of protein precipitation solution was added and mixed by vortexing at high speed for 20 seconds. The samples were chilled in ice for 5 minutes and the precipitated protein was removed by centrifugation at 300xg for 4 minutes. The clear supernatant containing DNA was removed carefully and transferred into new 1.5 ml eppendorf tubes. 300µl of room temperature iso-propanol was added and mixed by inverting the tubes several time to precipitate the genomic DNA. The precipitated DNA was obtained by centrifugation at 13000xg for 5 minutes at room temperature. The clear supernatant was removed carefully and DNA pellet was washed by 300µl of 70% ethanol then DNA was re-precipitated by centrifugation at 300xg for 5 minutes. The DNA pellet was air dried for 10-15 minutes and 50 µl of DNA re-hydration solution or nano free DNA's (ddH_2O) water was added and incubated for overnight at 2 – 8 °C. Then, the prepared genomic DNA was used.

3.7.2 Multiplex PCR for genotyping of MASP-2$^{-/-}$, C1q$^{-/-}$ and fB$^{-/-}$

Genotyping deficient mice were identified using multiplex PCR reaction. Each PCR reaction mixture consisted of (Table 10 or Table 11)

Table 10: **Standard PCR reaction mix used for genotyping**

Reaction Material	Final concentration
10X buffer	1X
25 mM MgCl2	1.5 mM
10 mM dNTP Mix	200 µM
First Primer 5 mM	0.2 µM
Second Primer 5 mM	0.2 µM
Third Primer 5 mM	0.2 µM
Taq DNA Polymerase	1.2 Unit
Template DNA	100 ng
ddH2O	Up to 30 µL

Table 11: **Fast PCR reaction mix used for genotyping**

Reaction Material	Final concentration
MyTaq Red Reaction buffer	10 µL
First Primer 5 mM	1µL
Second Primer 5 mM	1µL
Third Primer 5 mM	1µL
MyTaq DNA Polymerase	0.25µL
Template DNA	2µL
ddH2O	Up to 30 µL

Three primers were used of identification of (MASP-2$^{-/-}$) homozygous, heterozygous and wild type mice.

Table 12: MASP-2$^{-/-}$ identification primers

Primer	Primer sequence
M2screen F1	5'-CAT CTA TCC AAG TTC CTC AGA-3'
M2wto R1	5'-AGC TGT AGT TGT CAT TTG CTT GA-3'
Neo5 R1	5'-CTG ATC AGC CTC GAC TGT GC-3'.

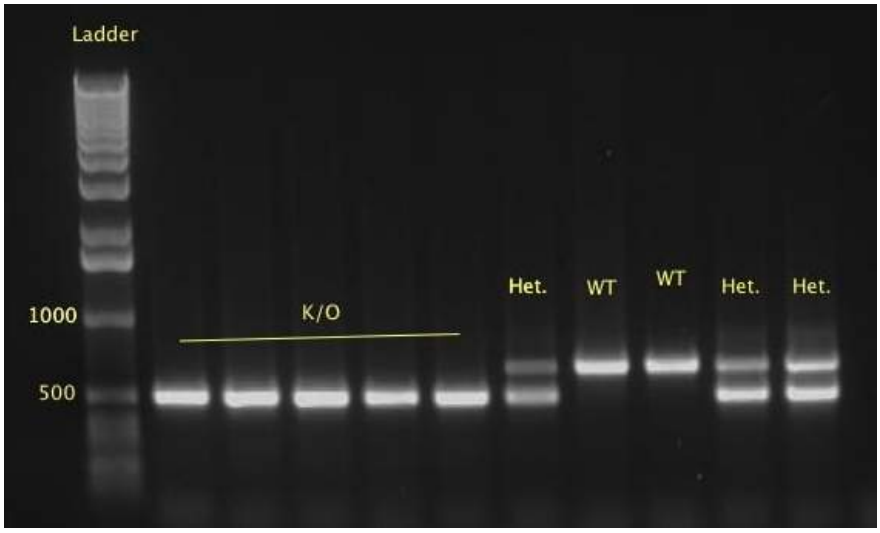

Figure 11: The size of the WT product is about 750bp, the K/O (MASP-2$^{-/-}$) is about 500bp and Heterozygote has both sizes.

Three primers were used of identification and Screening complement C1q K/O mice homozygous, heterozygous and wild type mice.

Table 13: $C1q^{-/-}$ identification primers

Primer	Primer sequence
mC1qA/5+	5'-GGG GCC TGT GAT CCA GAC AG-3'
mC1qN/2	5'-TAA CCA TTG CCT CCA GGA TGG-3'
Neo3	5'-GGG GAT CGG CAA TAA AAA GAC-3'

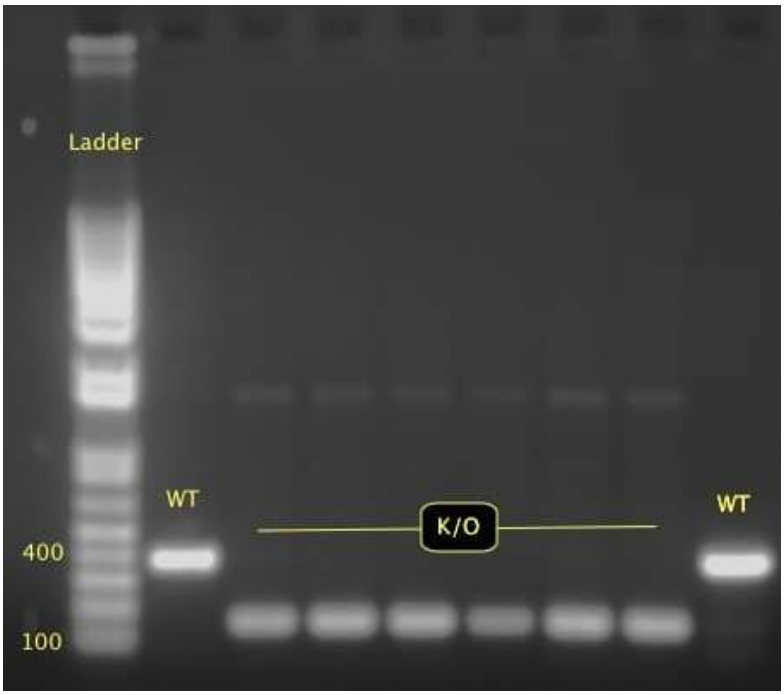

Figure 12: The size of the WT product is about 360bp, the K/O ($C1q^{-/-}$)is about 160bp.

Three primers were used of identification and Screening complement factor B K/O mice homozygous, heterozygous and wild type mice.

Table 14: $fB^{-/-}$ identification primers

Primer	Primer sesix pack abdominal musclesquence
Neo3-F5	5'-CTG TTG TGC CCA GTC ATA GCC GA-3'
FB-F2	5'-GAA GGA CCT AGA AAC AGC GCT CA-3'
FB-WTO-R1	5'-CTG ATC TAC CTT CTC AAT CAA GTT GGT GA-3'

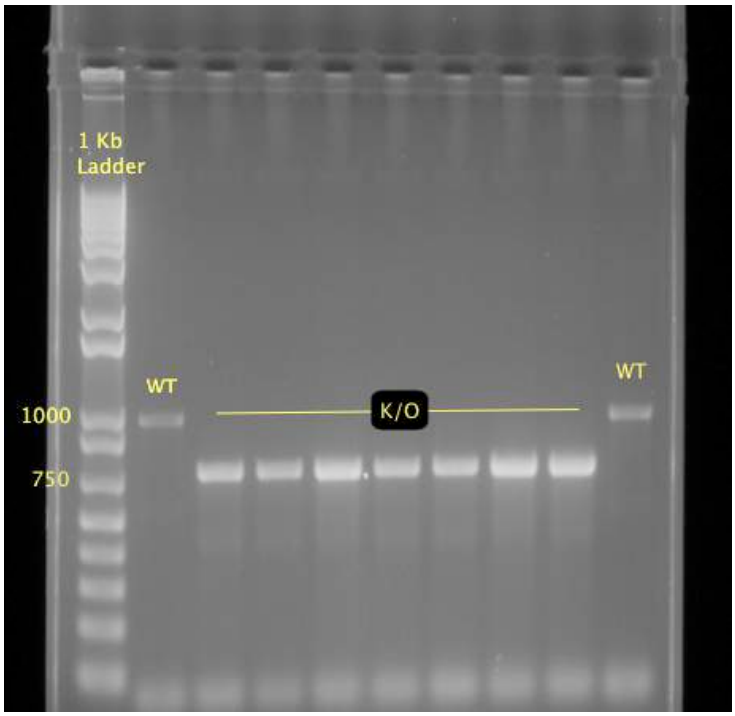

Figure 13: The size of the WT product is about 1000 bp, the K/O is about 750 bp.

Then, the PCR reaction was run at the following PCR conditions:

Table 15: PCR conditions

PCR conditions	Temperature	Time	Cycle
Initial denaturation	98°C	8 minutes	
Denaturation	94°C	45 sec	35
Primer annealing	58°C	30 Sec	
Extensions	72°C	90 Sec	
Final extension	72°C	10 minutes.	
Cooling down	4°C	∞	

Then the PCR samples were run using 1.5-2% Agarose gel (Table 16) to check the amplification of target region. Using Electrophoresis at 120 volts for approximately 45 minutes.

Table 16: Agarose gel Electrophoresis medium

Material	Composition
1.5-2% Agarose gel	1.5-2% Agarose gel dissolved in TAE buffer then add 0.5 µg/ml of ethidium bromide
TAE buffer (Tris- Acetic acid-EDTA)	2.0 M Tris-HCl, 1.0 mM acetic acid glacial and 50 mM EDTA-Na2 (PH: 8.5)

3.8 Subcutaneous injected mice

Mice were scruff (held the skin behind their neck) in one hand using the thumb and index finger and the tail was tided away by wrapping the tail around the smallest finger. Then the thumb was moved and the index finger adjusted slightly higher up to get an increase in space and to make sure there is a gap between the skin and the body muscles. The skin that was held acted as a pyramid or tent to allow for room for the needle of the syringe. Using this insulin syringe, doses into were injected into the subcutaneous cavity.

3.9 Intraperitoneal injected mice

Mice were scruff in one hand (held the skin behind their neck) between the thumb finger and index finger and the tail was tided away using the smallest finger. Then, the hand was turned over to let the mice go onto their back, keeping their tummy up and their head lower than their tail with an angle of approximately 45^{o}. After this, the organ was pushed slightly, moving from the bottom to the top and therefore placing the organ in the top part of the body to ensure the organs were not damaged.

Then an insulin syringe needle was used to inject the doses into the bottom left quarter of the abdominal cavity at an angle of 45^{o}.

3.10 Subcutaneous immunisation of mice with CRM_{197}

15 WT female C57BL/6J mice were used in each experiment as a control, run with an equal number from 15 female MASP-2$^{-/-}$, 15 female C1q$^{-/-}$ and 15-female fB$^{-/-}$ at 6-8 weeks old. Mice were immunised subcutaneously with 20µg/mouse of CRM_{197} protein, diluted in PBS. Alum was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and CRM_{197} protein and kept for 30 minutes at room temperature. Mice were immunised in a total volume of 100µl/ mouse. The immunisation was repeated every week for 4 weeks. Blood samples were collected prior to each immunisation during the course of immunisation to determine the antibody titre against CRM_{197}. Blood samples were collected from each individual mouse before immunisation experiments started, and were used as negative control. Then, before each immunisation, mice were bled from the saphenous vein, and at day 45 blood samples were taken by cardiac puncture (schedule 1 method). The blood samples were then kept on ice for 2 hours and spun-down at 11,000 rpm for 11 minutes at 4^{o}C. Sera were then stored in -80^{o}C until they were used.

3.11 Intraperitoneal immunisation of mice with CRM_{197}

15 WT female C57BL/6J mice were used in each experiment as a control, run with an equal number from 15 female MASP-2$^{-/-}$, 15 female C1q$^{-/-}$ and 15 female fB$^{-/-}$ at 6-8 weeks old. Mice were immunised i.p. with 20µg/mouse of CRM_{197} protein, diluted in PBS. Alum was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and CRM_{197} protein and kept for 30 minutes at room temperature. Mice were immunised in a

total volume of 100µl/ mouse. The immunisation was repeated every week for 4 weeks. Blood samples were collected from each individual mouse before immunisation experiments started, and were used as negative control. Then, before each immunisation, mice were bleeding from the saphenous vein, and at day 52 blood samples were taken by cardiac puncture (schedule 1 method). Blood samples were collected prior to each immunisation during the course of immunisation and at day 52 to determine the antibody titre against CRM_{197} protein. The blood samples were then and kept on ice for 2 hours then spun-down at 11,000 rpm for 11 minutes at 4^{o}C. Sera were then stored in -80^{o}C until they were used.

3.12 Intraperitoneal immunisation of mice with Pneumococcal Polysaccharide vaccine (PneumovaxII)

15 WT female C57BL/6J mice were used in each experiment as a control, run with an equal number from 15 female MASP-2$^{-/-}$, 15 female C1q$^{-/-}$ and 15 female fB$^{-/-}$ at 6-8 weeks old. Mice were immunised i.p. with 5µg/mouse and another mice were immunised with 1µg/mouse of PneumovaxII vaccine. Alum was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and PneumovaxII vaccine and kept for 30 minutes at room temperature. Mice were immunised in a total volume of (200µl/ mouse for 5µg/mouse group) or 40µl/mouse for 1µg/mouse groups. The immunisation was repeated every week for 3 weeks and at day 45. Blood samples were collected from each individual mouse before immunisation experiments started, and were used as negative control. Then, before each immunisation, mice were bleeding from the saphenous vein, and at day 52 blood samples were taken by cardiac puncture (schedule 1 method). Blood samples were collected prior to each immunisation during the course of immunisation and at day 52 to determine the antibody titre against PneumovaxII. The blood samples

were then and kept on ice for 2 hours then spun-down at 11,000 rpm for 11 minutes at 4^{o}C. Sera were then stored in -80^{o}C until they were used.

3.13 Intraperitoneal immunisation of mice with Pneumococcal Polysaccharide conjugate vaccine (Prevenar13)

15 WT female C57BL/6J mice were used in each experiment as a control, run with an equal number from 15 female MASP-2$^{-/-}$, 15 female C1q$^{-/-}$ and 15 female fB$^{-/-}$ at 6-8 weeks old. Mice were immunised i.p. with 150µl/mouse and another mice were immunised with 20µl/mouse of Prevenar13. Alum was used as adjuvant buffer after being diluted 1:3 in PBS. The final immunisation suspensions were mixed equally with alum buffer and Prevenar13 vaccine and kept for 30 minutes at room temperature. Mice were immunised in a total volume of 300µl/ mouse and another mice were immunised in a total volume of 40µl/mouse respectively. The immunisation was repeated every week for 3 weeks and at day 45. Blood samples were collected from each individual mouse before immunisation experiments started, and were used as negative control. Then, before each immunisation, mice were bleeding from the saphenous vein, and at day 52 blood samples were taken by cardiac puncture (schedule 1 method in animal licence issued under the Animal Scientific Procedures Act 1986 of the United Kingdom and with local ethical approval). Blood samples were collected prior to each immunisation during the course of immunisation and at day 52 to determine the antibody titre against Prevenar13. The blood samples were then and kept on ice for 2 hours then spun-down at 11,000 rpm for 11 minutes at 4^{o}C. Sera were then stored in -80^{o}C until they were used.

3.14 Spleen Collection and Preparation of splenocytes

After the final bleeding of mice by cardiac puncture, mice were culled (schedule 1 method in animal licence issued under the Animal Scientific

Procedures Act 1986 of the United Kingdom and with local ethical approval). IMS 75% spray was used to reduce the contamination that might come from skin or fur. Spleens from all immunised mice were removed under sterile conditions. Each spleen from immunised mice was aseptically cut into small pieces and was gently strained through 50μm cell strainer (BD) on a 50ml falcon tube and the tissue was pushed through the cell strainer using a plunger from a disposable 5ml syringe. After this, the cell was flushed through a strainer using 10 ml from the buffer (RPMI with 3% filtered bovine serum and 0.1% penicillin - streptomycin 10 mg/ml). Then the samples were span in the centrifuge at 300xg for 5 min at 4^{o}C. The supernatant was removed and the samples were re-suspended in 5 ml of ACK lysing buffer (erythrocyte lysis solution) and then incubated at room temperature for 5 minutes. After that, 5 ml of (RPMI with 3% filtered bovine serum and 0.1% penicillin - streptomycin 10 mg/ml) was added, and spun at 300xg for 5 min at 4^{o}C. The supernatant was disposed, and was then 5 ml of (RPMI with 3% filtered bovine serum and 0.1% penicillin - streptomycin 10 mg/ml) was added before the samples were spun down at 300xg for 5 min at 4^{o}C. Finally, the supernatant was removed once more, and 2 ml of frizzing buffer (90% filtered bovine serum and 10% DMSO-Sigma) was added using a cryo-tube and then the sample was incubated in containers, which decreased in temperature by 1^{o}C every minute. Eventually the samples froze at -80 ^{o}C, left 24-48 hours and then the samples were stored at -80 ^{o}C until they were used.

3.15 Immunofluorescent staining of Splenocytes

Frozen spleen cells were placed on an icebox and left to thaw out. Then a HANK's buffer, with 3% filtered fatal calf serum (FCS) as a washing and staining buffer, was added. After this, the cryo tubes were centrifuged at 4 ^{o}C and 300xg for 5 minutes. Then, the supernatant was removed. After that, 1 ml of washing buffer was added to each tube, and then centrifuged again at 4 ^{o}C and 300xg for 5 minutes. Again, the

supernatant was removed. 100µL of Fc blocking antibody (anti-mouse CD16/32) 1:200 in staining buffer was added to each tube and incubated in the dark on ice for 10 – 15 minutes. Then, the sample was spun down at 4 ^{o}C and 300xg for 5 minutes. The supernatant was removed and the pellet was kept. 300µL of washing buffer was added to each tube before they were centrifuged again at 4 ^{o}C and 300xg for 5 minutes. 100µL of mixed CD's Ab (anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD19, anti-mouse CD25 and anti-mouse CD45R/B220) diluted 1:300 in staining buffer was added (–ve control for each CD's Ab which is a mixture of all CD's antibodies except 1, and should have different –ve for different Ab). Then, all the samples were incubated in the dark on ice for 30 minutes. The samples were spun down at 4 ^{o}C and 300xg for 5 minutes. The supernatant was removed and the pellet was kept. 300µL of washing buffer was added to each tube before they were centrifuged again at 4 oC and 300xg for 5 minutes. 300µL of staining buffer was added to each tube and they were re-suspended by the tips. Then, each tube was transferred into a flow cytometry tube, which is suitable for a BD flowcytometery reader machine.

3.16 C3 deposition in WT, MASP-2$^{-/-}$and C1q$^{-/-}$ mice on CRM$_{197}$ protein

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plates were coated with 100µl/well of mannan (Sigma-Aldrich) 10µg/ml) in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and another half of plates were coated with 10µg/ml of CRM_{197} protein, and incubated overnight in fridge at 4^{o}C. The following day, the plates were washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in 1X TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. It was incubated at room temperature for 90 minutes. Then the plates were washed three times using 250µl/well of washing buffer (1X of TBS with 5 mM $CaCl_2$ and 0.05% Tween 20). After that, 100µl/well of serum (WT or MASP-2$^{-/-}$

or C1q$^{-/-)}$ were added after it had been serial diluted start with 1:80 in BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last well contained only HIS heated inactivated serum (heated at 56^{o}C/30minutes). The total were incubated at 37^{o}C for 2 hours. The plates were washed three times using washing buffer. 100µl/well of antihuman-C3c (Dako) was diluted 1:5000, in washing buffer and incubated for 90 minutes at 37^{o}C. The plates were washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plates were read at A_{405} nm using a BioRad microtitre reader.

3.17 C3 deposition in WT, MASP-2$^{-/-}$and C1q$^{-/-}$ mice on pneumococcal polysaccharide vaccine (PneumovaxII)

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plates were coated with 100µl/well of mannan (Sigma-Aldrich) 10µg/ml) in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and another half of plates were coated with 1µg/well of pneumococcal polysaccharide vaccine (PneumovaxII), and were incubated overnight in fridge at 4^{o}C. The following day, the plates were washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. Then, there were incubated at room temperature for 90 minutes. Then the plates were washed three times using 250µl/well of washing buffer (1X of TBS with 5 mM $CaCl_2$ and 0.05% Tween 20). After that, 100µl of serum (WT or MASP-2$^{-/-}$ or C1q$^{-/-}$) were added after it had been serial diluted start with 1:80 in BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last well contained only HIS heated inactivated serum (heated at 56^{o}C/30minutes). The total were incubated at 37^{o}C for 2 hours. The plates were washed three times using

washing buffer. 100µl/well of antihuman-C3c (Dako) was diluted 1:5000, in washing buffer was added and incubated for 90 minutes at 37^{o}C. The plates were washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plates were read at A405 nm using a BioRad microtitre reader.

3.18 C3 deposition in WT, MASP-2$^{-/-}$and C1q$^{-/-}$ mice on pneumococcal polysaccharide conjugate vaccine (Prevenar13)

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plates were coated with 100µl of mannan (Sigma-Aldrich) 10µg/ml) in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and another half of plates were coated with same volume of 1µg/well of pneumococcal polysaccharide vaccine from pneumococcal polysaccharide conjugate vaccine (Prevenar13), and incubated overnight in fridge at 4^{o}C. The following day, the plates were washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. It was incubated at room temperature for 90 minutes. Then the plates were washed three times using 250µl/well of washing buffer (1X of TBS with 5 mM $CaCl_2$ and 0.05% Tween 20). After that, 100µl/well of serum (WT or MASP-2$^{-/-}$ or C1q$^{-/-}$) was added after it had been serial diluted start with 1:80 in BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last well contained only HIS heated inactivated serum (heated at 56^{o}C/30minutes). The total were incubated at 37^{o}C for 2 hours. The plates were washed three times using washing buffer. 100µl/well of antihuman-C3c (Dako) was diluted 1:5000 in washing buffer was added and incubated for 90 minutes at 37^{o}C. The plates were washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted

to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plates were read at A_{405} nm using a BioRad microtitre reader.

3.19 C3 deposition in WT and Factor $B^{-/-}$ mice on CRM_{197} protein

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plate was coated with 100µl/well of zymosan (Sigma-Aldrich) 10µg/ml) in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and another half of plate was coated with 10µg/ml (1µg/well) of CRM_{197} protein; and incubated overnight in fridge at 4°C. The following day, the plate was washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. It was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum (WT or $fB^{-/-}$) were added after it had been serial diluted start with 1:20 in EGTA buffer (5 mM Barbital, 10 mM Nacl, 5 mM $MgCl_2$ and 10 mM EGTA). The last well contained only HIS heated inactivated serum (heated at 56°C / 30 minutes). The total was incubated at 37°C for 2 hours. The plate was washed three times using washing buffer. 100µl/well of antihuman-C3c (Dako) was diluted 1:5000, and the washing buffer was added and incubated for 90 minutes at 37°C. The plates were washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.20 C3 deposition in WT and Factor $B^{-/-}$ mice on pneumococcal polysaccharide vaccine (PneumovaxII)

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plate was coated with 100µl/well of zymosan (Sigma-Aldrich) 10µg/ml) in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and another half of plate was coated with 1µg/well of pneumococcal polysaccharide vaccine (PneumovaxII), and incubated overnight in fridge at 4°C. The following day, the plate was washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. It was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum (WT or $fB^{-/-}$) were added after it had been serial diluted start with 1:20 in EGTA buffer (5 mM Barbital, 10 mM Nacl, 5 mM $MgCl_2$ and 10 mM EGTA). The last well contained only HIS heated inactivated serum (heated at 56°C/30minutes). The total was incubated at 37°C for 2 hours. The plate was washed three times using washing buffer. 100µl/well of antihuman-C3c (Dako) was add after it had been diluted 1:5000, and the washing buffer was added and incubated for 90 minutes at 37°C. The plate was washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was diluted to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.21 C3 deposition in WT and Factor $B^{-/-}$ mice on pneumococcal polysaccharide conjugate vaccine (Prevenar13)

The half of NUNC MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plate was coated with 100µl/well of zymosan

(Sigma-Aldrich) 10µg/ml (1µg/well) in coating buffer; (15mM Na_2CO_3, 35mM NaHCO3, pH 9.6), and another half of plate was coated with same volume of 1µg/well of pneumococcal polysaccharide vaccine from pneumococcal polysaccharide conjugate vaccine (Prevenar13), and incubated overnight in fridge at 4^oC. The following day, the plate was washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. It was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum (WT or $fB^{-/-}$) was added after it had been serial diluted start with 1:20 in EGTA buffer (5 mM Barbital, 10 mM Nacl, 5 mM $MgCl_2$ and 10 mM EGTA). The last well contained only HIS heated inactivated serum (heated at 56^oC/30minutes). The total was incubated at 37^oC for 2 hours. The plate was washed three times using washing buffer. 100µl/well of antihuman-C3c (Dako) was diluted 1:5000, in the washing buffer was added and incubated for 90 minutes at 37^oC. The plate was washed again three times. After that, 100µl/well of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) was added after it had been diluted to 1:5000 was added and it was incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.22 CL-11, MBL-A, MBL-C binding assays

The ELISA plates was coated with 100µl/well of mannan 10µg/ml for the first half of the plat and another second half of the plat was coated with 100µl/well of Pneumococcal Polysaccharide vaccine (PneumovaxII) 10µg/ml; in coating buffer; (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated overnight in fridge at 4oC. The following day, the plates were washed once and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma- Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)]

was added. It was incubated at room temperature for 90 minutes. Then the plates were washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20 and 5mM $CaCl_2$). After that, 100µl/well of serum was added for first plat after it had been serial diluted start with 1:80 in BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4), and the last well contained only BBS without serum and was used as a negative control. The total were incubated at 37ºC for 2 hours. The plate was washed three times using washing buffer same as described above. 100µl/well of primary antibodies which are anti-mouse CL-11 produced in rat, anti-mouse MBL-A produced in rat (Hycult) and anti-mouse MBL-C produced in rat (Hycult) respectively; diluted 1:5000 in the washing buffer which used for plates, were added and incubated for 2 hours at 37ºC. The plates were washed again three times using washing buffer. After that, 100µl/well of secondary antibodies α-rat IgG alkaline phosphatase conjugate (Sigma-Aldrich); was used after diluted to 1:5000 were added and they were incubated for 90 minutes. Finally, 100µl/well of colorimetric substrate ϱ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) were used and incubated at room temperature. Then, the absorbance was measured using a BioRad microtitre plate reader at A_{405} nm.

3.23 Enzyme Linked Immune Sorbent Assays – ELISA:

ELISAs technique were used to measure the antibody titre in mice are immunised with CRM_{197} protein, or pneumococcal polysaccharide vaccine (PneumovaxII), or pneumococcal polysaccharides conjugated vaccine (Prevenar13); and the activation of complement to detect the level of C3b on the surface of them.

3.23.1 ELISA buffers

Table 17: Protocol of ELISA assays to prepare buffers

Buffer	Composition
Washing buffer	0.05% Tween 20, in 1X TBS
Coating buffer	15mM $NaCo_3$, 35mM $NaHCo_3$ (ph:9.6)
Blocking buffer	1% BSA, in 1X TBS
10X TBS (stock buffer)	10mM Tris-Hcl, NaCl (ph:7.4)
BBS buffer	4mM Barbital, 145 mM NaCl, 1mM $MgCl_2$, 2mM $CaCl_2$ (ph:7.4)
EGTA buffer	5 mM Barbital, 10 mM Nacl, 5 mM $MgCl_2$, 10 mM EGTA

3.24 Determination of antibody titre against CRM_{197} in immunised mice:

The ELISA plates were coated with 1µg/well of CRM_{197} in a coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and incubated overnight in a refrigerator at 4°C. The following day the plate was washed once, and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. Then the plate was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum was added after it had been serially diluted in a BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last row contained only BBS buffer. Part of the last row contained a +ve control (WT terminal bleed). The ELISA plate was incubated at room temperature for 2 hours. The plate was then washed three times using a washing buffer. 100 µl/well of anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted to 1:5000 was added, and was then incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated

at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.25 Determination of antibody titre against PneumovaxII in immunised mice:

The ELISA plates were coated with 1µg/well of PneumovaxII vaccine in a coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and incubated overnight in a refrigerator at 4^oC. The following day the plate was washed once, and 250µl/well of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. Then the plate was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum was added after it had been incubated with 5µg of CWPS at room temperature for 30 minutes and serially diluted in a BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last row contained only BBS buffer. Part of the last row contained a +ve control (WT terminal bleed). The ELISA plate was incubated at room temperature for 2 hours. The plate was then washed three times using a washing buffer. 100µl/well of anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted to 1:5000 was added, and was then incubated for 60 minutes. Finally, 100µl of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.26 Determination of antibody titre against Prevenar13 in immunised mice:

The ELISA plates were coated with 1µg/well of Prevenar13 vaccine in a coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6), and incubated

overnight in a refrigerator at 4^{o}C. The following day the plate was washed once, and 250µl of blocking buffer [1% (w/v) BSA (Sigma-Aldrich) in TBS buffer (10mM Tris, 140mM NaCl, pH 7.4)] was added. Then the plate was incubated at room temperature for 90 minutes. Then the plate was washed three times using 250µl/well of washing buffer (1X of TBS with 0.05% Tween 20). After that, 100µl/well of serum was added after it had been incubated with 5µg of CWPS at room temperature for 30 minutes and serially diluted in a BBS buffer (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and pH 7.4). The last row contained only BBS buffer. Part of the last row contained a +ve control (WT terminal bleed). The ELISA plate was incubated at room temperature for 2 hours. The plate was then washed three times using a washing buffer. 100µl/well of anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted to 1:5000 was added, and was then incubated for 60 minutes. Finally, 100µl/well of colorimetric substrate ρ-nitrophenyle phosphate (pNPP) (Sigma-Aldrich) was used and incubated at room temperature. Then, the plate was read at A_{405} nm using a BioRad microtitre reader.

3.27 Statistical analysis

Several programs were used for statistical analysis. GraphPad Prism version 6.0f for Mac (California USA) was used for the Student t-test and one-way ANOVA, while FlowJo version 8.8.3 for Mac was used for Flow cytometry.

Results and Discussions

4 Results and Discussions

4.1 C3 deposition assays

Measuring C3 activation as C3 deposition ELISAs in dilutions of serum on the surface of *S. pneumoniae* D39 (kindly provided from Prof. Peter Andrew's) and Pneumococcal vaccines is a standard methodology to quantify complement activation. MASP-2$^{-/-}$, C1q$^{-/-}$ and fB$^{-/-}$ mouse sera were used in these assays. A quantification of C3b or iC3b, or C3dg deposition was achieve by quantifying the deposition of C3 cleavage products in an ELISA using a C3c specific antibody as detection antibody.

In CRM_{197} coated ELISA plates, C3 deposition was not observed in sera of C1q$^{-/-}$ and MASP-2$^{-/-}$ mice in sera diluted in buffers containing calcium (Figure 14). Moreover, C3 deposition was not observed in fB$^{-/-}$ serum under alternative pathway (AP) conditions (Figure 17). On PneumovaxII vaccine, C3 deposition was observed in sera of C1q$^{-/-}$ and was not observed MASP-2$^{-/-}$ (Figure 15). However, C3 deposition was not observed in fB$^{-/-}$ serum under AP conditions (Figure 18). On Prevenar13 vaccine, C3 deposition was observed in sera of C1q$^{-/-}$ and fB$^{-/-}$ mice (Figure 16) and (Figure 19) respectively under lectin pathway (LP) conditions indicating that the LP works well on Prevenar13. The LP specify of complement activation is underlined since C3 deposition was not observed in MASP-2$^{-/-}$ serum under LP conditions (Figure 16).

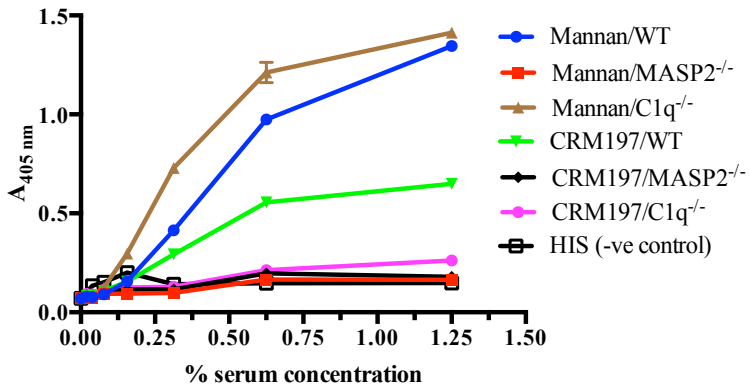

Figure 14: C3 depositions on CRM_{197} protein. Serum was serial diluted startingwith 1:80 in BBS buffer. HIS (-ve control) is WT mice serum heated at 56°C for 30minutes) - to destroy complement prior to performing neutralizing antibody assays) using as a negative control.

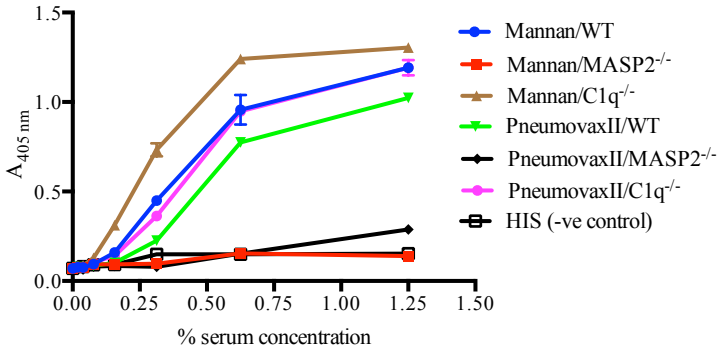

Figure 15: C3 depositions on PneumovaxII vaccine. Serum was serial diluted startingwith 1:80 in BBS buffer. HIS (-ve control) is WT mice serum heated at 56°C for 30minutes) - to destroy complement prior to performing neutralizing antibody assays) using as a negative control.

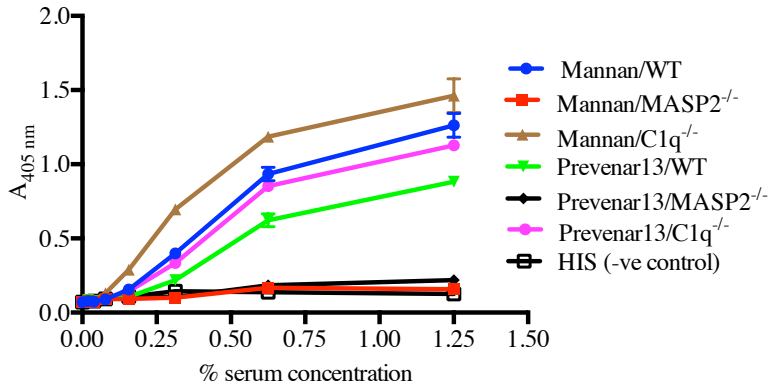

Figure 16: C3 depositions on Prevenar13 vaccine. Serum was serially diluted starting with 1:80 in BBS buffer. HIS (-ve control) is WT mice serum heated at 56°C for 30minutes) - to destroy complement prior to performing neutralizing antibody assays) using as a negative control.

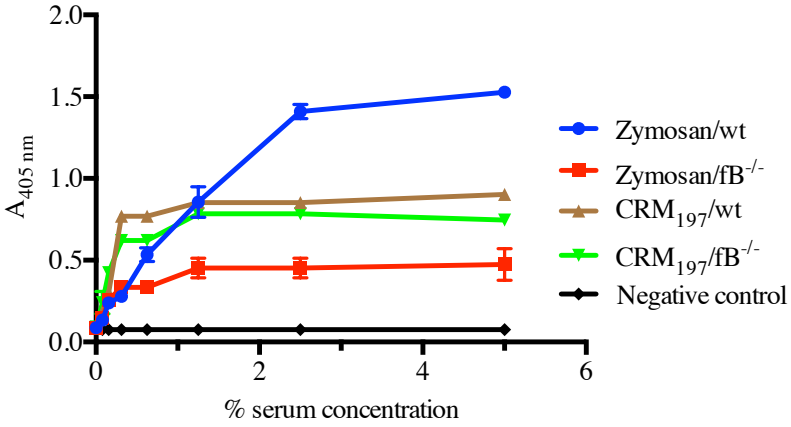

Figure 17: ELISA plate coated with 1µg of Zymosan (half plate) and another half of plate with 1µg of CRM_{197}. The blocking buffer was BSA-bovine serum albumin. Add 100µl of mouse serum (wt. - $fB^{-/-}$) the serial diluted of serum started with 1:20 in EGTA buffer. Add 100µl of α-human C3c complement produced in rabbit (1:5000) in washing buffer. Add 100µl of α-rabbit IgG ALK. Produced in goat (1:5000). Add 100µl of substrate.

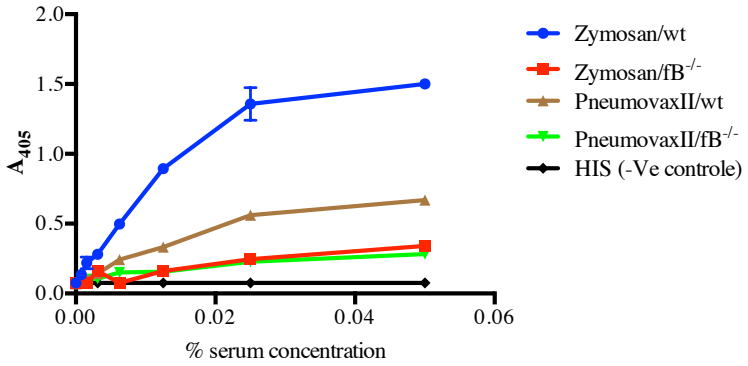

Figure 18: ELISA plate coated with 1µg of Zymosan (half plate) and another half of plate with 1µg of PneumovaxII vaccine. The blocking buffer was BSA- bovine serum albumin. Add 100µl of mouse serum (wt. - $fB^{-/-}$) the serial diluted of serum started with 1:20 in EGTA buffer. Add 100µl of α-human C3c complement produced in rabbit (1:5000) in washing buffer. Add 100µl of α-rabbit IgG ALK. Produced in goat (1:5000). Add 100µl of substrate.

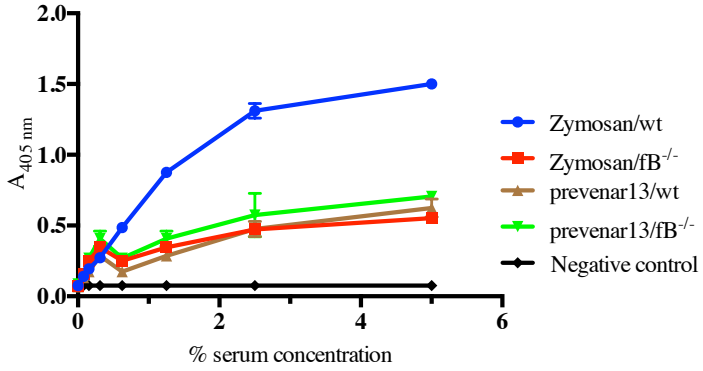

Figure 19: ELISA plate coated with 1µg of Zymosan (half plate) and another half of plate with 1µg of Prevenar13 vaccine. The blocking buffer was BSA- bovine serum albumin. Add 100µl of mouse serum (wt. - $fB^{-/-}$) the serial diluted of serum started with 1:20 in EGTA buffer. Add 100µl of α-human C3c complement produced in rabbit (1:5000) in washing buffer. Add 100µl of α-rabbit IgG ALK. Produced in goat (1:5000). Add 100µl of substrate.

4.2 MBL-A, MBL-C and CL-11 binding assays

The binding of LP recognition components to on PneumovaxII was determined using specific detection antibodies against the recognition subcomponents of the LP. The results shown in Figure 20 show that MBL-A, MBL-C and CL-11 have a high binding affinity towards pneumovaxII and neither MBL-a nor CL-11 bind with similarly high binding affinity to mannan.

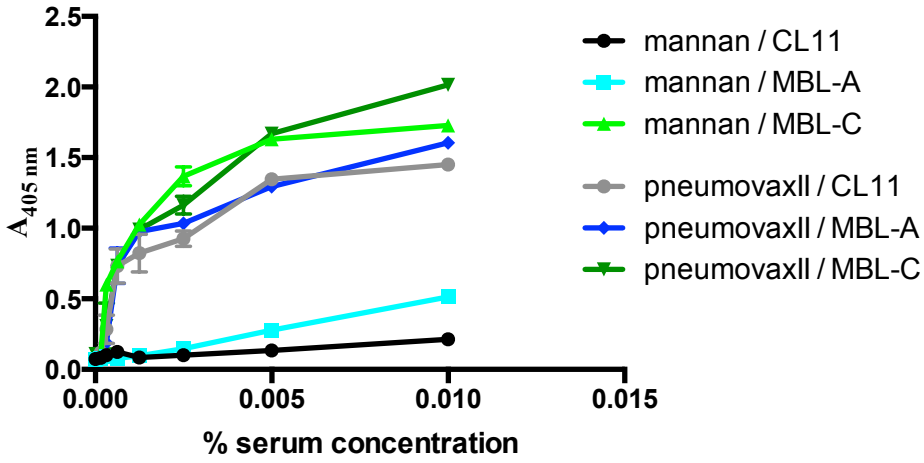

Figure 20: surface deposition of MBL-A, MBL-C and CL11 on PneumovaxII incubated with serial diluted mice sera (mannan coated used as a positive control).

4.3 CRM_{197} Protein

4.3.1 Introduction

CRM_{197} is a detoxified diphtheria toxin that has a substitution of glutamic acid for glycine at position 52 (*McNeela et al. 2004*). This mutation at position 52 results in the loss of ADP-ribosyltransferase activity, which is the main mechanism of toxin action *(Collier et al. 1997*). Indeed, some studies have shown that the structure of the diphtheria toxin is important in its immunological response *(Del Giudice and Rappuoli 1999*). Through inactivation of the protein toxin activity, CRM_{197} has been shown to exhibit moderate pro-inflammatory immunological activity *in-vivo* (*Dagan et al. 2010*). Intensive research into understanding the function and structure of CRM_{197} protein has promoted its use as a carrier protein for vaccines (*Pecetta et al. 2015*). However, there is clear evidence that suggests that carrier proteins could also suppress the immune response to conjugate vaccines. Interestingly, in the case of CRM_{197}, there is reduced carrier-induced suppression of the immune response compared to other conjugate proteins. Additionally, the linking of several capsular polysaccharides has been attributed to reduced immune suppression (*Pecetta et al. 2015*). Therefore, the current chapter investigates and assesses the potential of CRM_{197} to modulate the immune response, by enhancing or suppressing the immunogenic response. I investigated the interactions with components of either the three complement activation pathways to validate the results obtained in subsequent immunisation experiments. The CRM_{197} protein is widely used as a safe carrier for conjugate vaccines because of its ability to induce inflammatory response and its immune-stimulatory activity *(J Kimura et al. 2011*). Therefore, the experiments reported in this chapter are important because there are still questions regarding CRM_{197} interaction with the immune system. Moreover, the results presented here would confirm that there is a contribution provided by each complement pathway toward the efficacy of conjugate vaccines studies whereas the

absence of single complement pathway had little to no effect on the response to CRM_{197}.

4.3.2 Mice immunised at day1 and day 45

WT and complement deficient mice were immunised with 20µg of CRM_{197} protein at day 1 and boosted at day 45. Blood samples were collected from the saphenous vein prior to immunisation to determine the base line as a negative control. Blood was taken at days 7, 14, 21, 45 and the mice were culled and exsanguinated by cardiac puncture at day 52 (Figure 21).

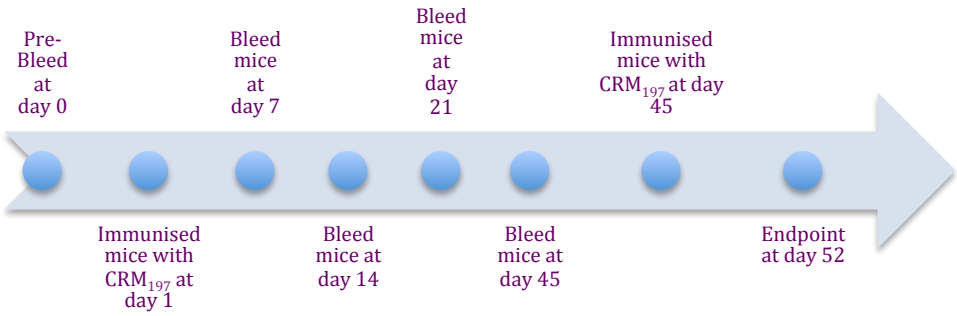

Figure 21: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with CRM_{197} protein at days 1 and 45.

4.3.2.1 MASP-$2^{-/-}$ mice immunised at day1 and boosterd at day 45

Measuring Ab titres in WT (as controls) and MASP-$2^{-/-}$ mice against CRM_{197} protein. Mice were immunised and sera collected as listed in Figure 21. The Ab titres increased in both WT and MASP-$2^{-/-}$ mice following immunisation (Figure 22), and no significant differences were

observed between MASP-2$^{-/-}$ and WT mice at time points after immunisation (Table 18) the Ab titres were increasing after each immunisation over time. CD's markers to characterise changes in the population of B and T cells were used (Figure 23).

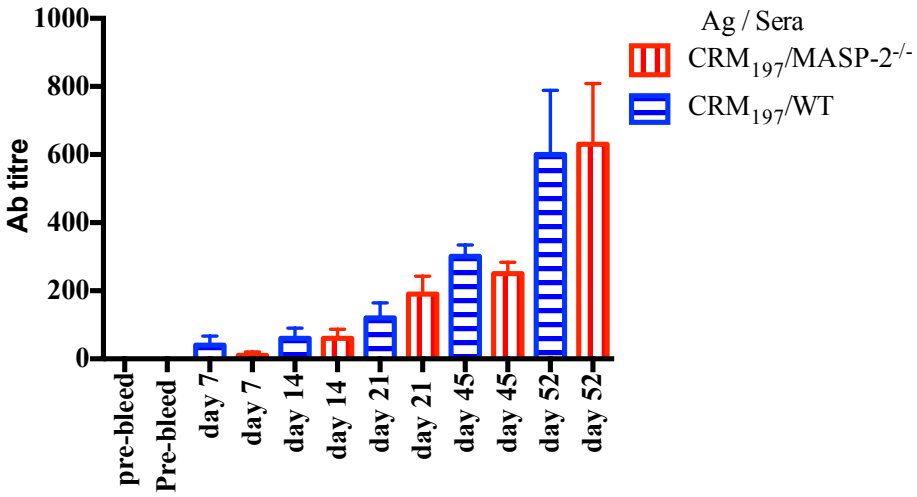

Figure 22: MASP-2$^{-/-}$ and WT control mice were immunised with CRM_{197} at day 1 and day 45 and serum collected at days shown at the x-axis

Table 18: Unpaired T-test for MASP-2$^{-/-}$ mice were immunised with CRM_{197} at days 1 and day 45,and t-test done between each point in the graph above. Unpaired t test with equal SD: ns: P value>0.05, *P value<0.05, **P value<0.005, ***P value<0.001,

CRM197	Wt/pre-bleed	MASP-2-/- / pre-bleed	Wt/ day 7	MASP-2-/- / day7	Wt/ day 14	MASP-2-/- / day 14	Wt/ day 21	MASP-2-/- / day 21	Wt/ day 45	MASP-2-/- / day 45	Wt/ day 52	MASP-2-/- / day52
Wt/pre-bleed	-	ns	ns	ns	ns	ns	**	**	**	**	***	***
MASP-2-/- / pre-bleed		-	ns	ns	ns	ns	**	**	**	**	***	***
Wt/day 7			-	ns	ns	ns	**	**	**	**	***	***
MASP-2-/- / day7				-	ns	ns	**	**	**	**	***	***
Wt/day14					-	ns	ns	ns	ns	ns	**	**
MASP-2-/- / day14						-	ns	ns	ns	ns	**	**
Wt/day21							-	ns	*	ns	*	*
MASP-2-/- / day21								-	ns	ns	*	*
Wt/day45									-	ns	*	*
MASP-2-/- / day45										-	ns	ns
Wt/day52											-	ns
MASP-2-/- / day52												-

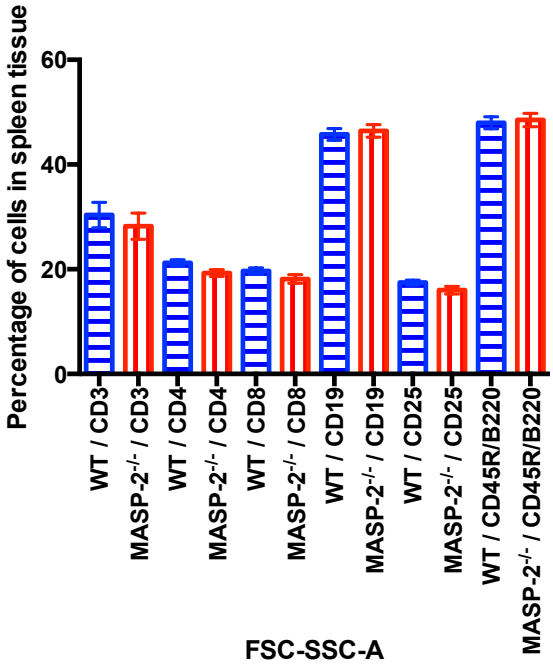

Figure 23: MASP-$2^{-/-}$ and WT control mice were immunised with CRM_{197} at day 1 and day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.2.2 $C1q^{-/-}$ mice immunised at day1 and day 45

C1q knockout and WT mice were immunised with 20µg of CRM_{197} protein as shown in Figure 21. The Ab titres were determined in blood samples taken on the days following immunisation as shown on the x-axis (Figure 24). Observing Table 19 to compare the differences between crossing WT and $C1q^{-/-}$, and identifies that there are no significant differences observed between $C1q^{-/-}$ and WT mice at time points after immunisation. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 25).

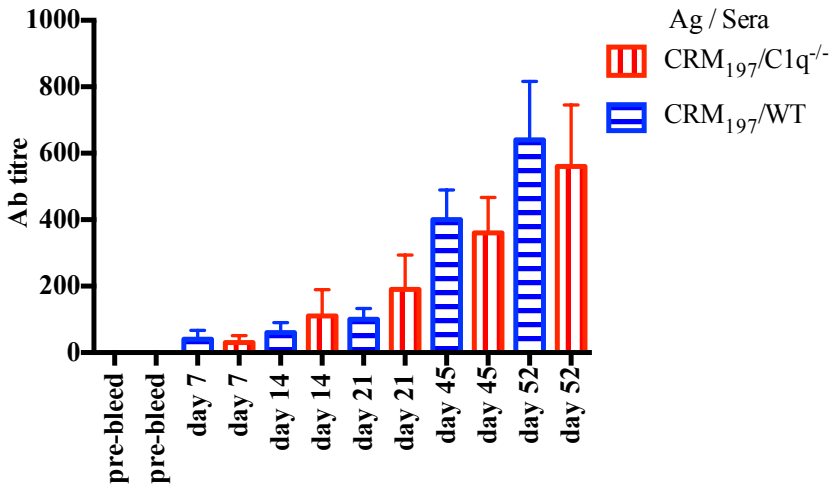

Figure 24: C1q$^{-/-}$ and WT control mice were immunised with CRM_{197} at day 1 and day 45 and serum collected at days shown at the x-axis

Table 19: Unpaired t test with equal SD for C1q$^{-/-}$ mice were immunised with CRM_{197} at days 1 and day 45,and t-test done between each point in the graph above (ns: P value>0.05, *P value<0.05, **P value<0.005, ***P value<0.001). , ****P value<0.0001,

CRM_{197}	Wt/ pre-bleed	C1q-/- / pre-bleed	Wt/ day 7	C1q-/- / day 7	Wt/ day 14	C1q-/- / day 14	Wt/ day 21	C1q-/- / day 21	Wt/ day 45	C1q-/- / day 45	Wt/ day 52	C1q-/- / day 52
Wt/pre-bleed	-	ns	ns	ns	ns	ns	**	**	**	**	***	***
C1q-/- / pre-bleed		-	ns	ns	ns	ns	**	**	**	**	***	***
Wt/day 7			-	ns	ns	ns	**	**	**	**	***	***
C1q-/- / day7				-	ns	ns	**	**	**	**	***	***
Wt/day14					-	ns	ns	ns	ns	ns	**	**
C1q-/- / day14						-	ns	ns	ns	ns	**	**
Wt/day21							-	ns	*	ns	*	*
C1q-/- / day21								-	ns	ns	*	*
Wt/day45									-	ns	*	ns
C1q-/- / day45										-	ns	ns
Wt/day52											-	ns
C1q-/- / day52												-

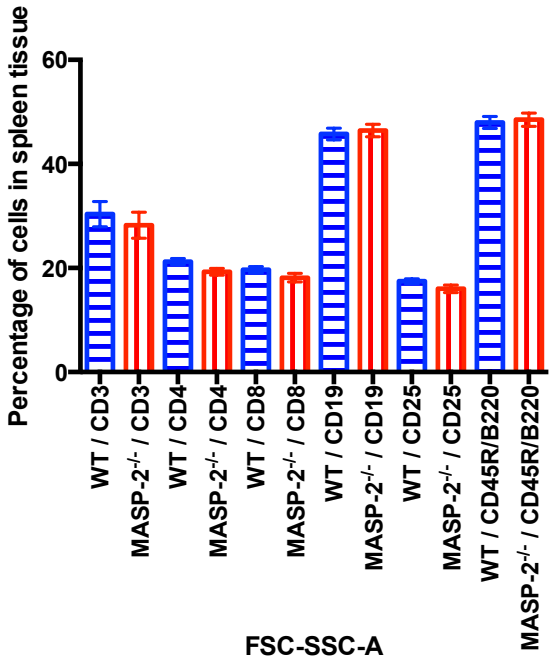

Figure 25: C1q$^{-/-}$ and WT control mice were immunised with CRM_{197} at day 1 and day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.2.3 Factor B$^{-/-}$ mice immunised at day1 and day 45

All of fB$^{-/-}$ mice were immunised and sera was collected as mention in Figure 21, and the Ab titre increased in both WT and fB$^{-/-}$ mice (Figure 26), and there are no significant differences between fB$^{-/-}$ and WT in the same days after each immunisation (Table 20). Following the immunisation, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 27).

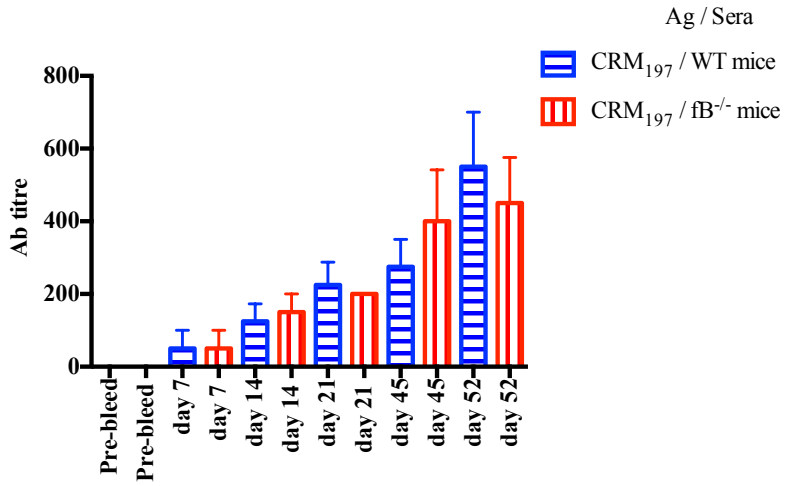

Figure 26: Factor $B^{-/-}$ and WT mice were immunised with CRM_{197} at day 1 and at day 45. Serum was collected at day 0, 7, 14, 21, 45 and day 52 as indicated on the x -axis, Serum was serially diluted in BBS and the antibody titres determined as described in Materials and Methods.

Table 20: Unpaired t test with equal SD for $fB^{-/-}$ and WT mice were immunised with CRM_{197} at day 1 and at day 45, serum was collected at day 0, 7, 14, 21, 45 and day 52 (ns: P value>0.05, *P value<0.05, **P value<0.005,),

CRM_{197}	Wt/ pre-bleed	fB-/- / pre-bleed	Wt/ day 7	fB-/- / day 7	Wt/ day 14	fB-/- / day 14	Wt/ day 21	fB-/- / day 21	Wt/ day 45	fB-/- / day 45	Wt/ day 52	fB-/- / day 52
Wt/pre-bleed	-	ns	ns	ns	*	*	*	*	**	**	**	**
fB-/- / pre-bleed		-	ns	ns	*	*	*	*	**	**	**	**
Wt/day 7			-	ns	ns	ns	ns	ns	**	**	**	*
fB-/- / day7				-	ns	ns	ns	ns	**	*	**	*
Wt/day14					-	ns	ns	ns	**	ns	**	*
fB-/- / day14						-	ns	ns	ns	*	*	*
Wt/day21							-	ns	ns	ns	*	ns
fB-/- / day21								-	ns	ns	*	ns
Wt/day45									-	ns	*	ns
fB-/- / day45										-	ns	ns
Wt/day52											-	ns
fB-/- / day52												-

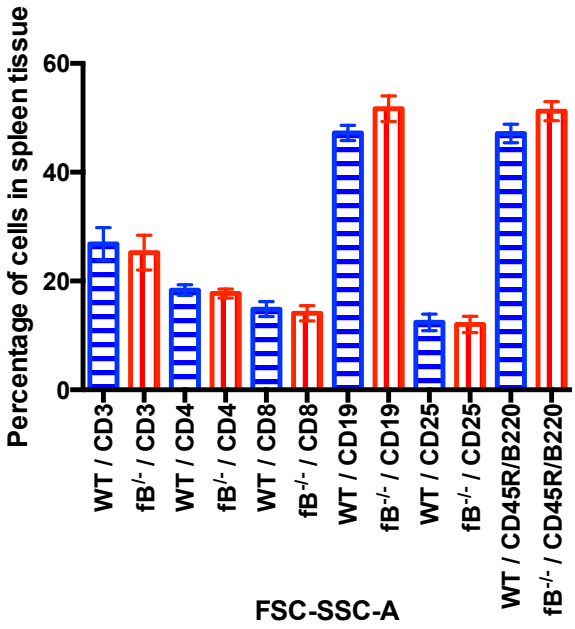

Figure 27: $fB^{-/-}$ and WT control mice were immunised with CRM_{197} at day 1 and day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.3 Mice immunised at day1, 8, 15 and day 22

The effects of multiple immunisations with 20µg of CRM_{197} protein on complement deficient mice (MASP-$2^{-/-}$ and $C1q^{-/-}$) as mention in (3.10) were blood samples collected prior to first immunisation as negative control. Then mice were immunised with 20 µg of CRM_{197} on day 1 and then booster on days 8, 15 and 22. Sera were collected at days 7, 14, 21, 28 and by cardiac puncture at day 45 (Figure 28).

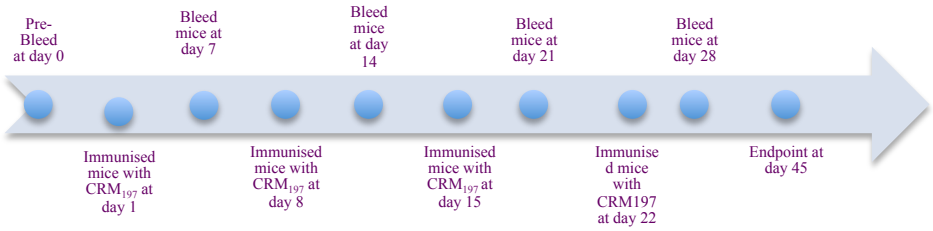

Figure 28: The immunisation schedule and the schedule to take blood samples following subcutaneous immunisation with CRM_{197} protein.

4.3.3.1 MASP-$2^{-/-}$ mice immunised at day1, 8, 15 and day 22

MASP-$2^{-/-}$ mice were subjected to multiple booster immunisations with 20µg of CRM_{197} protein on days 1, 8, 15 and day 22 using the s.c. application route. Blood samples were collected 1 day prior to each immunisation during the course of immunisations to determine the antibody titre against CRM_{197} (Figure 29). Blood samples were collected from each individual mouse before immunisation and used as negative controls. Then one day before each immunisation and at day 28 mice were bled from saphenous vein and at day 45 blood samples were taken by cardiac puncture (Figure 28). Table 21 shows that there is no significant differences observed between MASP-$2^{-/-}$ and WT mice at time points after each immunisation. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 30).

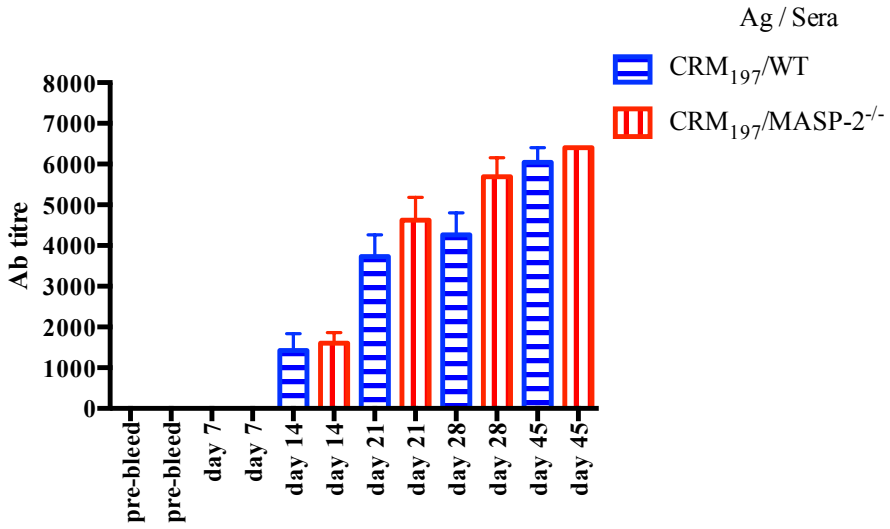

Figure 29: MASP-$2^{-/-}$ and WT mice were immunised with CRM_{197} at days 1, 8, 15 and 22, Serum was collected at time points listed on the x-axis, and serum serially diluted in BBS buffer to determine the antibody titres.

Table 21: Unpaired t test with equal SD for MASP-$2^{-/-}$ mice were immunised with CRM_{197} at days 1, 8, 15 and day 22, Serum was collected at time points listed in the table (ns: P value>0.05, *P value<0.05, **P value<0.005, ***P value<0.001),

CRM_{197}	Wt/pre-bleed	MASP-2-/- / pre-bleed	Wt/day 7	MASP-2-/- day 7	Wt/day14	MASP-2-/- day 14	Wt/day 21	MASP-2-/- day 21	Wt/day 28	MASP-2-/- day 28	Wt/day 45	MASP-2-/- day 45
Wt/pre-bleed	-	ns	ns	ns	*	***	***	***	***	***	***	***
MASP-2-/- / pre-bleed		-	ns	ns	*	***	***	***	***	***	***	***
Wt/day 7			-	ns	*	**	***	***	***	***	***	***
MASP-2-/- day7				-	*	**	***	***	***	***	***	***
Wt/day14					-	ns	*	**	**	***	***	***
MASP-2-/- day14						-	*	**	**	***	***	***
Wt/day21							-	ns	ns	*	**	**
MASP-2-/- day21								-	ns	ns	*	*
Wt/day28									-	ns	*	*
MASP-2-/- day28										-	ns	ns
Wt/day45											-	ns
MASP-2-/- day45												-

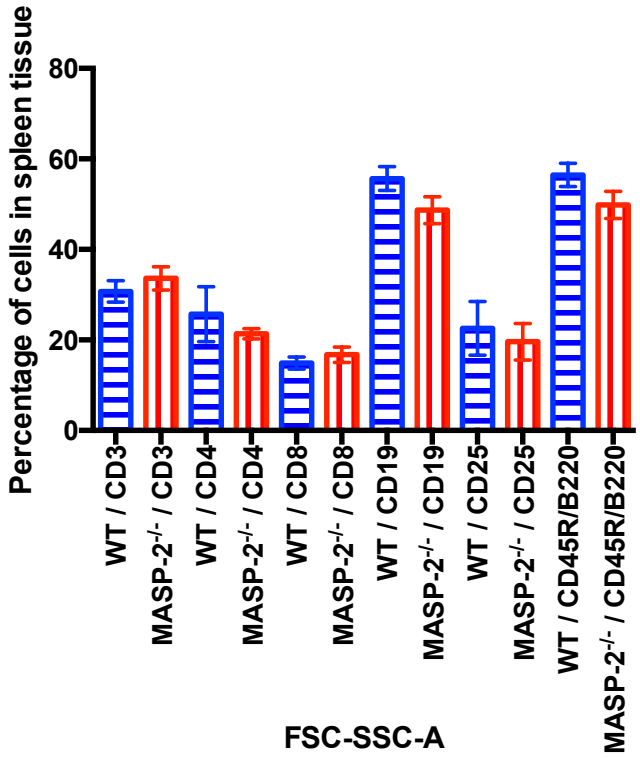

Figure 30: MASP-$2^{-/-}$ and WT control mice were immunised with CRM_{197} at days 1, 8, 15 and 22. Mice were culled at day 45 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.3.2 $C1q^{-/-}$ mice immunised at day1, 8, 15 and day 22

$C1q^{-/-}$ mice were challenged by multiple booster immunisations with 20µg of CRM_{197} protein at days 1, 8, 15 and day 22 using s.c. route. Blood samples were collected 1 day prior of each immunisation during the immunisation course to determine the antibody titre against CRM_{197} (Figure 31). Blood samples were collected from each individual mouse before immunisation course started and these samples used as negative control. Then one day before each immunisation and at days 28, bleeding mice from saphenous vein and at day 45 blood sample were taken by cardiac puncture (Figure 28). Observing Table 22 to compare the differences between crossing WT and $C1q^{-/-}$, there are no significant

differences observed between C1q$^{-/-}$ and WT mice at time points after immunisation. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 32).

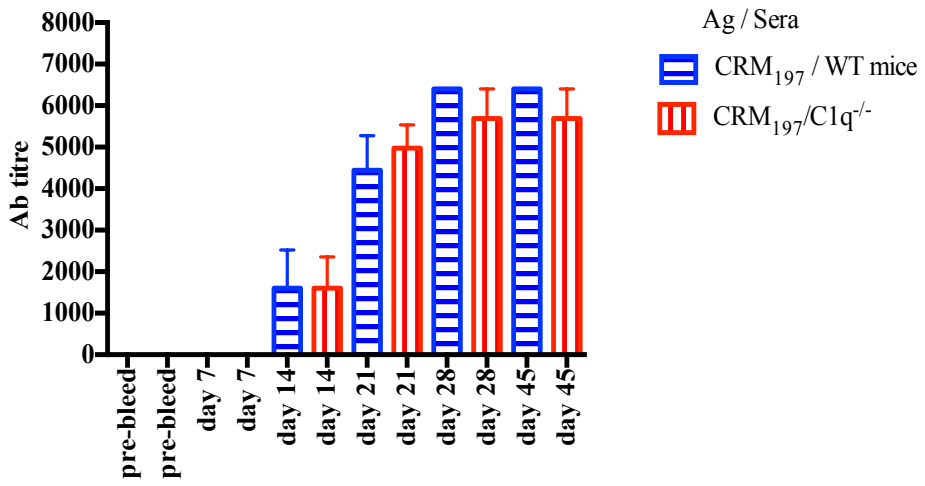

Figure 31: C1q$^{-/-}$ and WT control mice were immunised with CRM_{197} at days 1, 8, 15 and 22 and serum collected at days shown at the x-axis

Table 22: Unpaired t test with equal SD for C1q$^{-/-}$ mice were immunised with CRM_{197} at days 1, 8, 15 and day 22, Serum was collected at time points listed in the table (ns: P value>0.05, *P value<0.05, **P value<0.005, ***P value<0.001),

CRM_{197}	Wt/pre-bleed	C1q-/- / pre-bleed	Wt/day 7	C1q-/-/ day 7	Wt/day 14	C1q-/-/ day 14	Wt/day 21	C1q-/-/ day 21	Wt/day 28	C1q-/-/ day 28	Wt/day 45	C1q-/-/ day 45
Wt/pre-bleed	-	ns	ns	ns	ns	*	***	***	***	***	***	***
C1q-/- / pre-bleed		-	ns	ns	ns	*	***	***	***	***	***	***
Wt/day 7			-	ns	ns	ns	**	***	***	***	***	***
C1q-/-/ day 7				-	ns	ns	**	***	***	***	***	***

Wt/day 14					-	ns	*	*	***	**	***	**
C1q-/- day 14						-	*	*	***	**	***	**
Wt/day 21							-	ns	*	ns	*	ns
C1q-/- day 21								-	*	ns	*	ns
Wt/day 28									-	ns	ns	ns
C1q-/- day 28										-	ns	ns
Wt/day 45											-	ns
C1q-/- day 45												-

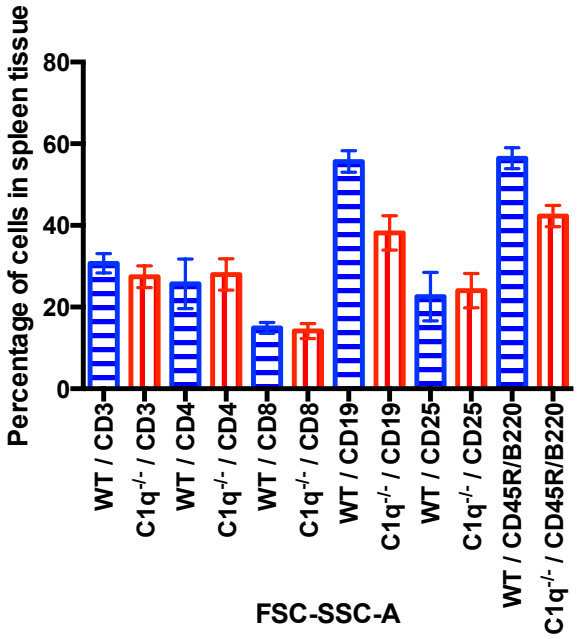

Figure 32: C1q$^{-/-}$ and WT control mice were immunised with CRM$_{197}$ at days 1, 8, 15 and 22. Mice were culled at day 45 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.3.3 Factor $B^{-/-}$ mice immunised at day1, 8, 15 and day 45

All of $fB^{-/-}$ mice were immunised multiple times with 20µg of CRM_{197} protein at days 1, 8, 15 and day 45 using i.p. route. Blood samples were collected 1 day prior to each immunisation and during the immunisation course to determine the antibody titre against CRM_{197} (Figure 34). Blood samples were collected from each individual mouse before immunisation course started and these samples were used as a negative control. Then 7 days after each immunisation and at on the 45th day before the mice were immunised, they were bled from saphenous vein and at day 52 blood sample was taken by cardiac puncture (Figure 33). In (Table 23), WT and $fB^{-/-}$ had the same the immune response for CRM_{197} protein comparing the differences between them at same days. Following the immunisation, at the end point, percentage of cells in spleen tissue of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 35).

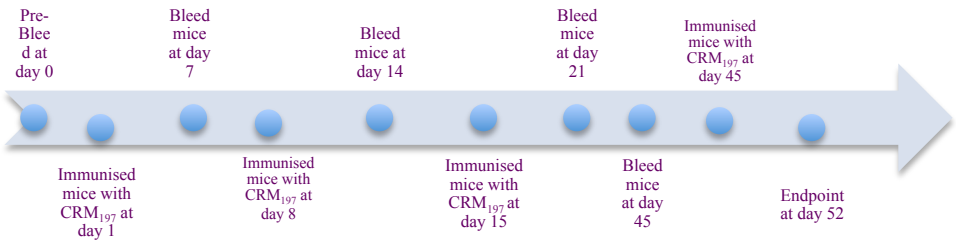

Figure 33: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with CRM_{197} protein

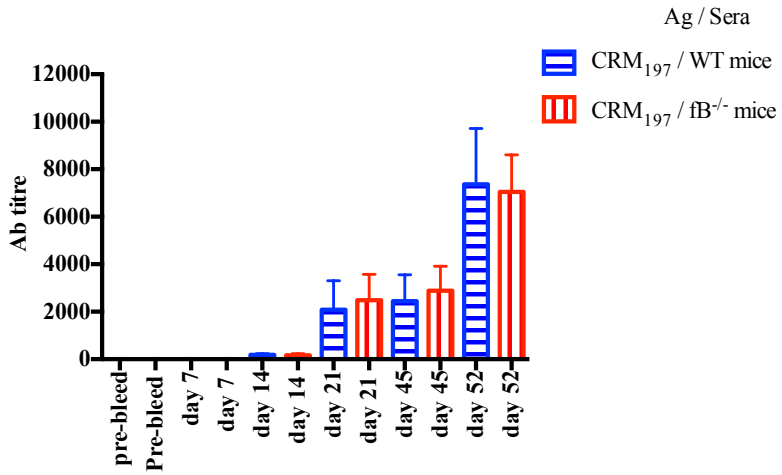

Figure 34:Factor $B^{-/-}$ and WT control mice were immunised with CRM_{197} at days 1, 8, 15 and 45 and serum collected at days shown at the x-axis.

Table 23: Unpaired t test with equal SD for $fB^{-/-}$ and WT mice were immunised with CRM_{197} at day 1, 8, 15 and day 45. Sera were collected at time points listed in the table (ns: P value>0.05, *P value<0.05, **P value<0.005, ***P value<0.001)

CRM_{197}	Wt/pre-bleed	fB-/- / pre-bleed	Wt/day 7	fB-/- / day 7	Wt/day 14	fB-/- / day 14	Wt/day 21	fB-/- / day 21	Wt/day 45	fB-/- / day 45	Wt/day 52	fB-/- / day 52
Wt/pre-bleed	-	ns	ns	ns	ns	ns	**	**	**	**	***	***
fB-/- / pre-bleed		-	ns	ns	ns	ns	**	**	**	**	***	***
Wt/day 7			-	ns	ns	ns	**	**	**	**	***	***
fB-/- / day7				-	ns	ns	**	**	**	**	***	***
Wt/day14					-	ns	*	*	*	*	**	**
fB-/- / day14						-	*	*	*	*	**	**
Wt/day21							-	ns	ns	ns	*	*
fB-/- / day21								-	ns	ns	*	*
Wt/day45									-	ns	ns	ns
fB-/- / day45										-	ns	ns
Wt/day52											-	ns
fB-/- / day52												-

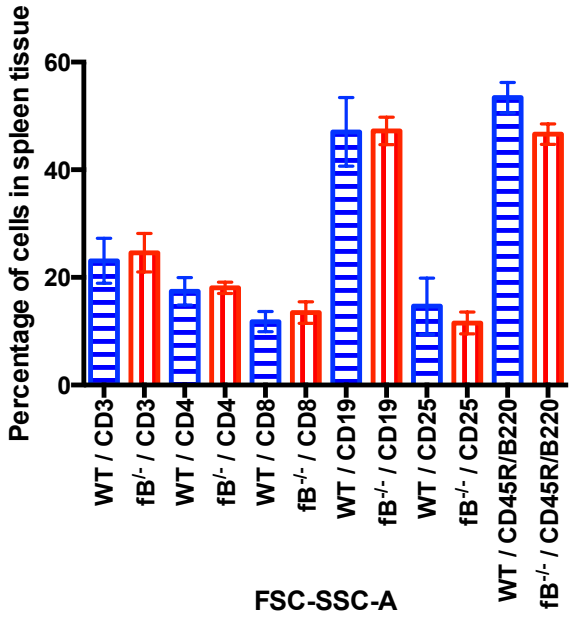

Figure 35: $fB^{-/-}$ and WT control mice were immunised with CRM_{197} at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.3.4 Discussion of the results following immnisation with CRM_{197}

The interaction between the CRM_{197} protein component of theconjugate vaccine with individual components of the complement system and the contribution of the activation pathways in the activity of conjugate vaccine was investigated. Therefore, the current chapter aimed to establish the specificity of the conjugate vaccines by exposing mice models with single deficiencies in all three complement components to free CRM_{197}. This chapter shows that the three activation pathways of complement system are not involved in the immunogenic activity of the carrier protein CRM_{197} used in conjugate vaccines. Deficiency in the lectin pathway in the MASP-$2^{-/-}$ mice was not associated with changes in immunogenic response. Specifically, the exposure to CRM_{197} was not associated with differential antibody production and infiltration of immune cells to the spleen. Similarly, deficiency in the classical pathway in the $C1q^{-/-}$ mice was not associated with changes in immunogenic response.

Furthermore, immunisation of MASP-2$^{-/-}$ mice with CRM_{197} at days 1 and 45 caused no significant differences in antibody production. However, immunisation of both wildtype and MASP-2$^{-/-}$ mice caused increased antibody production in a day dependent manner. Similarly, the immunisation with CRM_{197} failed to cause differences in immune cell infiltration of the spleen. The results suggest that MASP-2 plays no role in the CRM_{197} mediated response which is in agreement with the reported low immunogenic response to $CRAM_{197}$ compared to other carrier proteins (*McNeela et al. 2004*). Comparatively, immunisation of MASP-2$^{-/-}$ mice with CRM_{197} at days 1, 8, 15, and 22 caused no significant difference in antibody production. Interestingly, antibody production in MASP-2$^{-/-}$ mice increased progressively in each day following immunisation. These results also show that increasing the concentration of CRM_{197} failed to induce a dose dependent immune response through the lectin activation pathway, and confirms that the CRM_{197} protein component of the vaccine used does not interact with the lectin pathway but might be inducing an immunogenic response through other mechanisms (*McNeela et al. 2004*).

Similar to the results obtained following immunisation of MASP-2$^{-/-}$ mice, the C1q$^{-/-}$ mice were immunised with CRM_{197} showed no significant differences in antibody production compared to wildtype mice. Correspondingly, both C1q$^{-/-}$ and wildtype mice showed increased antibody production over the duration of the immunisation. Moreover, the numbers of immune cells in the spleen remained consistent between wildtype and C1q$^{-/-}$ mice following immunisation at days 1 and 45. The results obtained from days 1, 8, 15, and 22 immunisations were similar to the results from days 1 and 45 where antibody production between the C1q$^{-/-}$ and the wildtype mice were not different. Furthermore, the number of spleen infiltrating immune cells following immunisation of C1q$^{-/-}$ mice remained consistent. This result was expected because CRM_{197} elicited response has been shown to be less than immune response by other carrier proteins (*Del Giudice and Rappuoli 1999*). Finally, the immunisation of fB$^{-/-}$ mice at days 1 and 45 failed to cause

any significant differences in antibody production compared to the levels in the wildtype mice. As was found with MASP-2$^{-/-}$ and C1q$^{-/-}$ immunised mice the effect on migration of spleen immune cells following CRM_{197} exposure showed that CRM_{197} immunisation of fB$^{-/-}$ mice was unchanged. Immunisation of fB$^{-/-}$ caused a day dependent increase in antibody production in both fB$^{-/-}$ and wildtype mice. The immunisation of fB$^{-/-}$ mice with CRM_{197} at days 1, 8, 15, 45 did not cause any differences in the production of antibody, but CRM_{197} caused increased antibody production in a day dependent manner. Additionally, immunisation at days 1, 8, 15, 45 did not cause any significant changes in number of immune cells infiltrating the spleen in these mice.

Results from other studies suggests that CRM_{197} carrier protein may modulate the immunological response at high doses (*Del Giudice and Rappuoli 1999*), but the full mechanisms underlying this potential dose dependent effect are yet to be fully characterised. The current study shows that the concentration of CRM_{197} does not activate any of the complement pathways, as such excludes the complement activation pathways from the list of potential mechanisms of the dose dependent immunogenic response. Interestingly, the production of antibody increased as the days progressed. Indeed, the carrier dose-vaccine effects have been reported in infants following the administration of high concentration of CRM_{197} in a conjugate vaccine (*Burrage et al. 2002*). Although CRM_{197} contains a point mutation that alters the active domain that causes loss of toxicity and activity, the structural characteristics of CRM_{197} are preserved during the manufacturing process due to lack of detoxification process using formaldehyde (*Bröker et al. 2011*). Moreover, the reduction of peptone cross-linking observed in carrier proteins that undergo formaldehyde detoxification has been attributed to reduced immunogenic response. Indeed, unconjugated CRM_{197} has been shown to have reduced ability to bind NAD+, as such appears less immunogenic (*Pastan and FitzGerald 1991*). Therefore, the induction of the immune response at day dependent manner is reasonable. The fact that the number of vaccines utilising CRM_{197} carrier protein in childhood

and adult vaccination continues to increase equally makes the results presented in this chapter important. In many countries, children receive - 7 or 13 valent pneumococcal CRM_{197} conjugated vaccines. In general, 9-valent and 15-valent CRM_{197} conjugated vaccines could result in decreased, increased or no effect on immunogenic response. However, the results from the control experiments suggest that none of the three complement activation pathways can interact with the CRM_{197} component of these conjugate vaccines. Specifically, the reults show that immunogenic response to CRM_{197} protein was not affected by deficiency in the classical, lectin or alternative pathway, as MASP-$2^{-/-}$, $C1q^{-/-}$ and $fB^{-/-}$ mice, which did not exhibit any differential immunogenic response in comparison to the wildtype mice.

In general, the study expands the current knowledge on the in-vivo mechanisms of conjugate vaccine mediated immune response, and in particular shows that CRM_{197} protein does not interact with any of the complement pathway. It is well established that expression and production of the components of the three complement pathways are crucial in the complement activation in the innate immune response pathways, which acts through the polysaccharide component of conjugate vaccines. Though some immunogenic response elicited by CRM_{197} has been reported and the current study shows that a day dependent increase in antibody production may occur, the results obtained where the activation of complement system has been modified would be solely attributed to the effect of capsular polysaccharides on the complement pathways rather than any CRM_{197} mediated effect.

4.4 Pneumococcal polysaccharide vaccine (PneumovaxII)

4.4.1 Introduction

PneumovaxII is a polyvalent vaccine containing pneumococcal capsular polysaccharides that has proved immunogenic in humans *(Vella et al. 1992, Vliegenthart 2006*) and used in active immunisation for pneumococcal disease arising from 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F serotypes. PneumovaxII has been shown to induce immunologic memory and secretion of antibodies *(Jackson et al. 2007, Kroon et al. 2000, Musher et al. 2011*). The polysaccharides in the PneumovaxII stimulate the production of antibodies through the clonal expansion of B cells. The binding and cross-linking of the capsular polysaccharides to the B-cell receptor is central to this stimulation (*Bernasconi et al. 2002*). Unfortunately, the activity of polysaccharides cannot recruit CD4+ T cells through receptor recognition of peptide-major histocompatibility complex II, which in turn results in consistent level of antibody production between the initial exposure and multiple exposures (*Sami et al. 2007*). Moreover, the T cell independent activation of B cells in children makes them unresponsive to polysaccharide antigens and makes PneumovaxII ineffective in children under 2 years *(Marchese et al. 2006*). Clinical studies have reported a reduction in the carriage of pneumococci after vaccination (*Binks et al. 2015, Dagan et al. 1996*). In efficacy trials, PneumovaxII had almost 100% efficacy against pneumococcal infections (*Binks et al. 2015, Örtqvist et al. 1998*). More recently, animal studies have begun to shed light on the contribution of the dysfunctional immune response in the development of severe pneumococcal infection (*Glaser and Kiecolt-Glaser 2005, Kadioglu et al. 2000*). The results in this chapter present the Ab titre for mice were immunised with Pneumococcal Polysaccharide Vaccine (PneumovaxII).

4.4.2 Optimisation the dose and the route for PneumovaxII vaccine

To optimise the doses and the route of PneumovaxII vaccine experiments, 15 female WT mice were divided to 3 groups (Figure 37); group 1: WT mice were immunised with 1µg of PneumovaxII using an i.p. route. group 2: WT mice were immunised with 5µg of PneumovaxII using i.p. route. group 3: WT mice were immunised with 1µg of PneumovaxII using s.c. route. In all these three groups, the immunisation doses were prepared and done as described in (3.12). Blood samples were collected from each individual mouse one day prior to each immunisation during the immunisation course and the first samples were collected before the immunisation course start used as negative control. Then one day before each immunisation, bleeding mice from saphenous vein at days 7, 14 and at day 21 blood sample were taken by cardiac puncture (Figure 36). In (Table 24) comparing the differences between crossing the WT groups. In depend on this experiment, immunising mice with 1µg of PneumovaxII using i.p. route was chosen for the following experiments.

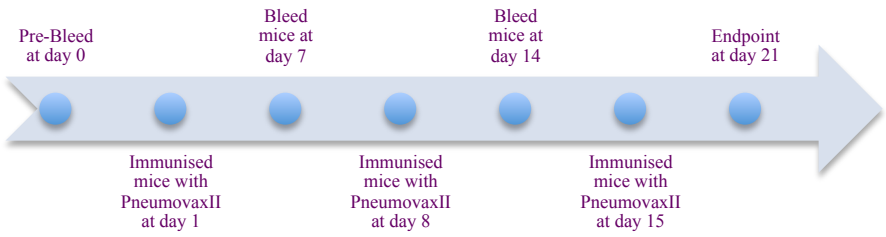

Figure 36: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with PneumovaxII vaccine to optimised the doses.

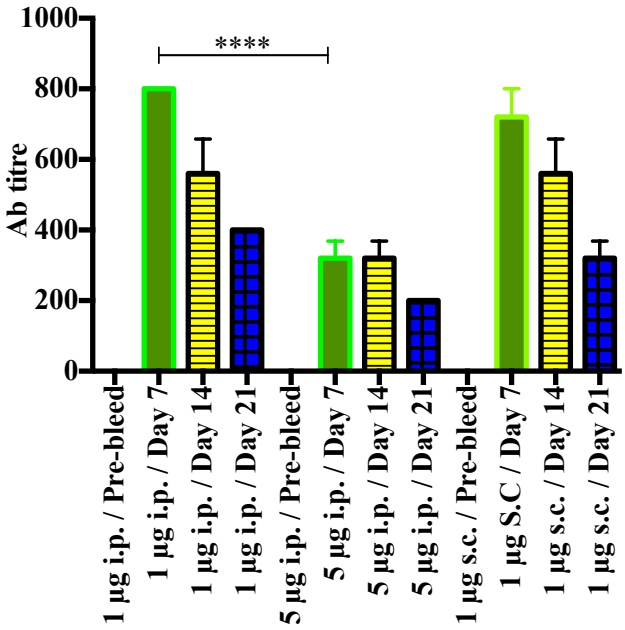

Figure 37: WT mice were immunised with immunised with PneumovaxII at days 1, 8, 15. The blood samples were collected on days 0, 7, 14, 21. Serum was serially diluted starting with 1:100. One-way ANOVA ****P value<0.0001, was used to compare the results obtained from mice were immunised with 1µg or 5µg of pneumovaxII vaccine to optimised the doses.

Table 24: Unpaired t test with equal SD for WT mice were immunised with 5 µg i.p. route or with 1µg of PneumovaxII using i.p. or s.c. routes (ns: P value>0.05, *: P value<0.05, **: P value<0.005),

WT mice	1µg i.p/ pre-bleed	1µg i.p/day 7	1µg i.p/day 14	1µg i.p/day 21	5µg i.p/ pre-bleed	5µg i.p/day 7	5µg i.p/day 14	5µg i.p/day 21	1µg s.c/pre-bleed	1µg s.c/day 7	1µg s.c/day 14	1µg s.c/day 21
1µg i.p/pre-bleed	-	**	**	**	ns	**	**	**	ns	**	**	**
1µg i.p/day 7		-	ns	**	**	**	**	**	**	ns	ns	**
1µg i.p/day 14			-	ns	**	ns	ns	**	**	ns	ns	ns
1µg i.p/day 21				-	**	ns	ns	**	**	*	ns	ns
5µg i.p/pre-bleed					-	**	**	**	ns	**	**	**
5µg i.p/day 7						-	ns	ns	**	*	ns	ns
5µg i.p/day 14							-	ns	**	*	ns	ns
5µg i.p/day								-	**	**	**	ns

21												
1µg s.c/pre-bleed									-	**	**	**
1µg s.c/day 7										-	ns	*
1µg s.c/day 14											-	ns
1µg s.c/day 21												-

4.4.3 Mice were immunised with PneumovaxII vaccine at day1 and day 45

WT and complement deficiency mice were immunised with 1µg of PneumovaxII at day 1 and boosted at day 45. Blood samples were collected from saphenous vein in the prior of experiments as a negative control. Then, blood samples were collected at days 7, 14, 21, 45 and by cardiac puncture at day 52 (Figure 38).

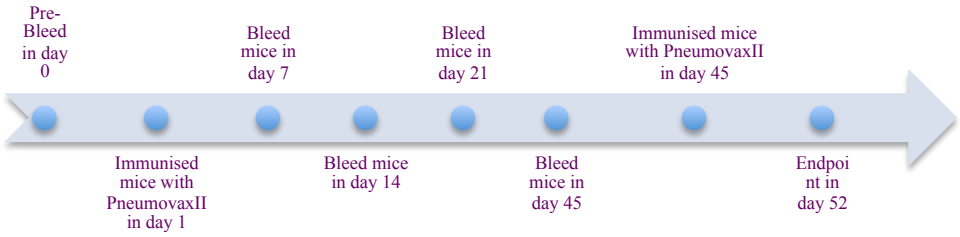

Figure 38: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with PneumovaxII vaccine.

4.4.3.1 MASP-2$^{-/-}$ mice were immunised with PneumovaxII vaccine at day 1 and day 45

All of MASP-2$^{-/-}$ mice were immunised with 1µg of PneumovaxII vaccine at days 1 and the vaccine boosted at day 45 using i.p. route. Blood samples were collected from each individual mouse 1 day prior to

immunisation course started and these samples used as negative control. Then, bleeding mice from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against PneumovaxII (Figure 39) In (Table 25), shows no significant differences were observed between MASP-2$^{-/-}$ and WT mice at time points after immunisation. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 40).

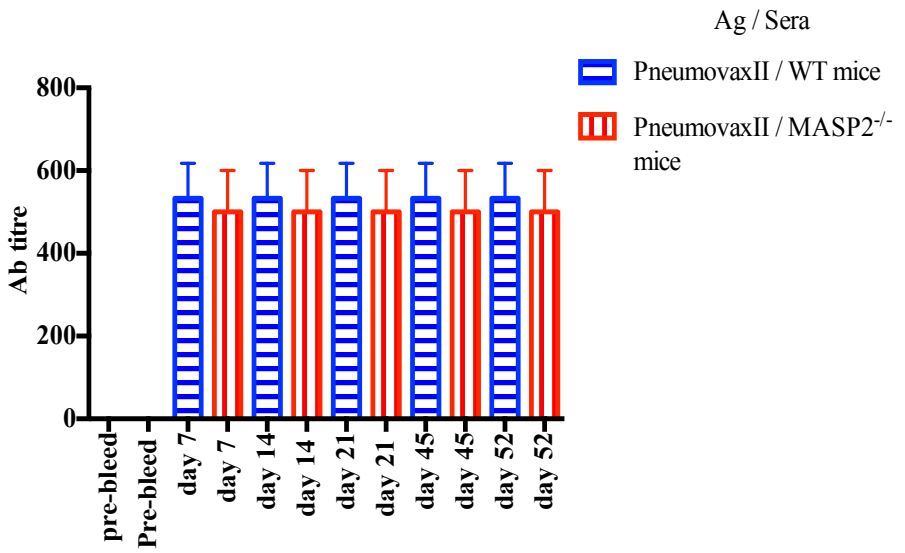

Figure 39: MASP-2$^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1 and 45 and serum collected at days shown at the x-axis

Table 25: Unpaired t test with equal SD for MASP-2$^{-/-}$ mice were immunised with 1µg of PneumovaxII at days 1 and day 45. Serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001),

Pneumovax II	Wt/pre-bleed	MASP-2$^{-/-}$ / pre-bleed	Wt/day 7	MASP-2$^{-/-}$ / day 7	Wt/day 14	MASP-2$^{-/-}$ / day 14	Wt/day 21	MASP-2$^{-/-}$ / day 21	Wt/day 45	MASP-2$^{-/-}$ / day 45	Wt/day 52	MASP-2$^{-/-}$ / day 52
Wt/pre-bleed	-	ns	***	***	***	***	***	***	***	***	***	***
MASP-2^{-}		-	***	***	***	***	***	***	***	***	***	***

$^{-/-}$ / pre-bleed												
Wt/day 7			-	ns	ns	ns	ns	ns	ns	ns	ns	ns
MASP-2$^{-/-}$ day7				-	ns	ns	ns	ns	ns	ns	ns	ns
Wt/day 14					-	ns	ns	ns	ns	ns	ns	ns
MASP-2$^{-/-}$ day 14						-	ns	ns	ns	ns	ns	ns
Wt/day 21							-	ns	ns	ns	ns	ns
MASP-2$^{-/-}$ day 21								-	ns	ns	ns	ns
Wt/day 45									-	ns	ns	ns
MASP-2$^{-/-}$ day 45										-	ns	ns
Wt/day 52											-	ns
MASP-2$^{-/-}$ day 52												-

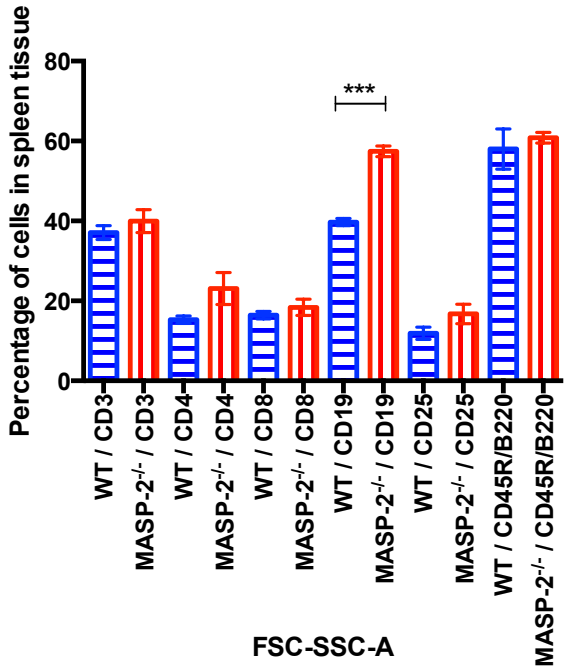

Figure 40: MASP-2$^{-/-}$ and WT control mice were immunised with PneumovaxII at day 1 and at day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis. Unpaired t test with equal SD (***: P value<0.001).

4.4.3.2 C1q$^{-/-}$ mice were immunised with PneumovaxII vaccine at day 1 and day 45

C1q$^{-/-}$ mice immunised with1µg of PneumovaxII vaccine at days 1 and the vaccine boosted at day 45 using i.p. route. Blood samples were collected from each individual mouse one-day prior to immunisation course started and these samples used as negative control. Then, bleeding mice from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against PneumovaxII (Figure 41). Observing Table 26 to compare the differences between crossing WT and C1q$^{-/-}$, there are clearly significant differences observed between C1q$^{-/-}$ and WT mice at time points after immunisation, which are show the C1q$^{-/-}$ mice are higher than WT mice. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 42).

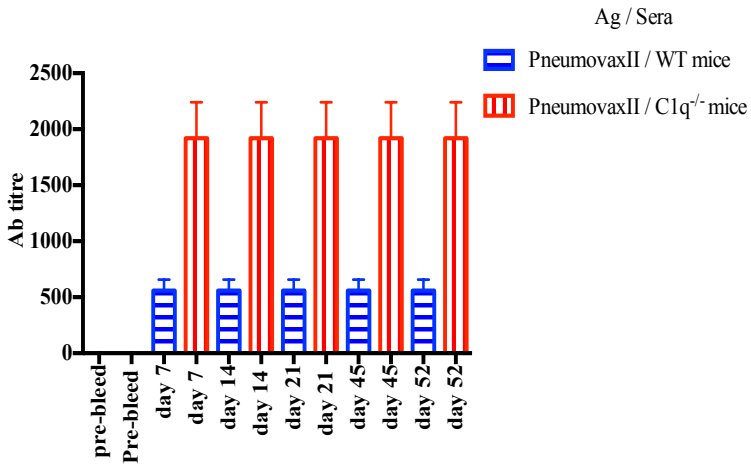

Figure 41: C1q$^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1 and 45 and serum collected at days shown at the x-axis

Table 26: Unpaired t test with equal SD for C1q$^{-/-}$ mice were immunised with 1µg of PneumovaxII at day 1 and day 45. Serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001 , ****: P value<0.0001),

pneumovaxII	Wt/ pre-bleed	C1q-/- / pre-bleed	Wt/ day 7	C1q-/- / day 7	Wt/ day 14	C1q-/- / day 14	Wt/ day 21	C1q-/- / day 21	Wt/ day 45	C1q-/- / day 45	Wt/ day 52	C1q-/- / day 52
Wt/pre-bleed	-	ns	***	****	***	****	***	****	***	****	***	****
C1q-/- / pre-bleed		-	***	****	***	****	***	****	***	****	***	****
Wt/day 7			-	**	ns	**	ns	**	ns	**	ns	**
C1q-/- / day7				-	**	ns	**	ns	**	ns	**	ns
Wt/day14					-	**	ns	**	ns	**	ns	**
C1q-/- / day14						-	**	ns	**	ns	**	ns
Wt/day21							-	**	ns	**	ns	**
C1q-/- / day21								-	**	ns	**	ns
Wt/day45									-	**	ns	**
C1q-/- / day45										-	**	ns
Wt/day52											-	**
C1q-/- / day52												-

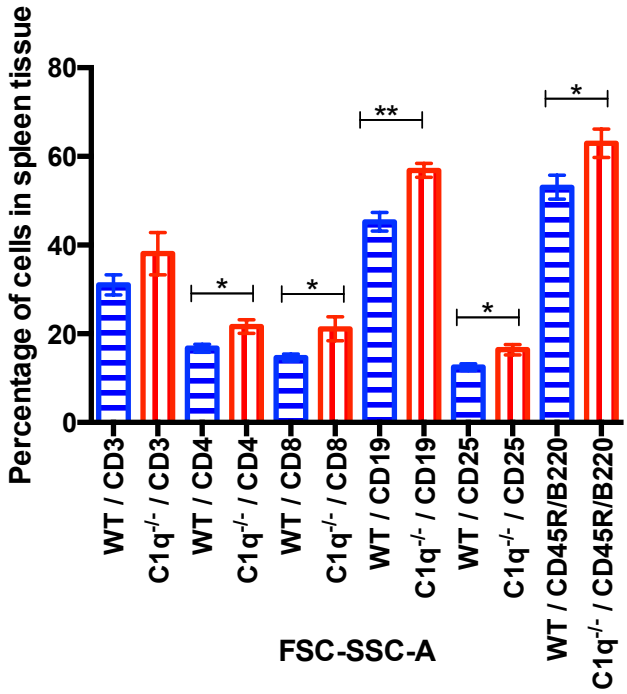

Figure 42: C1q$^{-/-}$ and WT control mice were immunised with PneumovaxII at day 1 and at day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis. Unpaired t test with equal SD (ns: P value>0.05, *: P value<0.05, **: P value<0.005).

4.4.3.3 Factor $B^{-/-}$ mice were immunised with PneumovaxII vaccine at day 1 and day 45

$fB^{-/-}$ mice immunised with1µg of PneumovaxII vaccine at days 1 and the vaccine boosted at day 45 using i.p. route. Blood samples were collected from each individual mouse 1 day prior to the immunisation course started and these samples used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample was taken by cardiac puncture to determine the antibody titre against PneumovaxII (Figure 43) In (Table 27), shows that WT mice had higher immune response than $fB^{-/-}$ comparing the differences crossing WT and $fB^{-/-}$. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 44).

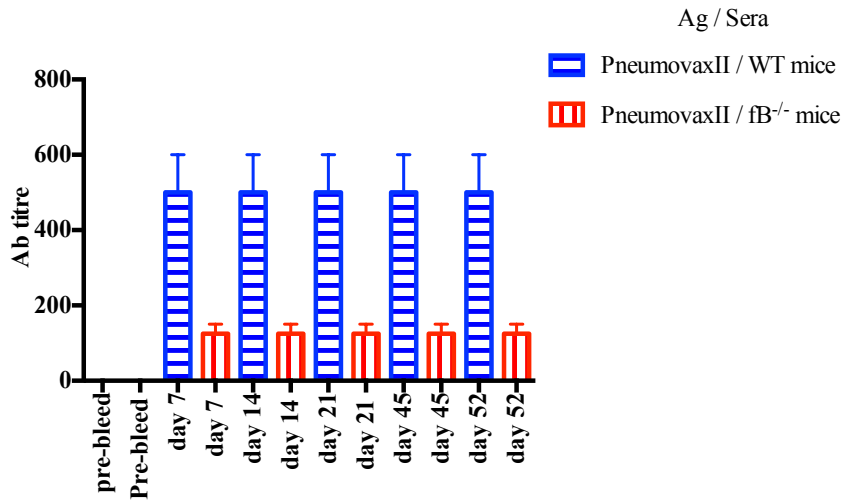

Figure 43: Factor $B^{-/-}$ and WT mice were immunised with pneumococcal polysaccharide vaccine at day 1 and at day 45. Serum collected at days shown at the x-axis

Table 27: Unpaired t test with equal SD for $fB^{-/-}$ mice were immunised with Pneumococcal Polysaccharide Vaccine (and WT mice as a control) at day 1 and at day 45. . Serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001),

Pneumovaxll	Wt/pre-bleed	fB-/- / pre-bleed	Wt/ day 7	fB-/- / day 7	Wt/ day14	fB-/- / day14	Wt/ day21	fB-/- / day 21	Wt/ day45	fB-/- / day 45	Wt/ day52	fB-/- / day 52
Wt/pre-bleed	-	ns	***	**	***	**	***	**	***	**	***	**
fB-/- / pre-bleed		-	***	**	***	**	***	**	***	**	***	**
Wt/day 7			-	*	ns	*	ns	*	ns	*	ns	*
fB-/- / day7				-	*	ns	*	ns	*	ns	*	ns
Wt/day14					-	*	ns	*	ns	*	ns	*
fB-/- / day14						-	*	ns	*	ns	*	ns
Wt/day21							-	*	ns	*	ns	*
fB-/- / day21								-	*	ns	*	ns
Wt/day45									-	*	ns	*
fB-/- / day45										-	*	ns
Wt/day52											-	*
fB-/- / day52												-

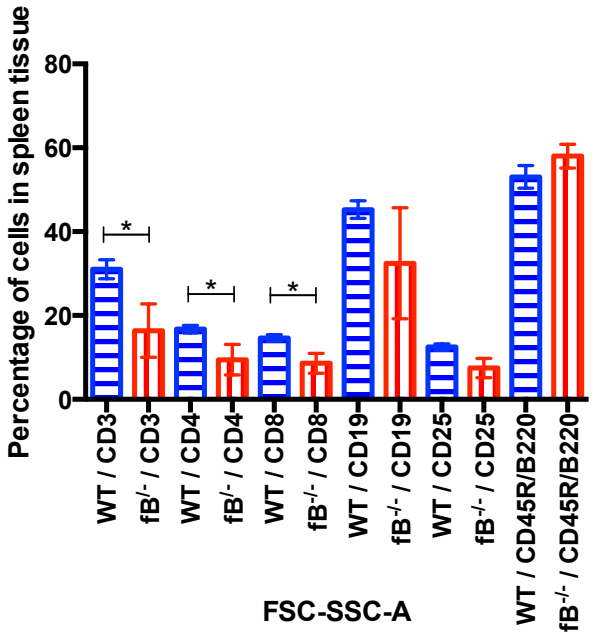

Figure 44: $fB^{-/-}$ and WT control mice were immunised with PneumovaxII at day 1 and at day 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.4.4 mice were immunised with PneumovaxII vaccine at day1, 8, 15 and day 45

WT and complement deficiency mice were immunised with 1 µg of PneumovaxII at days 1, 8, 15 and at day 45. Blood samples were collected from saphenous vein in the prior of experiments as a negative control. Then at days 7, 14, 21, 45 and by cardiac puncture at day 52 (Figure 45).

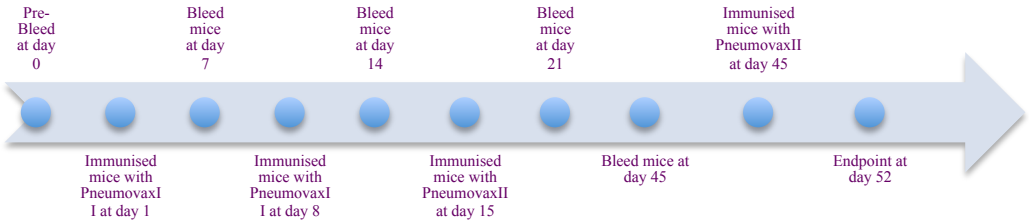

Figure 45: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with PneumovaxII vaccine.

4.4.4.1 MASP-2$^{-/-}$ mice were immunised with PneumovaxII vaccine in at day1, 8, 15 and day 45

MASP-2$^{-/-}$ mice immunised with1µg of PneumovaxII vaccine at days 1, 8, 15 and at day 45 using i.p. route (Figure 45). Blood samples were collected from each individual mouse 1 day prior to the immunisation course started and these samples were used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against PneumovaxII (Figure 46). Observing Table 28 to compare the differences between crossing WT and MASP-2$^{-/-}$, there are clearly significant differences observed between MASP-2$^{-/-}$ and WT mice at and

after day 21 when the immune response in WT mice are dropped down. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flow cytometry analysis (Figure 47).

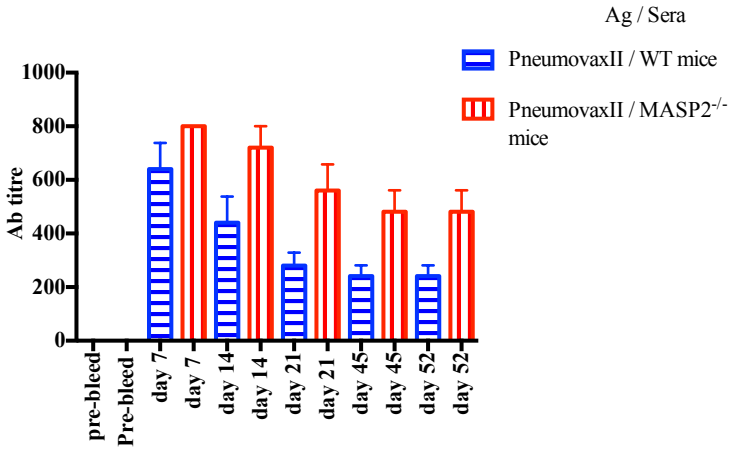

Figure 46: MASP-2$^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1, 8, 15 and at day 45. Serum was collected at days shown at the x-axis

Table 28: Unpaired t test with equal SD for MASP-2$^{-/-}$ mice were immunised with 1µg of PneumovaxII vaccine at days 1, 8, 15 and 45. Sera collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001),

Pneumovax II	Wt/ pre-bleed	MASP-2$^{-/-}$ / pre-bleed	Wt/ day 7	MASP-2$^{-/-}$ / day 7	Wt/ day14	MASP-2$^{-/-}$ / day 14	Wt/ day21	MASP-2$^{-/-}$ / day 21	Wt/ day45	MASP-2$^{-/-}$ / day 45	Wt/ day52	MASP-2$^{-/-}$ / day 52
Wt/pre-bleed	-	ns	***	***	***	***	***	***	***	***	***	***
MASP-2$^{-/-}$ / pre-bleed		-	***	***	***	***	***	***	***	***	***	***
Wt/day 7			-	ns	ns	ns	*	ns	**	ns	**	ns
MASP-2$^{-/-}$ / day7				-	**	ns	***	*	***	**	***	**
Wt/day14					-	ns	ns	ns	ns	ns	ns	ns
MASP-2$^{-/-}$ / day14						-	**	ns	***	ns	***	ns
Wt/day21							-	*	ns	*	ns	*
MASP-2$^{-/-}$ / day21								-	*	ns	*	ns
Wt/day45									-	*	ns	*
MASP-2$^{-/-}$ / day45										-	*	ns
Wt/day52											-	*
MASP-2$^{-/-}$ / day52												-

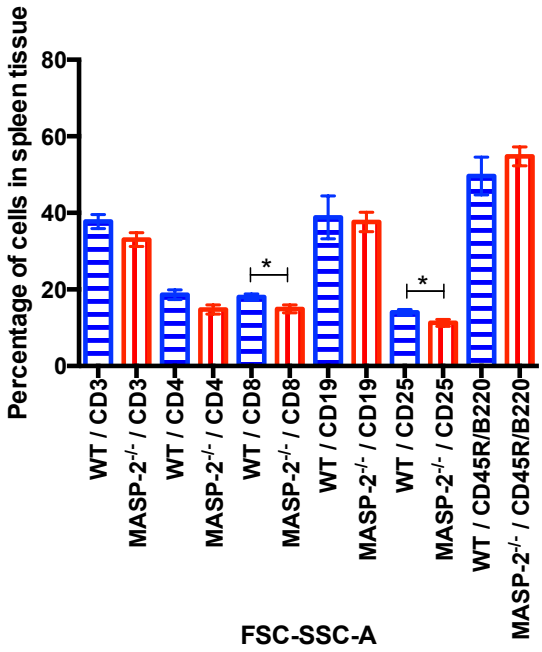

Figure 47: MASP-$2^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.4.4.2 $C1q^{-/-}$ mice were immunised with PneumovaxII vaccine at day 1, 8, 15 and day 45

$C1q^{-/-}$ mice immunised with1µg of PneumovaxII vaccine at days 1, 8, 15 and at day 45 using i.p. route (Figure 45). Blood samples were collected from each individual mouse one day prior to the immunisation and these samples were used as background controls. To determine the antibody titre against PneumovaxII blood was taken from the saphenous vein at days 7, 14, 21, 45 and at day 52, mice were killed and exsanguinated by cardiac puncture. (Figure 48). (Table 29) shows that $C1q^{-/-}$ mice had higher immune response than WT mice. Following the immunisation, at

the end point of the experiments, sub-populations of lymphocytes infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 49).

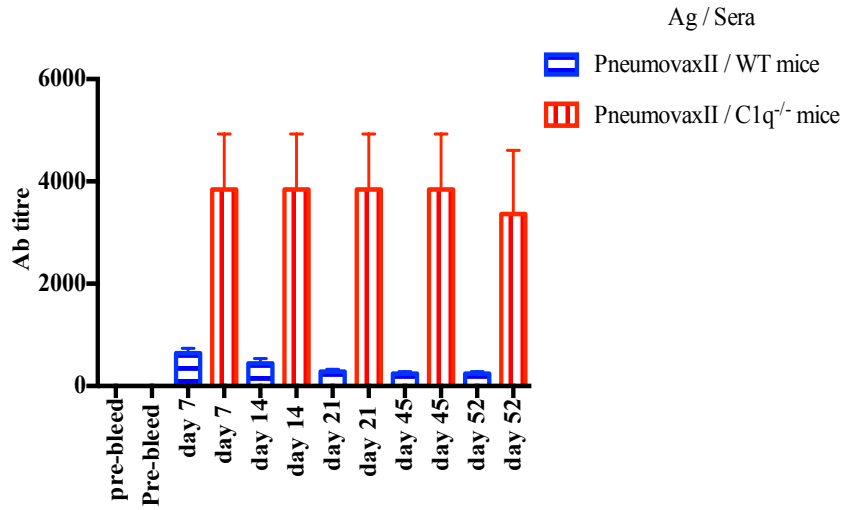

Figure 48: Antibody titres against PneumovaxII were determined after immunisation at days 1, 8, 15 and day 45. The high titres achieved in the C1q deficient mice compare to the C1q sufficient WT control mice.

Table 29: Unpaired t test with equal SD for $C1q^{-/-}$ mice were immunised with 1µg of PneumovaxII vaccine at days 1, 8, 15 and day 45. Sera collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005),

Pneumovax II	Wt/pre-bleed	$C1q^{-/-}$ / pre-bleed	Wt/ day 7	$C1q^{-/-}$ / day7	Wt/ day14	$C1q^{-/-}$ / day14	Wt/ day21	$C1q^{-/-}$ / day21	Wt/ day45	$C1q^{-/-}$ / day45	Wt/ day52	$C1q^{-/-}$ / day52
Wt/pre-bleed	-	ns	***	***	***	***	***	***	***	***	***	***
$C1q^{-/-}$ / pre-bleed		-	***	***	***	***	***	***	***	***	***	***
Wt/day 7			-	*	ns	*	*	*	**	**	**	**
$C1q^{-/-}$ / day7				-	*	ns	*	ns	**	ns	**	ns
Wt/day14					-	*	ns	*	ns	*	ns	*
$C1q^{-/-}$ / day14						-	*	ns	**	ns	**	ns
Wt/day21							-	*	ns	**	ns	**
$C1q^{-/-}$ / day21								-	**	ns	**	ns
Wt/day45									-	**	ns	**
$C1q^{-/-}$ / day45										-	**	ns
Wt/day52											-	**
$C1q^{-/-}$ / day52												-

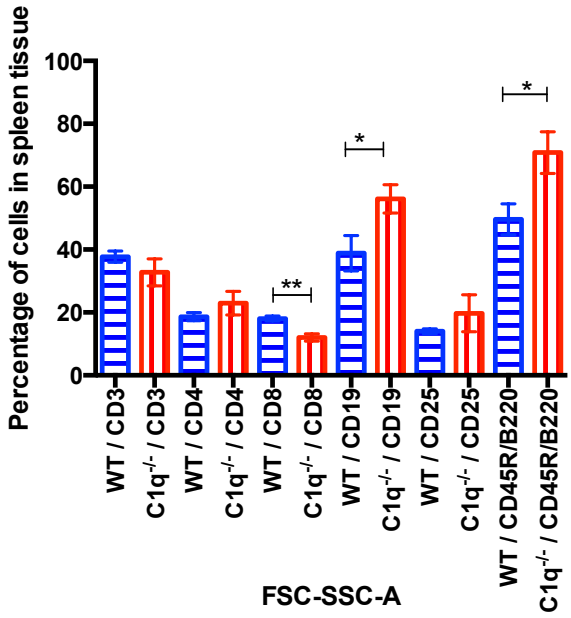

Figure 49: C1q$^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.4.4.3 Factor B$^{-/-}$ mice were immunised with PneumovaxII vaccine in at day1, 8, 15 and day 45

fB$^{-/-}$ mice immunised with1µg of PneumovaxII vaccine at days 1, 8, 15 and at day 45 using i.p. route (Figure 45). Blood samples were collected from each individual mouse one-day prior to the immunisation course was started and these samples were used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against PneumovaxII (Figure 50). Table 30 shows that there were significant differences at early stage, which is on at days 7 and 14, then there were no differencess at late stage. Following the immunisation, at the end point of the experiments, sub-populations of lymphocytes

infiltrating the spleen were assessed using monoclonal antibodies and flowcytometry analysis (Figure 51).

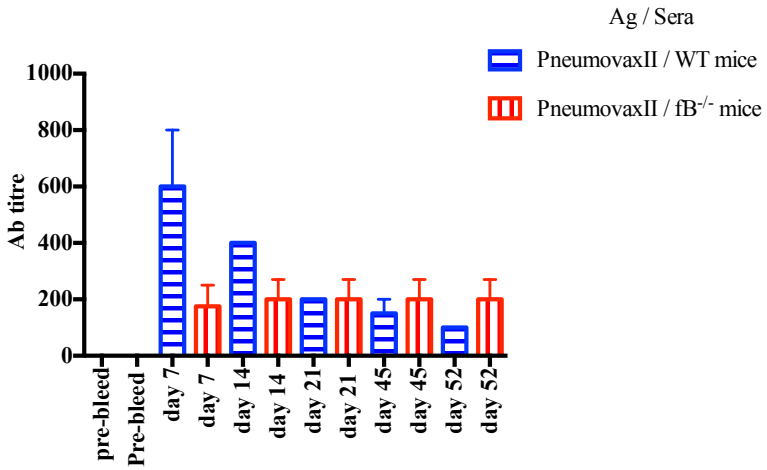

Figure 50: Factor $B^{-/-}$ mice were immunised with pneumococcal polysaccharide vaccine (WT mice used as a control) at day 1, 8, 15 and at day 45. Serum was collected at day 0, 7, 14, 21, 45 and day 52. Ab titres were determined as described.

Table 30: Unpaired t test with equal SD for $fB^{-/-}$ mice were immunised with 1µg of PneumovaxII vaccine at days 1, 8, 15 and day 45. Sera collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005),

pneumovaxII	Wt/ pre-bleed	$fB^{-/-}$ / pre-bleed	Wt/ day 7	$fB^{-/-}$ / day 7	Wt/ day 14	$fB^{-/-}$ / day 14	Wt/ day 21	$fB^{-/-}$ / day 21	Wt/ day 45	$fB^{-/-}$ / day45	Wt/ day 52	$fB^{-/-}$ / day 52
Wt/pre-bleed	-	ns	***	*	***	*	***	*	***	*	***	*
$fB^{-/-}$ / pre-bleed		-	***	*	***	*	***	*	***	*	***	*
Wt/day 7			-	**	ns	**	*	*	**	*	**	**
$fB^{-/-}$ / day7				-	*	ns	*	ns	ns	ns	ns	ns
Wt/day14					-	*	ns	*	ns	*	ns	*
$fB^{-/-}$ / day14						-	ns	ns	ns	ns	ns	ns
Wt/day21							-	ns	ns	ns	ns	ns
$fB^{-/-}$ / day21								-	ns	ns	ns	ns
Wt/day45									-	ns	ns	ns
$fB^{-/-}$ / day45										-	ns	ns
Wt/day52											-	ns
$fB^{-/-}$ / day52												-

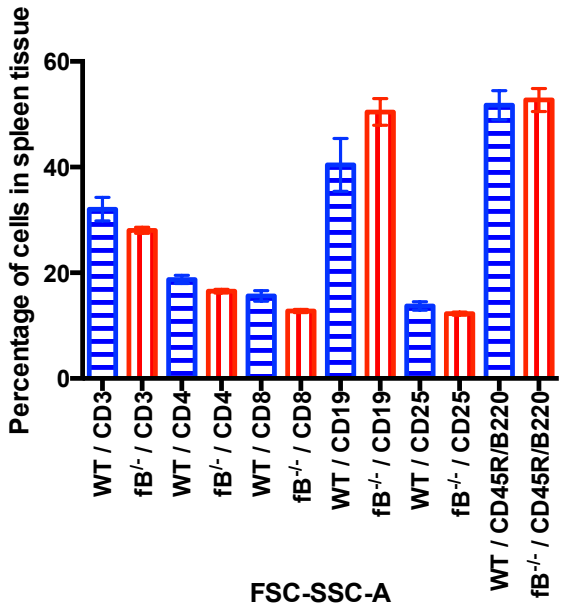

Figure 51: $fB^{-/-}$ and WT control mice were immunised with PneumovaxII at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

4.4.5 Discussion of the results following immnisation with PneumovaxII

Complement activation is an integral part of the protection against pneumococcal infection, as mice deficient in different components of the complement system present with increased susceptibility and severity of pneumococcal disease (*Alper et al. 1970, Ross and Densen 1984*). Indeed, infection with pneumococci has been shown to elicit a defined complement activation profile *in-vitro* and many *in-vivo* experiments have shown that complement activation is critical for an adequate host defence *(Ali et al. 2012, Bruyn et al. 1992, Winkelstein 1981*). Mechanistically, glycoproteins derived from pneumococci can activate the classical pathway through the interaction with the C1q molecule (*Stephens et al. 1977*). Similarly, carbohydrates on the structural proteins of pneumococci bind to MBL to activate the lectin pathway (*Bouwman* et *al. 2006*). Independently, pneumococci can also activate the alternative pathway through mechanisms that remain poorly understood. The contribution of individual components of the complement system and their activation pathways to the development of PneumovaxII mediated immunity remains elusive. Here employed mouse models with targeted deficiencies in each of the three complement pathways to investigate their role in PneumovaxII vaccine triggered immunity.

Initial experiment to determine the optimal dose and route of administration showed that 1µg elicited a more potent immunogenic response compared to the responses to a higher dose, here 5µg. PneumovaxII was expected to induce dose dependent response, the results however suggest that high doses may induce anergy which reduces the titres through a negative feedback mechanism. The activation of negative feedback mechanisms is an intrinsic feature of the immune system to prevent overshooting immune responses. The route of administration did not affect the immunogenic response observed. Though results from other studies suggested that immunogenic response

to vaccines could be modulated by route of administration, the findings from this study suggests that the two common administration routes used in PneumovaxII immunisation are equally effective in mediating an immunogenic response. Subsequently experiments used PneumovaxII at concentration of 1µg. My work shows that different activation pathways of the complement system play distinct roles in the induction of immunity by PneumovaxII to protect against pneumococcal infection. Deficiency in MASP-2 caused defective lectin pathway activity in the MASP-$2^{-/-}$ mice used in this study, but this did not affect the extent of vaccine-induced immunogenicity. Specifically, no significant differences were observed between wild type mice and MASP-$2^{-/-}$ mice. This suggests that the lectin pathway is not required for PneumovaxII induced immune response and that blocking the lectin pathway therapeutically does nor affect the efficacy of vaccination. Surprisingly, a deficiency of the classical complement activation pathway in $C1q^{-/-}$ mice resulted in increased immunological response to PneumovaxII vaccination. This points to a negative control of immunogenic response by C1q, which an observation that merits further investigation. Interestingly, the deficiency in the alternative complement activation as seen in $fB^{-/-}$ mice resulted in lower antibody titres o PneumovaxII. This result is in line with previous reports showing that the alternative pathway plays a supporting role in eliciting good antibody titres following vaccination.

The alternative complement pathway is phylogenetically the oldest activation pathway of the complement system (*Zarkadis et al. 2001*). Moreover, the activation of the alternative pathway is tightly regulated by the complement regulators factor H and I (*de Córdoba et al. 2004*). While no complete fB and fD deficiency has been reported, partial dysfunction in fB and fD has been reported previously (*Biesma et al. 2001, Newman et al. 1978, Rougier et al. 1998*), associated with recurrent infection. Hence, the results confirm the importance of alternative pathway in vaccine mediated immunity and suggests the importance of determining approaches to immunise patients with impaired activation of the alternative complement pathway. In contrast to

the specific interactions between proteins and carbohydrates that mark the activation of the classical and lectin pathway, the alternative pathway relies on the C3 tickover to function (*Bexborn et al. 2008*). However, the divergent immunogenic response seen in MASP-2$^{-/-}$ and C1q$^{-/-}$ mice suggests that these pathways are not involved in the immune response to PneumovaxII. In light of the supportive roles of the lectin pathway and the classical pathway in facilitating the development of adaptive immunity, I would have expected that MASP-2-/- and C1q-/- mice show reduced antibody titres in response to PneumovaxII .

Considering that the results showed that dysfunction in the classical pathway was associated with significantly higher antibody titre response, I speculate that perhaps C1q binds directly to PneumovaxII and reduces the availability of the antigen structures presented to trigger an antibody response. However, further research is required to establish the regulatory mechanism contributing to high responsiveness of C1q deficient mice to this vaccine.

Conversely, the interaction of antibodies with their respective antigens elicits the classical pathway. In the case of C1q component, the exposure of C1q binding site in the multi-molecular C1 complex results in the binding of this component to the Fc regions of immunoglobulins (*Ghebrehiwet et al. 1996*). The classical pathway may be playing a negative regulatory role in PneumovaxII mediated immunogenic response (*Sjöberg et al. 2009*). The finding that deficiency in the classical activation pathway resulted in increased immune titre response correlates with some of the described molecular mechanisms that underpins the activation this pathway. In line with this hypothesis, C1q$^{-/-}$ mice showed increased antibody secretion, with a corresponding increased in CD4, CD8, CD19, CD25, CD45R/B220 cells in the spleen. First, this result could be attributed to the effect of classical pathway deficiency on the levels of different IgG subclasses that are known to elicit divergent effects on the infiltration and activity of immune cells (*Griggs and Zinkewich-Peotti 2009*). Indeed, IgG2 and IgG3 can activate

the classical pathway, but IgG4 has been shown to lack complement activation capabilities (*Griggs and Zinkewich-Peotti 2009*). Therefore, C1q mediated control of the levels of these IgG could contribute towards the immunity conferred by PneumovaxII. Studies on the function of IgG1 has shown that it suppresses antibody response to infection (*Hebell et al. 1991*). C1q is required for classical complement activation by IgG1, IgG2 and IgG3, and $C1q^{-/-}$ mice was previously reported to have dysfunctional antibody response to infections (*Boes 2000*). Moreover, C1q has been shown to inhibit T cell activation and proliferation through surface gC1q receptor expressed on T-cells (*Kittlesen et al. 2000*). This receptor is equally expressed by B-cells, where it could be playing important role in tolerance (*Waggoner et al. 2007*). The inhibitory effect of C1q is thought to be a function of self-tolerance to antigens, which could explain the different effect in $C1q^{-/-}$ mice compared to the $fB^{-/-}$ mice. Furthermore, C1q play important role in clearing apoptotic bodies (*Taylor et al. 2000*). Indeed, if these apoptotic body are not cleared they may elicit immunogenic response leading to autoantibody production (*Baumann et al. 2002, Munoz et al. 2005*). This protective function hypothesis corresponds to the increased antibody secretion observed in this study following PneumovaxII injection. It is equally thought that C1q may work through immature B cells to maintain the threshold for antigen specific B cells selection (*Carroll 2004*). Therefore, the PneumovaxII induced immunity could be controlled through the negative regulatory effect of C1q and positively controlled by fB mediated activation of alternative complement pathway.

MASP-2 deficiency had no effect on the number of immune cells in the spleen, except for the number of CD19 positive spleen cells which were increased. This result supports the finding that in these mice, antibody production remained unaltered to that seen in WT control mice. Expression of CD19 mainly serves in a signalling process linking the innate immunity to the adaptive immunity (*Fearon and Locksley 1996*). Therefore, this increased number of CD19 cells would suggests enhanced B-cell antigen receptor signalling which in turn is critical for effective

vaccine mediated immunity, but the lack of any significant differences in antibody titres and majority of other immune cells measured, suggest that the lectin activation pathway does not play part in this response following PneumovaxII treatment. Moreover, the lectin pathway in context with vaccine mediated immunity might be minor, since also the levels of other immune cells, including CD3, CD4, CD25, CD45r/B220 positive cells was not different between the WT controls and the MASP-2 knockout mice. Combined, the results indicate that MASP-2 and corresponding lectin pathway is not involved in PneumovaxII mediated immunity.

Though the results showed that deficiency in the lectin activation pathway did not affect the immune response to PneumovaxII to induce immunogenic response, the results suggests that compensatory mechanisms initiated by other complement pathways that could remedy the deficiency in lectin pathway might be playing a role. Particularly, the increased response seen could be because of the activity of mannan-binding lectin function through other compensatory mechanisms. Indeed, all the mannan-binding lectins has been shown to recognise glucans, lipophosphoglycans and glycoinositol that contain sugar molecules in their terminal hexose (*Gadjeva et al. 2001*).

Furthermore, MASP-$2^{-/-}$ mice immunised at day 1, 8, 15 and 45 showed increased antibody production at late stages of immunisation. Multiple exposure of MASP-$2^{-/-}$ mice caused increased anti-body production by day 21, suggesting increased exposure could induce the production of antibody through other complement pathway. Interestingly, the results from the multiple exposure was contrary to the result obtained in the immunisation at day 1 and day 45 that caused no change in antibody production. This suggests that increased regime results in the activation of other complement pathway. As such deficiency in lectin pathway could be compensated by increasing the immunisation regime for PneumovaxII. Indeed, the results suggest that increasing the days of immunisation could activate other pathways. Interestingly, $C1q^{-/-}$ mice

immunised for days 1, 8, 15 and 45 resulted in increased antibody production. The multiple exposure of $C1q^{-/-}$ mice resulted in increased antibody production, which was similar to the antibody production seen in $C1q^{-/-}$ mice immunised at day 1 and 45. Hence, the results further confirm that the function of C1q in the classical pathway is majorly a negative regulatory role. Conversely, immunisation of $fB^{-/-}$ at days 1, 8, 15 and 45 decreased antibody production at days 7 and 14 that was similar to reduced antibody production following exposure of $fB^{-/-}$ mice at only day 1 and 45. Though the result of double exposure was similar to the results observed below day 21, by day 45 and 52 the antibody production has normalised to similar levels to the wildtype mice. Therefore, the impairment in immunogenic response in mice deficient in alternative pathway can be reduced by other pathways by increasing the dose.

In general, the study expands the current knowledge on the in-vivo mechanisms of PneumovaxII mediated immune response, and in particular suggests that the activation of the alternative complement pathway could be playing significant role. Previous studies have shown that the presence of the three complement activation pathways is crucial in the innate immune response and that acute pneumococcal infection elicits the gene expression of complement pathways *(Ali et al. 2012, Bruyn et al. 1992 and Hyman et al. 1975*). However, there are no reports assessing the induction of complement gene expression following vaccination with PneumovaxII. In the current study we found that deficiency in the activation of the alternative pathway of the complement system reduced immunogenic response, suggesting that PneumovaxII could be acting by up-regulating the expression of the molecules associated with this pathway. Though, the expression of fB following this PneumovaxII injection was not measured, the findings are analogous to other studies that have shown that fB is expressed following injection of lipopolysaccharides in mice (*Finck et al. 1998*). The mechanisms of regulation and activation of the alternative pathway during vaccine-induced immunity against pneumococcal infection are not fully

understood. It is possible that the vaccine active components may activate non-specific alternative pathway. Therefore, further experiments into the links between complement pathway and other aspect of vaccine immunity is needed. Exploring the biological consequences of the PneumovaxII mediated activation of alternative pathway in the defence against pneumococcal disease would shed some light on the potential treatment of this infection in people with classical and lectin pathway deficiencies. Moreover, pneumococcal capsule can hinder opsonophagocytosis by inhibiting both the classical and alternative complement pathways (*Hyams et al. 2010*). Several mechanisms involved in the evasion of the complement system has been described, including the Psp A inhibition of opsonisation through the reduction of C3 deposition (*Poolman 2004*). Similarly, the activity of the alternative pathway could be affected by Psp C pneumococcal surface protein C, which binds to factor H to inhibit crucial proteolytic process (*Poolman 2004*). Therefore, the current findings allows us to make the conclusion that the fB activation contribute to the PneumovaxII induced immunogenic response, but the role of the MASP-2 and C1q components in the activation and control of the alternative complement pathway might be minimal. Adequate function of both the innate and adaptive immune response is crucial in the defence against pneumococcal infection as mice models with engineered deficiencies in the function of classical, lectin and alternative pathway are susceptible to pneumococcal infections (*Ochsenbein and Zinkernagel 2000, Paterson and Mitchell 2006, Schmitt et al. 2015*).

Regardless, a fully functional fB and the alternative pathway is clearly important for PneumovaxII induced immunogenic response and subsequent protection against pneumococcal infection given that antibody and spleen infiltration by T-cells are important for protection against infection. It is interesting to consider that though MASP-2 and C1q deficiencies has been reported in humans, complete fB deficiency is yet to be reported. Therefore, the finding that alternative complement pathway is central to PneumovaxII protection suggests that it could be

effective in the treatment of pneumococcal infections in patients with dysfunctional classical and lectin complement pathways. Heterozygous deficiencies of classical and lectin pathway are common (*Garred et al. 2003*), and have been linked with increased risk of pneumococcal infection. In summary, the results presented in this chapter demonstrate that fB is crucial in the immunogenic response to PneumovaxII. The injection of this vaccine in complement deficient mice showed that antibody release and immune cell infiltration were attenuated in alternative pathway deficient mice. This pathway supports the concept that PneumovaxII confers immunity through alternative pathway dependent complement activation. However, although deficiencies in the lectin pathway and classical pathway activation has neutral and positive impact on antibody titres, respectively, spleen infiltration with CD3, CD4, CD8, CD19, CD 25 and CD45/B220 was reduced in MASP-$2^{-/-}$ and $C1q^{-/-}$ mice. Our results suggest that alternative and classical pathways may contribute differently in the immune response against PneumovaxII.

4.5 Pneumococcal polysaccharide conjugate vaccine (Prevenar13)

4.5.1 Introduction

Prevenar13 is a conjugate vaccine containing pneumococcal capsular polysaccharides and the carrier protein CRM_{197} that improves immunogenicity, used as an adjuvant in active immunisation of adults above 50 years of age and children between the ages of 6 weeks and 17 years (*Jackson et al. 2013*). Prevenar13 is mainly used for protection against invasive pneumococcal disease arising from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. In humans as well as in animal models, exposure to Prevenar13 elicits immunogenic response with corresponding release of anti-vaccine antibodies *(Gruber et al. 2012, Sucher et al. 2011*). The capsular polysaccharides conjugated to the CRM_{197} can independently stimulate the production of antibodies through the clonal expansion of B cells. Mechanistically, pneumococcal capsular polysaccharides bind to active receptors on the surface of B cells to induce antibody production (*Bernasconi et al. 2002*). However, the CRM_{197} carrier protein has been shown to elicit immunogenic response through mechanisms that remains to be fully understood (*Pecetta et al. 2015, Pecetta et al. 2016, Peeters et al. 1991*). Indeed, initial experiments using different component of polysaccharide conjugated vaccine at two different doses showed that all components of this conjugate vaccine can elicit immunogenic response. The effect on each component was shown to increase in a dose dependent manner. Interestingly, whole conjugate vaccine causes significant increase in serum antibody concentration compared to the corresponding concentration of either capsular polysaccharides or CRM_{197} carrier protein. This result suggests that CRM_{197} protein might be promoting immunogenic response to pneumococcal polysaccharides through mechanisms that are independent of complement activation, (see 4.3). Therefore, the results discussed in this chapter are based on the premise

that CRM_{197} can independently induce the activation of adaptive immune response.

4.5.2 Optimisation of the dose of Prevenar13 vaccine

ELISA technique was used to optimise the doses for the Prevenar13 vaccine experiments, WT mice were divided into two groups (Figure 52); the first group of WT mice was immunised with 20µl of Prevenar13 using i.p. route. The second group of WT mice was immunised with 150µl of Prevenar13 using the i.p. route (weekly, over three weeks) for all these groups, blood samples were collected from each individual mouse at day 21 by cardiac puncture. My experiments aimed to study the different immune response to all single Prevenar13 components (i.e. CRM_{197}, Pneumococcal Polysaccharides and whole Prevenar13 vaccine) in WT mice and complement deficiency mice. From this experiment 20µl of Prevenar13 vaccine was choosing to immunise complement deficient mice because that with 20µl of Prevenar13 vaccine immunising mice was the immune response was good.

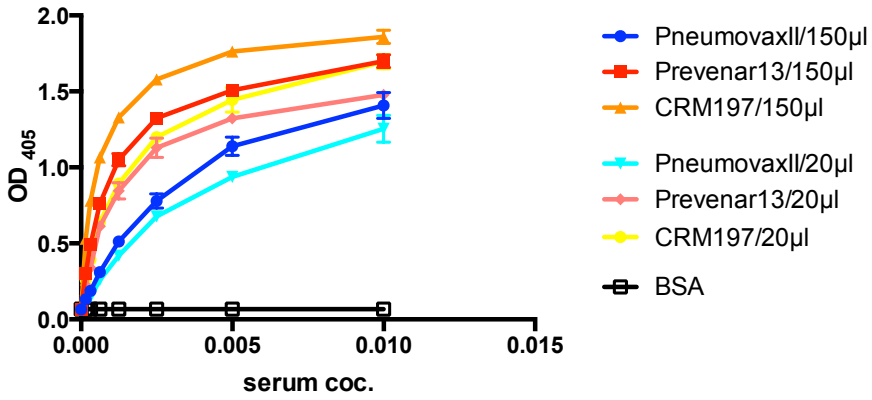

Figure 52: Two groups of WT mice were immunised with either 150µl and 20µl Then the immune response of Prevenar13 components was compared in both groups, to see if there immune response by lower doses. As be seen in this figure mice group immunised with 20µl was given a good responded.

4.5.3 Mice immunised at day 1 and day 45

WT and complement deficient mice were immunised with Prevenar13 vaccine at day 1 and booster at day 45. Blood samples were collected from saphenous vein in the prior of experiments as a negative control. Then at days 7, 14, 21, 45 and by cardiac puncture at day 52 (Figure 53).

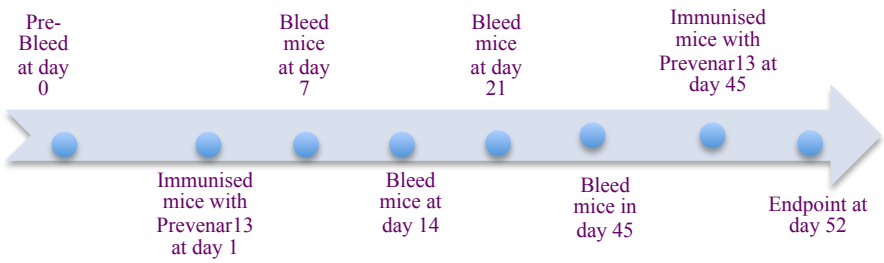

Figure 53: The experimental time schedule for intraperitoneal immunisation with Prevenar13 vaccine and blood collection points.

4.5.3.1 Antibody titres against the whole Prevenar13 vaccine

4.5.3.1.1 The immune intraperitoneal response to whole Prevenar13 vaccine in MASP-2$^{-/-}$ mice

MASP-2$^{-/-}$ mice were immunised with Prevenar13 on day 1 and the vaccine booster on day 45 using i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against whole Prevenar13 vaccine (Figure 54). Observing Table 31 to compare the differences between crossing WT and MASP-2$^{-/-}$, and

identifies that there are significant differences observed between MASP-$2^{-/-}$ and WT mice at early response however, at day 52 there is no significant differences was observed between MASP-$2^{-/-}$ and WT mice.

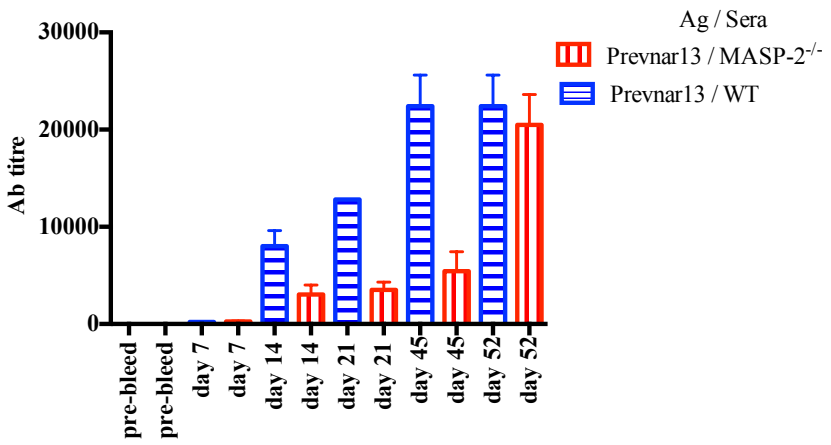

Figure 54: The immune response to the whole vaccine in MASP-$2^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 31: Unpaired t test with equal SD for MASP-$2^{-/-}$ mice were immunised with Prevenar13. The immune response to whole vaccine mice were immunised with Prevenar13 at days 1 and day 45, and serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005)

Prevenar 13	Wt/pre-bleed	MASP-$2^{-/-}$ / pre-bleed	Wt/ day 7	MASP-$2^{-/-}$ / day 7	Wt/ day 14	MASP-$2^{-/-}$ / day 14	Wt/ day 21	MASP-$2^{-/-}$ / day 21	Wt/ day 45	MASP-$2^{-/-}$ / day45	Wt/ day 52	MASP-$2^{-/-}$ / day 52
Wt/pre-bleed	-	ns	*	*	**	*	***	**	***	**	***	***
MASP-$2^{-/-}$ / pre-bleed		-	*	*	***	*	***	**	***	**	****	***
Wt/day 7			-	ns	**	*	***	**	***	**	***	***
MASP-$2^{-/-}$ / day 7				-	***	*	***	**	***	**	***	***
Wt/day14					-	*	*	*	**	ns	**	*
MASP-$2^{-/-}$ / day14						-	***	ns	***	ns	***	***
Wt/day 21							-	***	*	*	*	ns
MASP-$2^{-/-}$ / day21								-	***	ns	***	***
Wt/day 45									-	**	ns	ns
MASP-$2^{-/-}$ / day45										-	**	**
Wt/day 52											-	ns
MASP-$2^{-/-}$ / day52												-

4.5.3.1.2 The immune response to whole Prevenar13 in C1q$^{-/-}$ mice

All of C1q$^{-/-}$ mice were immunised with Prevenar13 on day 1 and the vaccine boostered on day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against whole Prevenar13 vaccine (Figure 55). Observing Table 32 to compare the differences between crossing WT and C1q$^{-/-}$ mice, and identifies that there are no significant differences observed between C1q$^{-/-}$ and WT mice at time points after immunisation.

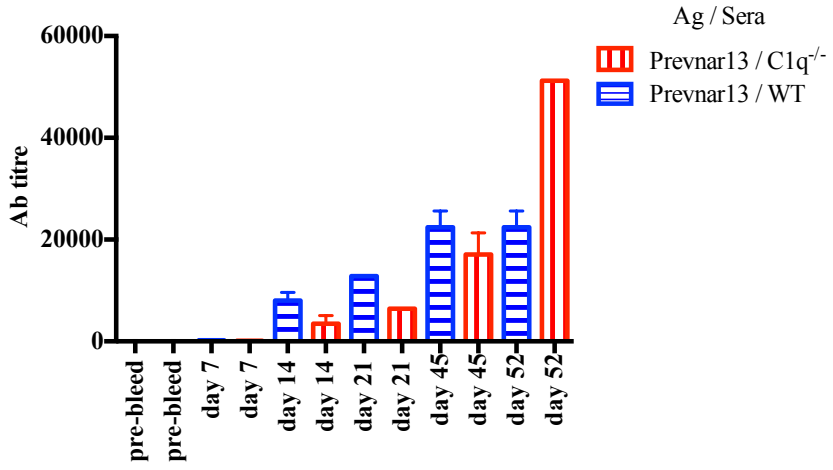

Figure 55: The immune response to the whole vaccine in C1q$^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 32: Unpaired t test with equal SD for $C1q^{-/-}$ mice were immunised with Prevenar13. The immune response to whole vaccine mice were immunised with Prevenar13 at days 1 and 45, and serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001)

Prevenar 13	Wt/pre-bleed	$C1q^{-/-}$ / pre-bleed	Wt/day 7	$C1q^{-/-}$ / day7	Wt/ day14	$C1q^{-/-}$ / day14	Wt/day 21	$C1q^{-/-}$ / day21	Wt/day 45	$C1q^{-/-}$ / day 45	Wt/day 52	$C1q^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	**	**	**	***	**	***	**	***	***
$C1q^{-/-}$ / pre-bleed		-	*	**	**	**	***	**	***	**	***	***
Wt/day 7			-	ns	**	ns	***	**	***	**	***	***
$C1q^{-/-}$ / day7				-	**	ns	***	*	**	*	**	**
Wt/day14					-	ns	*	**	**	**	**	**
$C1q^{-/-}$ / day14						-	***	ns	**	*	**	**
Wt/day21							-	ns	*	ns	*	**
$C1q^{-/-}$ / day21								-	*	ns	*	**
Wt/day45									-	ns	ns	*
$C1q^{-/-}$ / day45										-	ns	*
Wt/day52											-	ns
$C1q^{-/-}$ / day52												-

4.5.3.1.3 The immune response to Prevenar13 in Factor $B^{-/-}$ mice

$fB^{-/-}$ mice were immunised with Prevenar13 at day 1 and day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to the immunisation course to be used as background standard. Mice were bled from the saphenous vein at days 7, 14, 21, 45 and on day 52 blood sample were collected by cardiac puncture to determine the antibody titre against whole Prevenar13 vaccine (Figure 56). Observing Table 33 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there are high significant differences observed between $fB^{-/-}$ and WT mice at days 14, 21 and 45. Moreover, there are no significant differences observed between $fB^{-/-}$ and WT mice at day 52.

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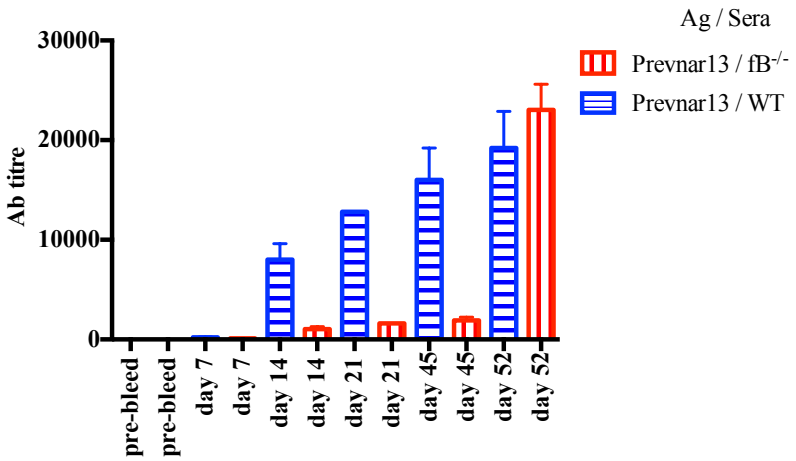


Figure 56: The immune response to the whole vaccine in $fB^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 33: Unpaired t test with equal SD for $fB^{-/-}$ mice were immunised with Prevenar13. The immune response to whole vaccine mice were immunised with Prevenar13 at days 1 and 45, and serum collected at days mention in the table (ns: P value>0.05, *: P value<0.05, **: P value<0.005, ***: P value<0.001)

Prevenar 13	Wt/pre-bleed	$fB^{-/-}$ / pre-bleed	Wt/day 7	$fB^{-/-}$ / day7	Wt/day14	$fB^{-/-}$ / day14	Wt/day21	$fB^{-/-}$ / day21	Wt/day45	$fB^{-/-}$ / day45	Wt/day52	$fB^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	**	**	**	***	**	***	**	***	***
$fB^{-/-}$ / pre-bleed		-	*	**	**	**	***	***	***	***	***	***
Wt/day 7			-	ns	**	*	***	**	***	**	***	***
$fB^{-/-}$ / day 7				-	**	*	***	**	***	**	***	***
Wt/day 14					-	**	*	**	*	**	*	**
$fB^{-/-}$ / day14						-	**	ns	**	*	***	***
Wt/day 21							-	***	*	***	*	**
$fB^{-/-}$ / day 21								-	**	ns	**	***
Wt/day 45									-	**	ns	ns
$fB^{-/-}$ / day 45										-	**	***
Wt/day 52											-	ns
$fB^{-/-}$ / day 52												-

4.5.3.2 Antibody titre for CRM_{197} component

4.5.3.2.1 The immune response to CRM_{197} component in MASP-$2^{-/-}$ mice

All of MASP-$2^{-/-}$ mice were immunised with Prevenar13 at days 1 and 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one-day prior to the immunisation course and these samples used as negative control. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against CRM_{197} (Figure 57). Observing Table 34 to compare the differences between crossing WT and MASP-$2^{-/-}$ mice, and identifies that there is significant differences observed only at day 52 between MASP-$2^{-/-}$ and WT mice.

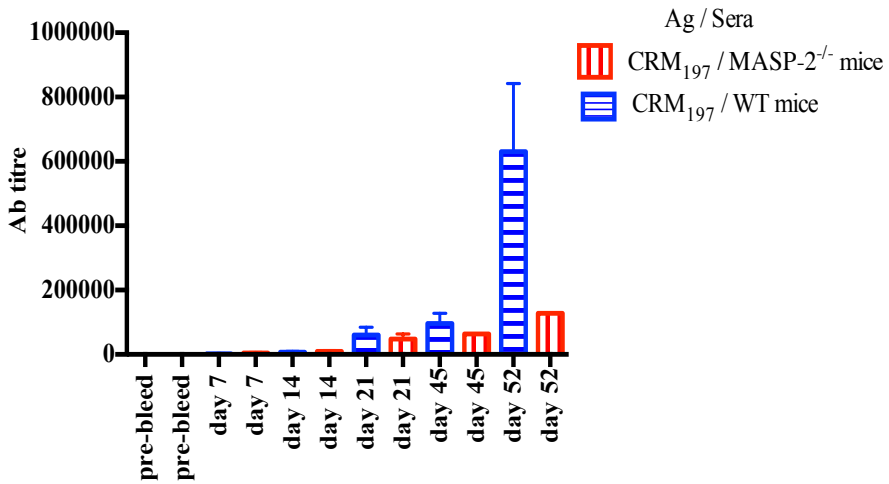


Figure 57: The immune response to CRM_{197} protein in $C1q^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 34: The immune response to CRM_{197} component in MASP-$2^{-/-}$ mice were immunised with Prevenar13 at days 1 and 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

Prevenar13 / CRM_{197}	Wt/pre-bleed	MASP-$2^{-/-}$ / pre-bleed	Wt/ day 7	MASP-$2^{-/-}$ / day7	Wt/ day14	MASP-$2^{-/-}$ / day14	Wt/ day21	MASP-$2^{-/-}$ / day21	Wt/ day45	MASP-$2^{-/-}$ / day45	Wt/ day52	MASP-$2^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	*	*	*	*	**	*	**	***	**
MASP-$2^{-/-}$ / pre-bleed		-	*	*	*	*	*	**	*	**	***	**
Wt/day 7			-	ns	ns	*	ns	**	*	**	***	**
MASP-$2^{-/-}$ / day7				-	ns	ns	ns	**	*	**	***	**
Wt/day14					-	ns	ns	**	*	**	***	**
MASP-$2^{-/-}$ / day14						-	ns	**	*	**	***	**
Wt/ day21							-	ns	ns	ns	*	ns
MASP-$2^{-/-}$ / day21								-	ns	ns	*	*
Wt/day45									-	ns	*	ns
MASP-$2^{-/-}$ / day45										-	*	ns
Wt/day52											-	*
MASP-$2^{-/-}$ / day52												-

4.5.3.2.2 The immune response to CRM_{197} component in $C1q^{-/-}$ mice

All of $C1q^{-/-}$ mice were immunised with Prevenar13 at day 1 and boosted at day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to immunisation and these samples used as background controls. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against CRM_{197} (Figure 58). Observing Table 35 to compare the differences between crossing WT and $C1q^{-/-}$ mice, and identifies that there is significant differences observed only at day 52 between $C1q^{-/-}$ and WT mice.

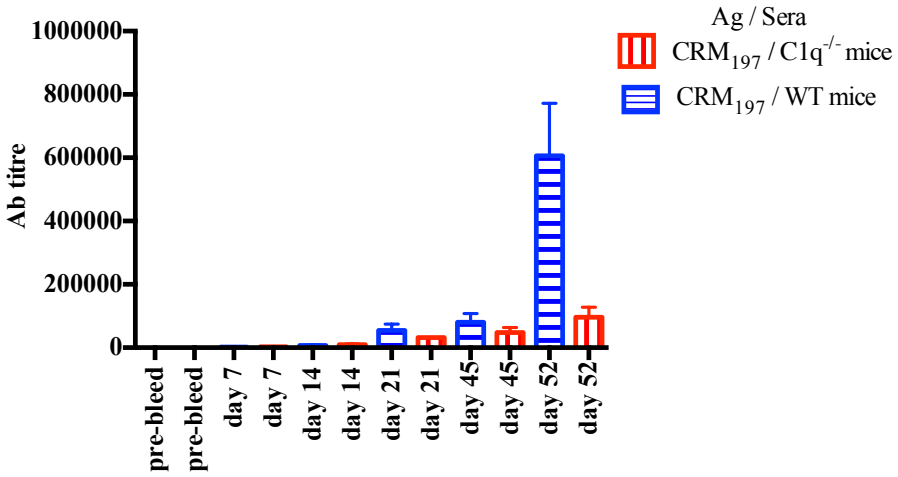


Figure 58: The immune response to CRM_{197} component in mice were immunised with Prevenar13 at day 1 and day 45. Serum collected at days mention in the graph

Table 35: The immune response to CRM_{197} component in $C1q^{-/-}$ mice were immunised with Prevenar13 at days 1 and 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

Prevenar 13 / CRM_{197}	Wt/ pre-bleed	$C1q^{-/-}$ / pre-bleed	Wt/ day 7	$C1q^{-/-}$ / day7	Wt/ day14	$C1q^{-/-}$ / day14	Wt/ day21	$C1q^{-/-}$ / day21	Wt/ day45	$C1q^{-/-}$ / day45	Wt/ day52	$C1q^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	*	*	*	*	**	*	**	***	**
$C1q^{-/-}$ / pre-bleed		-	*	*	*	*	*	**	*	**	***	**
Wt/day 7			-	ns	ns	*	ns	**	*	**	***	**
$C1q^{-/-}$ / day7				-	ns	ns	ns	**	*	**	***	**
Wt/day14					-	ns	ns	**	*	**	***	**
$C1q^{-/-}$ / day14						-	ns	**	*	**	***	**
Wt/day21							-	ns	ns	ns	*	ns
$C1q^{-/-}$ / day21								-	ns	ns	*	*
Wt/day45									-	ns	*	ns
$C1q^{-/-}$ / day45										-	*	ns
Wt/day52											-	*
$C1q^{-/-}$ / day52												-

4.5.3.2.3 The immune response to CRM_{197} component in Factor $B^{-/-}$ mice

All of $fB^{-/-}$ mice were immunised with Prevenar13 vaccine at day 1 and boosted at day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to immunisation and these samples used as background controls. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against CRM_{197} (Figure 59). Observing Table 36 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there is significant differences observed only at day 52 between $fB^{-/-}$ and WT mice.

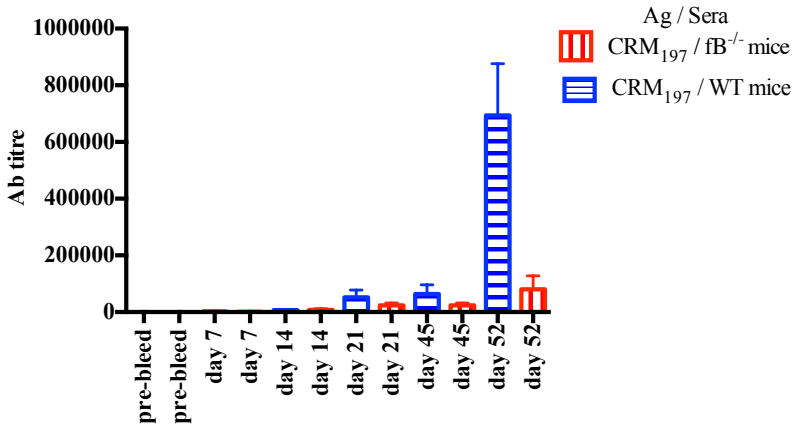


Figure 59: The immune response to CRM_{197} protein in $fB^{-/-}$ and WT mice were immunised with Prevenar13 at days 1 and 45. Serum was collected at days listed on the x-axis

Table 36: The immune response to CRM_{197} component in $fB^{-/-}$ mice were immunised with Prevenar13 at days 1 and 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

Prevenar13/ CRM_{197}	Wt/pre-bleed	$fB^{-/-}$ / pre-bleed	Wt/ day 7	$fB^{-/-}$ / day7	Wt/day14	$fB^{-/-}$ / day14	Wt/ day21	$fB^{-/-}$ / day21	Wt/ day45	$fB^{-/-}$ / day45	Wt/ day52	$fB^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	*	*	*	*	**	*	**	***	**
$fB^{-/-}$ / pre-bleed		-	*	*	*	*	*	**	*	**	***	**
Wt/day 7			-	ns	ns	*	ns	**	*	**	***	**
$fB^{-/-}$ / day7				-	ns	ns	ns	**	*	**	***	**
Wt/day14					-	ns	ns	**	*	**	***	**
$fB^{-/-}$ / day14						-	ns	**	*	**	***	**
Wt/day21							-	ns	ns	ns	*	ns
$fB^{-/-}$ / day21								-	ns	ns	*	*
Wt/day45									-	ns	*	ns
$fB^{-/-}$ / day45										-	*	ns
Wt/day52											-	*
$fB^{-/-}$ / day52												-

4.5.3.3 Antibody titres to Polysaccharides component

4.5.3.3.1 The immune response to Polysaccharides components in MASP-$2^{-/-}$ mice

MASP-$2^{-/-}$ mice were immunised with Prevenar13 at day 1 and the vaccine booster on day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to immunisation course and these samples used as background controls. Then, they were bled from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were collected by cardiac puncture to determine the antibody titres against Pneumococcal Polysaccharides (Figure 60). Observing Table 37 to compare the differences between crossing WT and

MASP-2$^{-/-}$ mice, and identifies that there is high significant differences observed at day 45 between MASP-2$^{-/-}$ and WT mice.

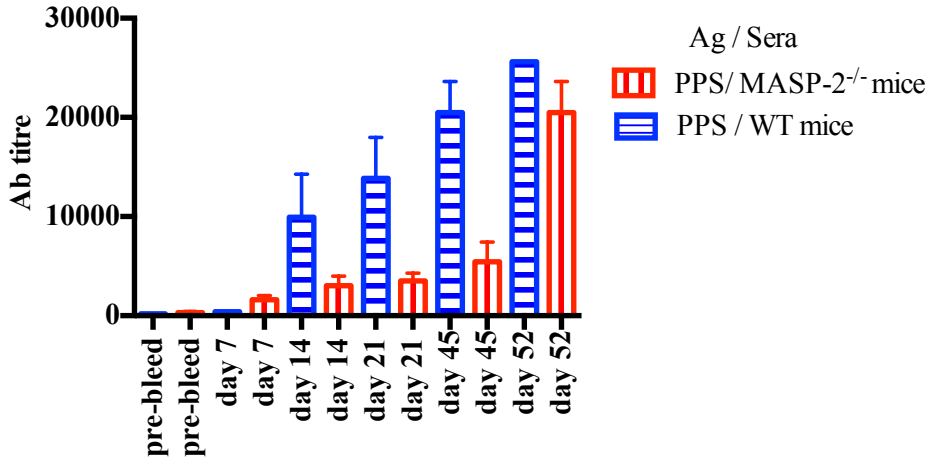


Figure 60: The immune response to pneumococcal polysaccharides (PPS) in MASP-2$^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 37: The immune response to Polysaccharides Alone in MASP-2$^{-/-}$ mice were immunised with Prevenar13 at day 1 and day 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, , ****: P value<0.0001)

Prevenar13 / Pneumovaxll	Wt/pre-bleed	MASP-2$^{-/-}$ / pre-bleed	Wt/ day 7	MASP-2$^{-/-}$ / day7	Wt/ day14	MASP-2$^{-/-}$ / day14	Wt/ day21	MASP-2$^{-/-}$ / day21	Wt/ day45	MASP-2$^{-/-}$ / day45	Wt/ day52	MASP-2$^{-/-}$ / day52
Wt/pre-bleed	-	ns	ns	*	*	*	**	**	****	**	****	****
MASP-2$^{-/-}$ / pre-bleed		-	ns	*	*	*	**	**	****	**	****	****
Wt/day 7			-	*	*	*	**	**	****	**	****	****
MASP-2$^{-/-}$ / day7				-	ns	ns	*	ns	***	**	****	***
Wt/day14					-	ns	ns	ns	ns	ns	*	*
MASP-2$^{-/-}$ / day14						-	*	ns	***	ns	****	***
Wt/day21							-	ns	ns	ns	ns	ns
MASP-2$^{-/-}$ / day21								-	***	ns	****	***
Wt/day45									-	**	ns	ns
MASP-2$^{-/-}$ / day45										-	****	**
Wt/day52											-	ns
MASP-2$^{-/-}$ / day52												-

4.5.3.3.2 The immune response to Polysaccharides components in C1q$^{-/-}$ mice

C1q$^{-/-}$ mice were immunised with Prevenar13 vaccine on days 1 and 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to immunisation course and these samples used as background controls. Then, they were bled from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample was taken by cardiac puncture to determine the antibody titre against Pneumococcal Polysaccharides (Figure 61). Observing Table 38 to compare the differences between crossing WT and C1q$^{-/-}$, and identifies that there are high significant differences observed at late days 45 and 52.

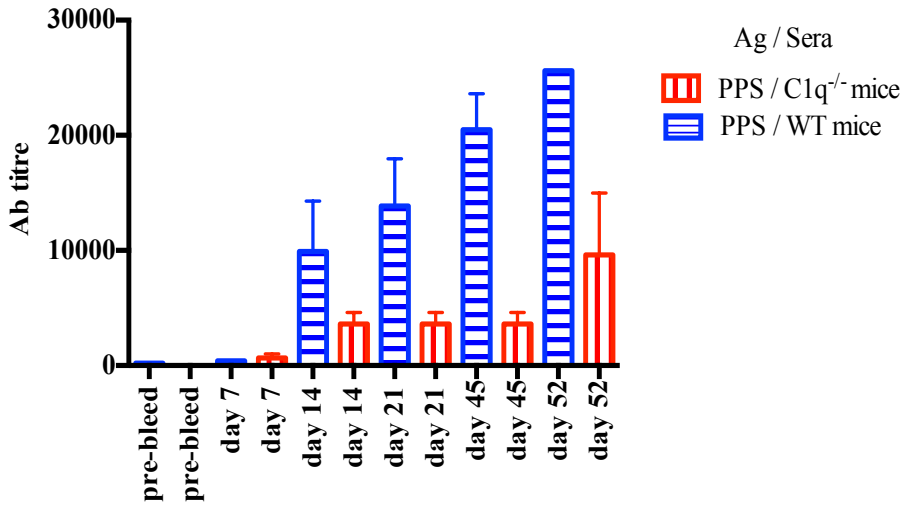


Figure 61: The immune response to pneumococcal polysaccharides (PPS) in C1q$^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 38: The immune response to Polysaccharides Alone in $C1q^{-/-}$mice were immunised with Prevenar13 at day1 and day 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, , ****: P value<0.0001)

Prevenar13 / Pneumovaxll	Wt/pre-bleed	$C1q^{-/-}$ / pre-bleed	Wt/day 7	$C1q^{-/-}$ / day7	Wt/ day14	$C1q^{-/-}$ / day14	Wt/ day21	$C1q^{-/-}$ / day21	Wt/ day45	$C1q^{-/-}$ / day45	Wt/ day52	$C1q^{-/-}$ / day52
Wt/pre-bleed	-	ns	ns	ns	*	*	**	**	****	**	****	**
$C1q^{-/-}$ / pre-bleed		-	ns	ns	*	*	**	**	****	**	****	**
Wt/day 7			-	ns	*	*	**	**	****	**	****	**
$C1q^{-/-}$ / day7				-	*	*	**	**	****	**	****	**
Wt/day14					-	ns	ns	ns	ns	ns	*	ns
$C1q^{-/-}$ / day14						-	ns	ns	**	ns	****	ns
Wt/day21							-	ns	ns	ns	ns	ns
$C1q^{-/-}$ / day21								-	**	ns	****	ns
Wt/day45									-	**	ns	**
$C1q^{-/-}$ / day45										-	****	ns
Wt/day52											-	*
$C1q^{-/-}$ / day52												-

4.5.3.3.3 The immune response to Polysaccharides components in Factor $B^{-/-}$ mice

$fB^{-/-}$ mice were immunised with Prevenar13 at day 1 and the vaccine booster on day 45 using the i.p. route (Figure 53). Blood samples were collected from each individual mouse one day prior to immunisation course and these samples used as background controls. Then, they were bled from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample was collected by cardiac puncture to determine the antibody titres against Pneumococcal Polysaccharides (Figure 62). Observing Table 39 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there are very high significant differences observed at and after day 14.

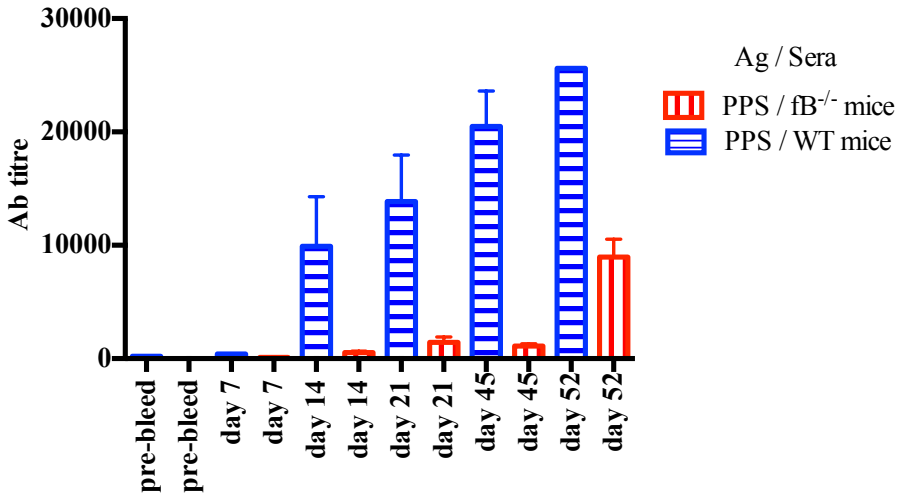


Figure 62: The immune response to pneumococcal polysaccharides (PPS) in fB$^{-/-}$ and WT mice were immunised with Prevenar13 at day 1 and day 45. Serum was collected at days listed on the x-axis

Table 39: The immune response to Polysaccharides Alone in fB$^{-/-}$ mice were immunised with Prevenar13 at day 1 and day 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, , ****: P value<0.0001)

Prevenar13/ Pneumovaxll	Wt/pre-bleed	fB-/- / pre-bleed	Wt/ day 7	fB-/- / day7	Wt/ day14	fB-/- / day14	Wt/ day21	fB-/- / day21	Wt/ day45	fB-/- / day45	Wt/ day52	fB-/- / day52
Wt/pre-bleed	-	ns	ns	ns	*	*	**	*	****	***	****	***
fB-/- / pre-bleed		-	ns	ns	*	*	**	*	****	***	****	***
Wt/day 7			-	ns	*	ns	**	*	****	**	****	***
fB-/- / day7				-	ns	*	*	*	***	**	****	***
Wt/day14					-	*	ns	*	ns	*	*	*
fB-/- / day14						-	*	ns	***	*	****	**
Wt/day21							-	*	ns	*	ns	*
fB-/- / day21								-	***	ns	****	**
Wt/day45									-	***	ns	*
fB-/- / day45										-	****	**
Wt/day52											-	****
fB-/- / day52												-

4.5.3.4 Flowcytometry analysis of splenocytes

B and T cells population were characterised by flow cytometry using CD's markers to analyse the splenocytes. Immunostaining with antibodies specific for either CD3, CD4, CD8, CD19, CD25 and CD45R/B220 in mice were immunised with Prevenar13 on days 1 and 45. MASP-2$^{-/-}$ group comparing with WT mice, there were high significant differences naïve T-cells (CD3), activated B cells (CD19 and CD45R/B220) (Figure 63). However, there are no differences between WT and C1q$^{-/-}$ or fB$^{-/-}$ mice (Figure 64)(Figure 65) respectively.

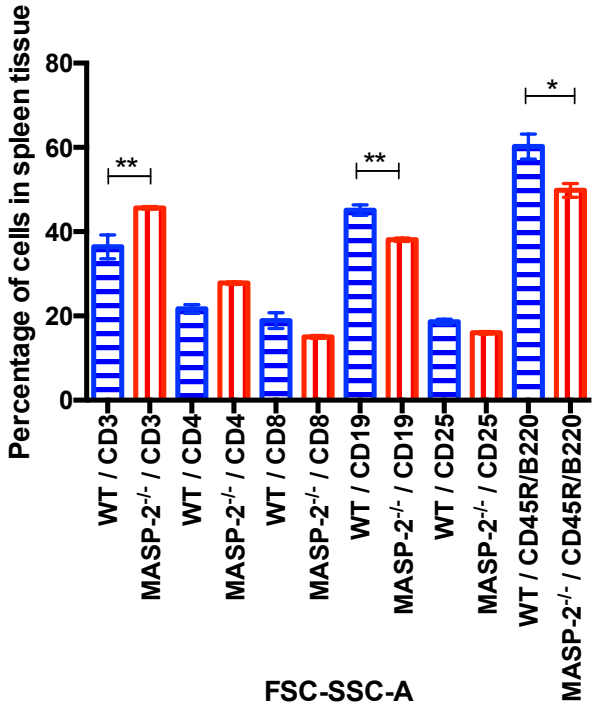


Figure 63: MASP-2$^{-/-}$ and WT control mice were immunised with Prevenar13 at days and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

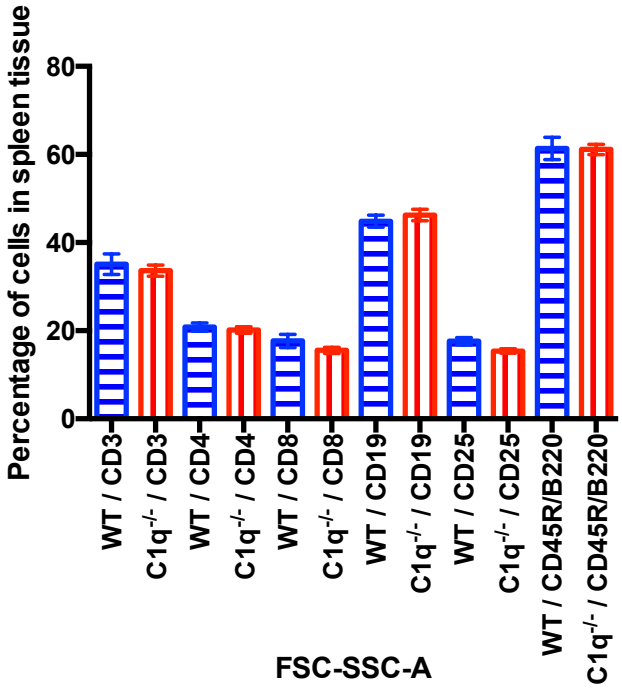


Figure 64: C1q$^{-/-}$ and WT control mice were immunised with Prevenar13 at days and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis.

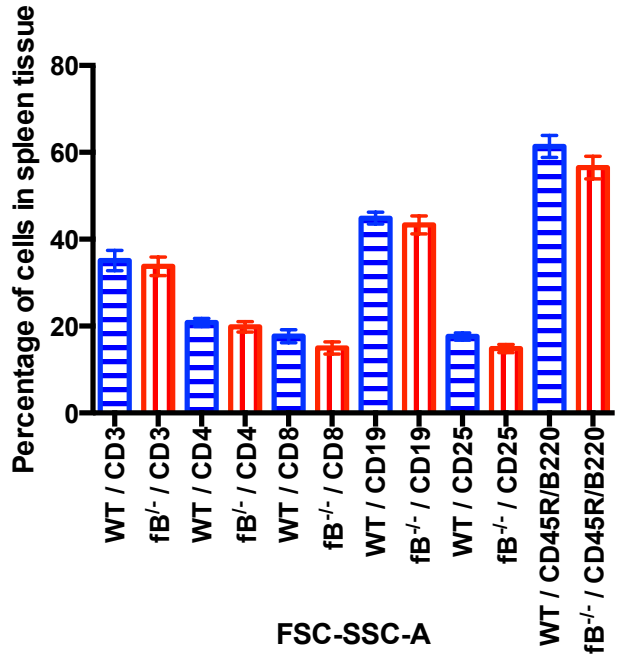


Figure 65: Mice were immunised with Prevenart13 at days 1 and day 45. Spleen tissue collected at day 52.

4.5.4 Mice immunised at days 1, 8, 15 and 45

WT and complement deficiency mice were immunised with Prevenar13 vaccine at day 1, 8, 15 and at day 45. Blood samples were collected from saphenous vein in the experiments as a negative control. Then at days 7, 14, 21, 45 and by cardiac puncture at day 52 (Figure 66).

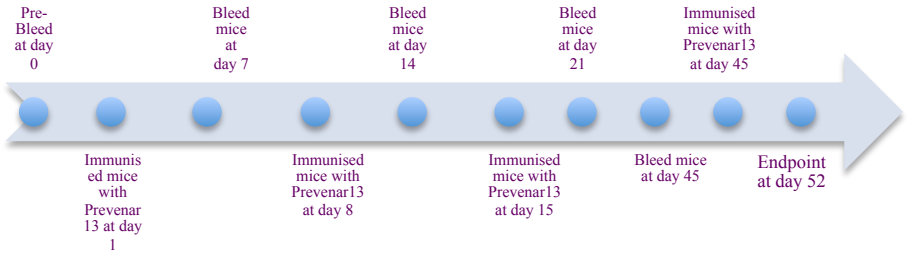


Figure 66: The immunisation schedule and the schedule to take blood samples following intraperitoneal immunisation with Prevenar13 vaccine.

4.5.4.1 Antibody titres to whole Prevenar13 vaccine

4.5.4.1.1 The immune response against whole Prevenar13 vaccine in MASP-2$^{-/-}$

MASP-2$^{-/-}$ mice were immunised with Prevenar13 vaccine at days 1, 8, 15 and at day 45 using the i.p. administration route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples were used as a baseline controls. Then mice were bled from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titre against whole Prevenar13 vaccine (Figure 67). Observing

Table 40 to compare the differences between crossing WT and MASP-2-/- mice, and identifies that there are no significant differences observed between WT and MASP-2$^{-/-}$ mice at time points after immunisation.

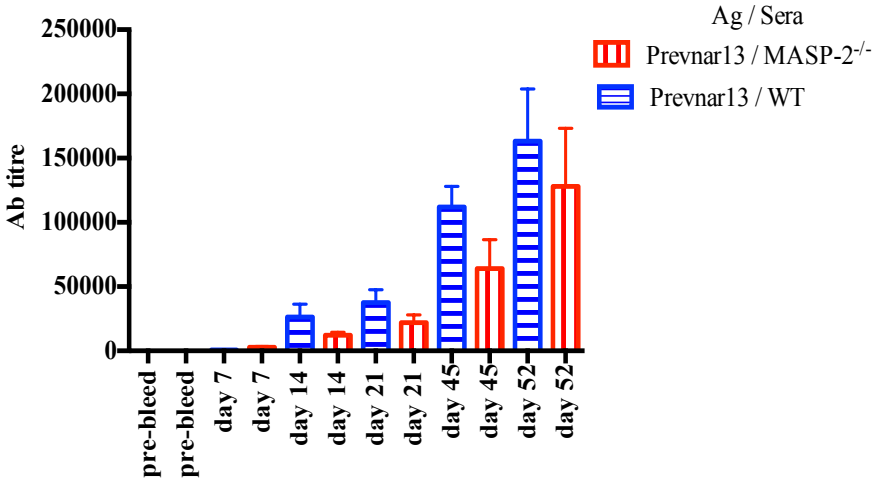


Figure 67: The immune response to the whole Prevenar13 in MASP-2$^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 40: The immune response to whole vaccine in MASP-2$^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45.and serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

Prevenar13	Wt/ pre-bleed	MASP-2$^{-/-}$ / pre-bleed	Wt/ day 7	MASP-2$^{-/-}$ / day7	Wt/day 14	MASP-2$^{-/-}$ / day14	Wt/day 21	MASP-2$^{-/-}$ / day21	Wt/day 45	MASP-2$^{-/-}$ / day45	Wt/day 52	MASP-2$^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	**	**	**	***	**	***	**	***	***
MASP-2-/- / pre-bleed		-	*	*	**	**	***	**	***	***	***	***
Wt/day 7			-	ns	**	*	***	**	***	**	***	***
MASP-2-/- / day7				-	*	ns	*	*	***	*	***	***
Wt/day14					-	ns	ns	ns	**	*	***	**
MASP-2-/- / day14						-	ns	ns	**	ns	***	***
Wt/day21							-	ns	**	ns	***	*
MASP-2-/- / day21								-	**	ns	**	*
Wt/day45									-	ns	ns	ns
MASP-2-/- / day45										-	ns	ns
Wt/day52											-	ns

MASP-2-/- / day52												-

4.5.4.1.2 The immune response to whole Prevenar13 vaccine in C1q$^{-/-}$ mice

C1q$^{-/-}$ mice were immunised with Prevenar13 vaccine at days 1, 8, 15 and at day 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as background controls. Mice were bled from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were collected by cardiac puncture to determine the antibody titres against the whole Prevenar13 vaccine (Figure 68). Observing Table 41 to compare the differences between crossing WT and C1q$^{-/-}$ mice, and identifies that there are no significant differences observed between C1q$^{-/-}$ and WT mice at time points after immunisation.

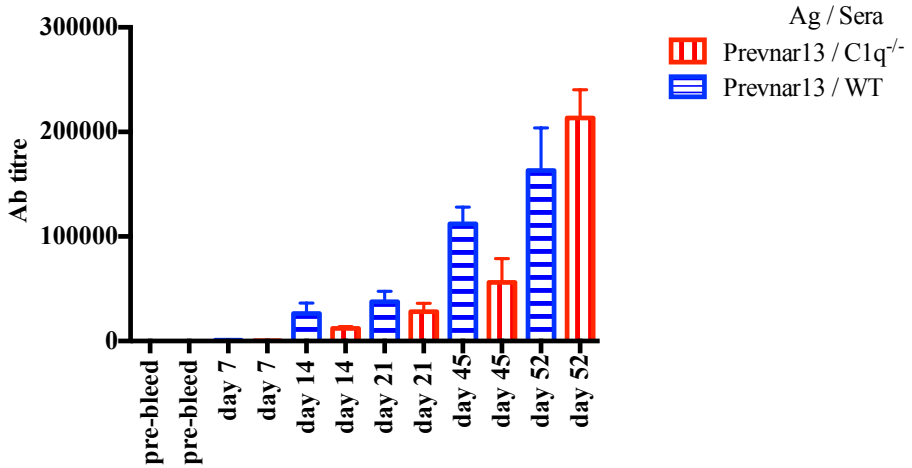


Figure 68: The immune response to the whole Prevenar13 in C1q$^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 41: The immune response to whole vaccine in $C1q^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, , ****: P value<0.0001)

Prevenar 13	Wt/ pre-bleed	$C1q^{-/-}$ / pre-bleed	Wt/ day 7	$C1q^{-/-}$ / day 7	Wt/ day 14	$C1q^{-/-}$ / day 14	Wt/ day 21	$C1q^{-/-}$ / day 21	Wt/ day 45	$C1q^{-/-}$ / day 45	Wt/ day 52	$C1q^{-/-}$ / day 52
Wt/pre-bleed	-	ns	*	**	**	**	***	**	***	**	***	****
$C1q^{-/-}$ / pre-bleed		-	*	*	**	**	***	**	***	***	***	****
Wt/day 7			-	ns	**	*	***	**	***	**	***	****
$C1q^{-/-}$ / day7				-	*	**	**	**	***	**	***	****
Wt/day14					-	ns	ns	ns	**	ns	***	***
$C1q^{-/-}$ / day14						-	ns	ns	**	ns	**	****
Wt/day21							-	ns	**	ns	***	***
$C1q^{-/-}$ / day21								-	**	ns	**	****
Wt/day45									-	ns	ns	*
$C1q^{-/-}$ / day45										-	*	**
Wt/day52											-	ns
$C1q^{-/-}$ / day52												-

4.5.4.1.3 The immune response to whole Prevenar13 vaccine in Factor $B^{-/-}$ mice

$fB^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as background controls. Then, mice were bled from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample were taken by cardiac puncture to determine the antibody titres against whole Prevenar13 vaccine (Figure 69). Observing Table 42 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there are no significant differences observed between $fB^{-/-}$ and WT mice until day 45 which is observed high significant differences in WT mice.

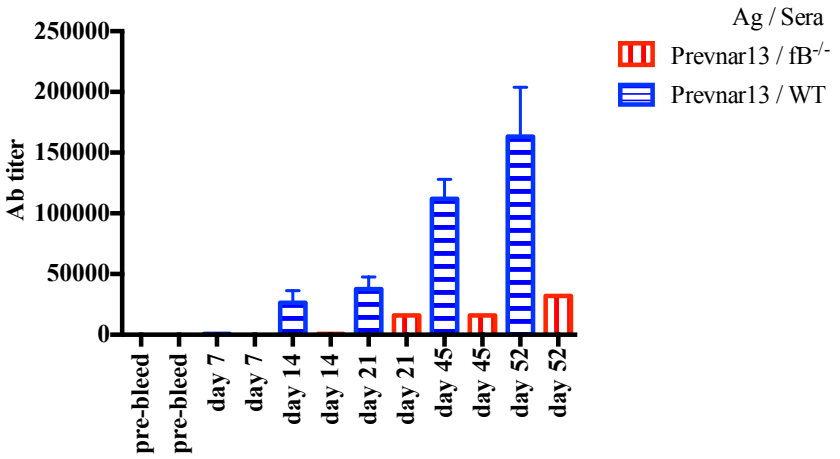


Figure 69: The immune response to the whole Prevenar13 in $fB^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 42: The immune response to whole vaccine in $fB^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and day 45. Serum was collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

Prevenar13	Wt/ pre-bleed	$fB^{-/-}$ / pre-bleed	Wt/ day 7	$fB^{-/-}$ / day7	Wt/ day14	$fB^{-/-}$ / day14	Wt/ day21	$fB^{-/-}$ / day21	Wt/day45	$fB^{-/-}$ / day45	Wt/ day52	$fB^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	**	**	**	***	**	***	**	***	***
fB-/- / pre-bleed		-	*	*	**	**	***	**	***	***	***	***
Wt/day 7			-	ns	**	*	***	**	***	**	***	***
fB-/- / day7				-	**	*	**	**	***	**	***	***
Wt/day14					-	ns	ns	ns	**	ns	***	ns
fB-/- / day14						-	*	*	***	*	***	***
Wt/day21							-	ns	**	ns	***	ns
fB-/- / day21								-	**	ns	**	ns
Wt/day45									-	**	ns	**
fB-/- / day45										-	*	ns
Wt/day52											-	**
fB-/- / day52												-

4.5.4.2 Antibody titres for CRM_{197} component

4.5.4.2.1 The immune response to CRM_{197} component in MASP-$2^{-/-}$ mice

MASP-$2^{-/-}$ mice were immunised with Prevenar13 on days 1, 8, 15 and on day 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as background control. Then, bleeding mice from saphenous vein at days 7, 14, 21, 45 and at day 52 blood sample was collected by cardiac puncture to determine the antibody titres against CRM_{197} (Figure 70). Observing Table 43 to compare the differences between crossing WT and MASP-$2^{-/-}$ mice, and identifies that there are no significant differences observed between MASP-$2^{-/-}$ and WT mice at time points after immunisation.

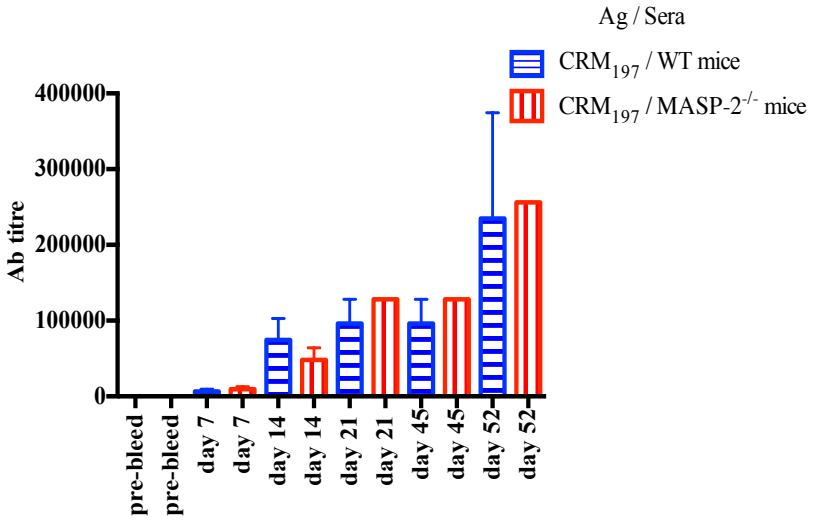


Figure 70: The immune response to CRM_{197} protein in MASP-$2^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 43: The immune response to CRM_{197} component in MASP-$2^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum were collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, , ****: P value<0.0001)

Prevenar13/ CRM_{197}	Wt/pre-bleed	MASP-$2^{-/-}$/ pre-bleed	Wt/ day 7	MASP-$2^{-/-}$/ day7	Wt/ day14	MASP-$2^{-/-}$/ day14	Wt/ day21	MASP-$2^{-/-}$/ day21	Wt/ day45	MASP-$2^{-/-}$/ day45	Wt/ day52	MASP-$2^{-/-}$/ day52
Wt/pre-bleed	-	ns	*	*	**	**	**	***	**	***	***	****
MASP-2-/- / pre-bleed		-	*	*	**	**	**	***	**	***	***	****
Wt/day 7			-	ns	**	**	**	***	**	***	***	***
MASP-2-/- / day7				-	**	**	**	***	**	***	**	***
Wt/day14					-	ns	ns	ns	ns	ns	*	**
MASP-2-/- / day14						-	ns	*	ns	*	ns	**
Wt/day21							-	ns	ns	ns	ns	*
MASP-2-/- / day21								-	ns	ns	ns	*
Wt/day45									-	ns	ns	*
MASP-2-/- / day45										-	ns	*
Wt/day52											-	ns
MASP-2-/- / day52												-

4.5.4.2.2 The immune response to CRM_{197} component in C1q$^{-/-}$ mice

C1q$^{-/-}$ mice were immunised with Prevenar13 vaccine on days 1, 8, 15 and on day 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as background controls. Then, mice were bled from the saphenous vein on days 7, 14, 21, 45 and on day 52 blood sample were taken by cardiac puncture to determine the antibody titres against CRM_{197} (Figure 71). Observing Table 44 to compare the differences between crossing WT and C1q$^{-/-}$ mice, and identifies that there are no significant differences observed between C1q$^{-/-}$ and WT mice at time points after immunisation.

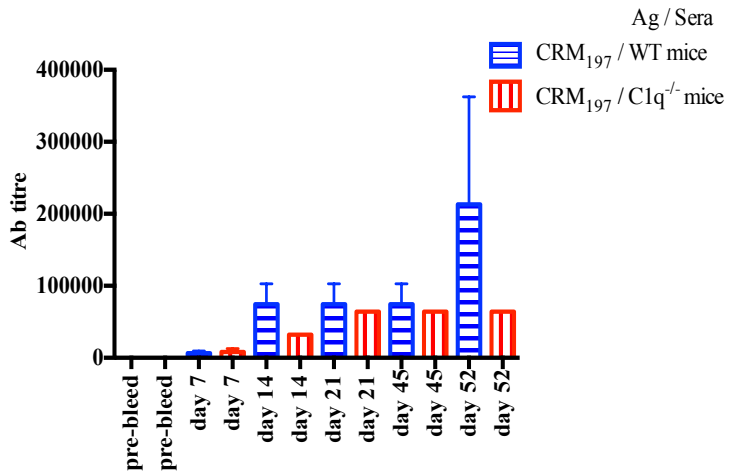


Figure 71: The immune response to CRM_{197} protein in $C1q^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 44: The immune response to CRM_{197} component in $C1q^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum were collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, ****: P value<0.0001)

Prevenar13 / CRM197	Wt/pre-bleed	C1q-/- / pre-bleed	Wt/ day 7	$C1q^{-/-}$ / day7	Wt/day 14	C1q-/- / day14	Wt/day 21	C1q-/- / day21	Wt/day 45	C1q-/- / day45	Wt/ day52	C1q-/- / day52
Wt/pre-bleed	-	ns	*	*	**	**	**	**	**	**	***	**
C1q-/- / pre-bleed		-	*	*	**	**	**	**	**	**	***	**
Wt/day 7			-	ns	**	**	**	***	**	**	***	**
C1q-/- / day7				-	**	**	**	***	**	**	**	**
Wt/ day14					-	ns	ns	ns	ns	ns	*	ns
C1q-/- / day14						-	ns	*	ns	*	ns	*
Wt/day21							-	ns	ns	ns	ns	ns
C1q-/- / day21								-	ns	ns	ns	ns
Wt/day45									-	ns	ns	ns
C1q-/- / day45										-	ns	ns
Wt/day52											-	ns
C1q-/- / day52												-

4.5.4.2.3 The immune response to CRM_{197} component in $fB^{-/-}$ mice

$fB^{-/-}$ mice were immunised with Prevenar13 on days 1, 8, 15 and 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as negative controls. Then, mice were bled from the saphenous vein on days 7, 14, 21, 45 and on day 52 blood sample were taken by cardiac puncture to determine the antibody titres against CRM_{197} (Figure 72). Observing Table 45 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there is only significant differences is observed between $fB^{-/-}$ and WT mice at day 14 after immunisation.

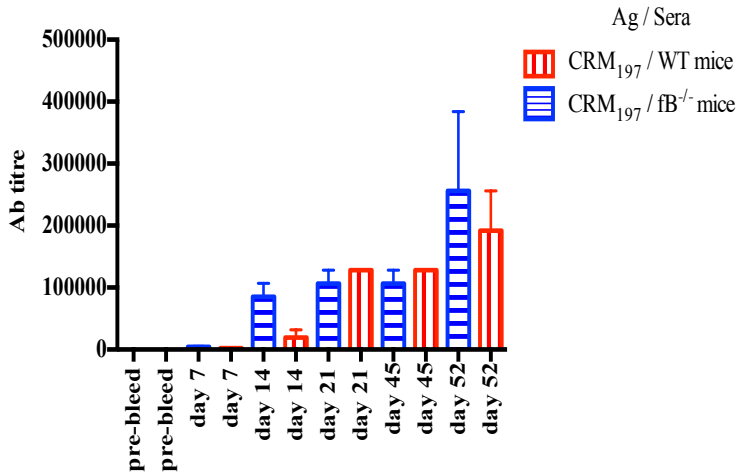


Figure 72: The immune response to CRM_{197} protein in $fB^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 45: The immune response to CRM_{197} component in $fB^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, ****: P value<0.0001)

Prevenar13 / CRM_{197}	Wt/ pre-bleed	$fB^{-/-}$ / pre-bleed	Wt/ day 7	$fB^{-/-}$ / day7	Wt/ day14	$fB^{-/-}$ / day14	Wt/ day21	$fB^{-/-}$ / day21	Wt/ day45	$fB^{-/-}$ / day45	Wt/ day52	$fB^{-/-}$ / day52
Wt/pre-bleed	-	ns	*	*	*	**	**	***	**	***	***	****
fB-/- / pre-bleed		-	*	*	*	**	**	***	**	***	***	****
Wt/day 7			-	ns	*	**	**	***	**	***	***	***
fB-/- / day7				-	*	**	**	***	**	***	**	***
Wt/day14					-	**	ns	ns	ns	ns	*	**
fB-/- / day14						-	**	**	**	**	**	**
Wt/day21							-	ns	ns	ns	ns	*
fB-/- / day21								-	ns	ns	ns	*
Wt/day45									-	ns	ns	*
fB-/- / day45										-	ns	*
Wt/day52											-	ns
fB-/- / day52												-

4.5.4.3 Antibody titres for Pneumococcal Polysaccharides components

4.5.4.3.1 The immune response to Pneumococcal Polysaccharides components in MASP-$2^{-/-}$ mice

MASP-$2^{-/-}$ mice were immunised with Prevenar13 vaccine on days 1, 8, 15 and on day 45 using i.p. route (Figure 66). Blood samples were collected from each individual mouse 1 day prior to the immunisation course and these samples used as background controls. Then, bleeding mice from the saphenous vein at days 7, 14, 21, 45 and at day 52 blood

sample were collected by cardiac puncture to determine the antibody titres against Pneumococcal Polysaccharides (Figure 73). In (Table 46) comparing the differences between WT and MASP-2$^{-/-}$ mice, and identifies that there is high significant differences observed at day 52.

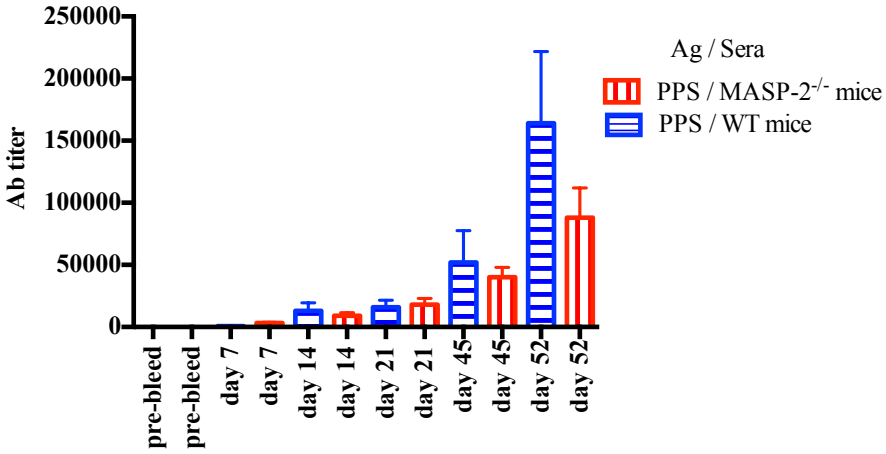


Figure 73: The immune response to pneumococcal polysaccharides (PPS) in MASP-2$^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 46: The immune response to Polysaccharides component in MASP-2$^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, ****: P value<0.0001)

Prevenar13/ PneumovaxII	Wt/pre-bleed	MASP-2-/- / pre-bleed	Wt/ day 7	MASP-2-/- / day7	Wt/ day14	MASP-2-/- / day14	Wt/ day21	MASP-2-/- / day21	Wt/ day45	MASP-2-/- / day45	Wt/ day52	MASP-2-/- / day52
Wt/pre-bleed	-	ns	ns	*	*	*	**	**	****	**	****	****
MASP-2-/- / pre-bleed		-	ns	*	*	*	**	**	****	**	****	****
Wt/day 7			-	*	*	*	**	**	****	**	****	****
MASP-2-/- / day7				-	ns	ns	*	ns	***	*	****	***
Wt/day14					-	ns	ns	ns	*	*	**	*
MASP-2-/- / day14						-	ns	ns	*	*	**	*
Wt/day21							-	ns	*	*	**	*
MASP-2-/- / day21								-	*	ns	**	*
Wt/day45									-	ns	**	ns

MASP-2-/- / day45										-	**	ns
Wt/day52											-	*
MASP-2-/- / day52												-

4.5.4.3.2 The immune response to Pneumococcal Polysaccharides components in C1q$^{-/-}$ mice

C1q$^{-/-}$ mice were immunised with Prevenar13 vaccine at days 1, 8, 15 and at day 45 using i.p. route (Figure 66). Blood samples were collected from each individual mouse one day prior to the immunisation course and these samples used as negative controls. Then, mice were bled from the saphenous vein on days 7, 14, 21, 45 and on day 52 blood sample were collected by cardiac puncture to determine the antibody titres against Pneumococcal Polysaccharides (Figure 74). Observing Table 47 to compare the differences between crossing WT and C1q$^{-/-}$, and identifies that there is high significant differences observed at day 52.

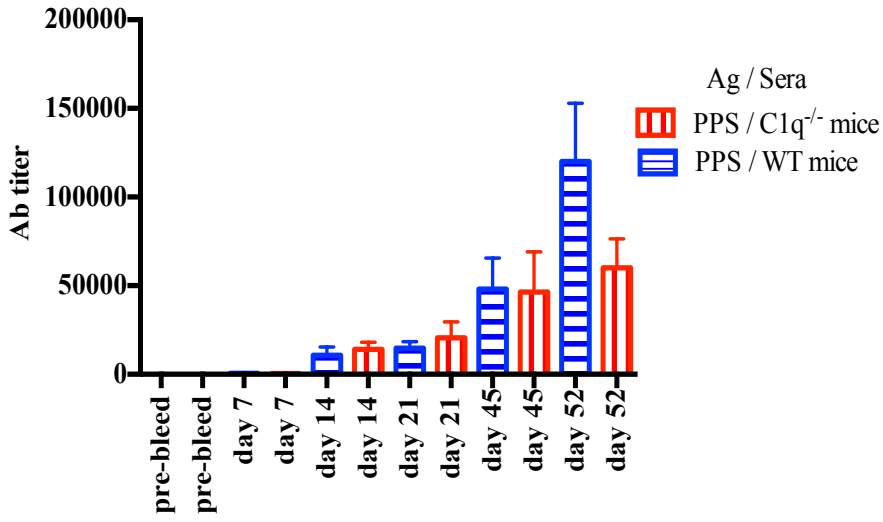


Figure 74: The immune response to pneumococcal polysaccharides (PPS) in C1q$^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 47: The immune response to Polysaccharides component in $C1q^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, ****: P value<0.0001)

Prevenar13 / Pneumovax II	Wt/pre-bleed	C1q-/- / pre-bleed	Wt/day 7	C1q-/- / day7	Wt/day 14	C1q-/- / day14	Wt/day 21	C1q-/- / day21	Wt/day 45	C1q-/- / day45	Wt/day 52	C1q-/- / day52
Wt/pre-bleed	-	ns	ns	ns	*	*	**	**	****	**	****	**
C1q-/- / pre-bleed		-	ns	ns	*	*	**	**	****	**	****	**
Wt/day 7			-	ns	*	*	**	**	****	**	****	**
C1q-/- / day7				-	*	*	**	**	****	**	****	**
Wt/day14					-	ns	ns	ns	*	*	**	*
C1q-/- / day14						-	ns	ns	*	ns	**	*
Wt/day21							-	ns	*	ns	**	ns
C1q-/- / day21								-	*	ns	**	ns
Wt/day45									-	ns	**	ns
C1q-/- / day45										-	**	ns
Wt/day52											-	*
C1q-/- / day52												-

4.5.4.3.3 The immune response to Pneumococcal Polysaccharides component in Factor $B^{-/-}$ mice

$fB^{-/-}$ mice were immunised with Prevenar13 vaccine on days 1, 8, 15 and 45 using the i.p. route (Figure 66). Blood samples were collected from each individual mouse 1 day prior to the immunisation course and these samples used as background controls. Then, mice were bled from the saphenous vein on days 7, 14, 21, 45 and on day 52 blood sample were taken by cardiac puncture to determine the antibody titres against Pneumococcal Polysaccharides (Figure 75). Observing Table 48 to compare the differences between crossing WT and $fB^{-/-}$ mice, and identifies that there is high significant differences observed at day 52.

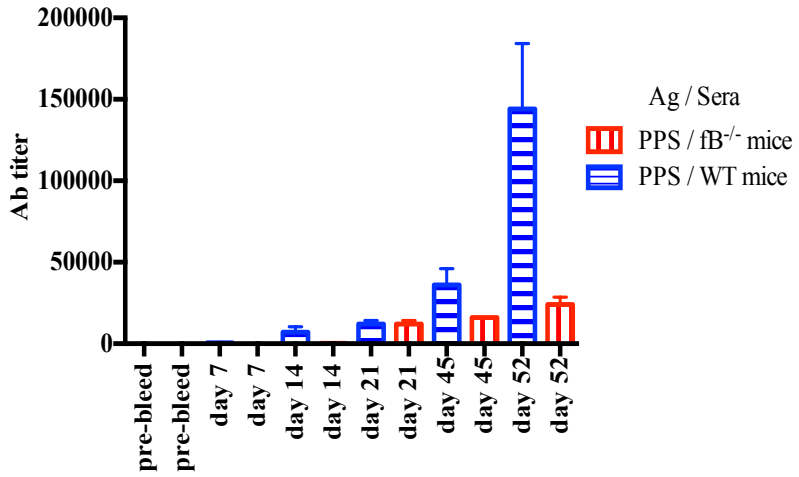


Figure 75: The immune response to pneumococcal polysaccharides (PPS) in $fB^{-/-}$ and WT mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Serum was collected at days listed on the x-axis

Table 48: The immune response to Polysaccharides component in $fB^{-/-}$ mice were immunised with Prevenar13 at days 1, 8, 15 and day 45. Serum collected at days mention in the table. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001, ****: P value<0.0001)

Prevenar13 / Pneumovax II	Wt/pre-bleed	fB-/- / pre-bleed	Wt/ day 7	fB-/- / day7	Wt/ day14	fB-/- / day14	Wt/ day21	fB-/- / day21	Wt/ day45	fB-/- / day45	Wt/ day52	fB-/- / day52
Wt/pre-bleed	-	ns	ns	ns	*	*	**	*	****	***	****	***
fB-/- / pre-bleed		-	ns	ns	*	*	**	*	****	***	****	***
Wt/day 7			-	ns	*	ns	**	*	****	**	****	***
fB-/- / day7				-	ns	*	*	*	***	**	****	***
Wt/day14					-		ns	ns	*	ns	**	*
fB-/- / day14						-	ns	ns	*	ns	**	*
Wt/day21							-	ns	*	ns	**	*
fB-/- / day21								-	*	ns	**	ns
Wt/day45									-	ns	**	ns
fB-/- / day45										-	**	ns
Wt/day52											-	*
fB-/- / day52												-

4.5.5 Flowcytometry analysis of splenocytes

B and T cells population, we performed flow-cytometry using CD's markers for the splenocytes. Immunostaining with antibodies for CD3, CD4, CD8, CD19, CD25 and CD45R/B220 in mice were immunised with Prevenar13 at days 1, 8, 15 and at day 45. MASP-2-/- group comparing with WT mice, there were high significant differences naïve and activated T-cells (CD3 and CD4), activated B-cells (CD19 and CD45R/B220) (Figure 76). However, there are high significant differences in activated T_h cells and B activated cells markers between WT and $C1q^{-/-}$ (Figure 77) and in $fB^{-/-}$ mice, the significant differences was just in T_h marker (Figure 78).

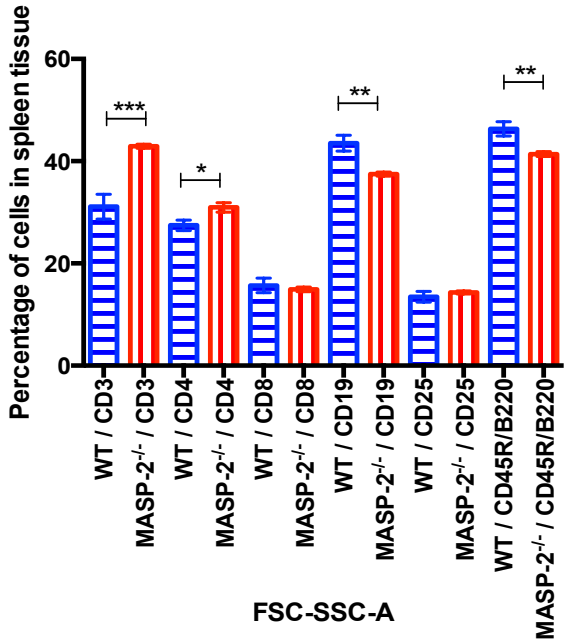


Figure 76: MASP-$2^{-/-}$ and WT control mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005 ***: P value<0.001)

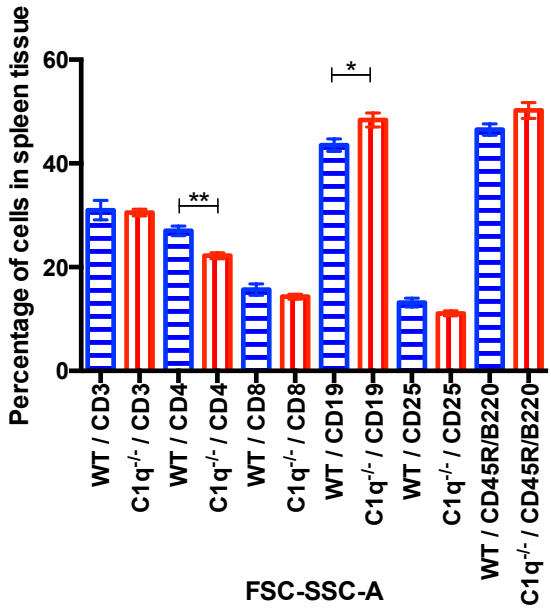


Figure 77: C1q-/- and WT control mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05, **: P value<0.005)

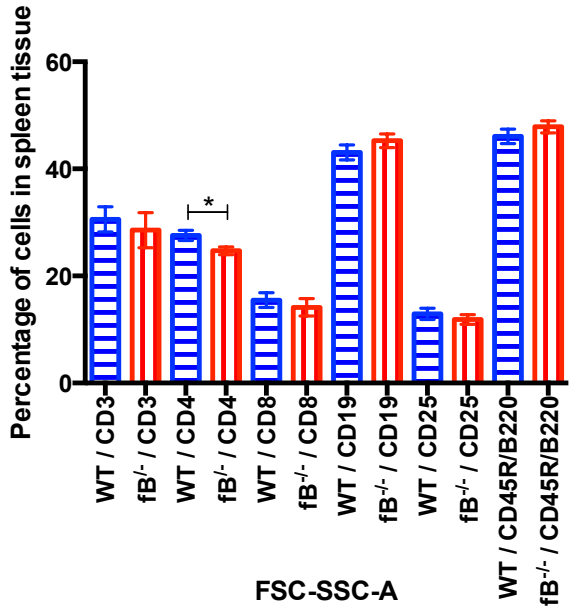


Figure 78: $fB^{-/-}$ and WT control mice were immunised with Prevenar13 at days 1, 8, 15 and 45. Mice were culled at day 52 and spleen cells isolated and their composition determined using CD3, CD4, CD8, CD19 CD25 and CD45R/B220 specific antibodies by FACS analysis. Unpaired t test with equal SD; (ns: P value>0.05, *: P value<0.05)

4.5.6 Discussion of the results following immnisation with Prevenar13

Based on the optimisation experiments presented in (CRM_{197} Protein – Result's chapter), the contribution of CRM_{197} protein in the interaction between whole Prevenar13 and the three complement activation pathways can be excluded from the results generated following immunisation of MASP-$2^{-/-}$, $C1q^{-/-}$ and $fB^{-/-}$ mice with whole Prevenar13 vaccine. Furthermore, optimisation experiments reported here showed that immunogenic response to CRM_{197} is independent of complement pathways, because mice with deficiencies in classical, lectin and alternative pathways had immunogenic response similar to wildtype mice. Complement dependent phagocytosis is indispensable in the host defence against bacterial infections, including pneumococcal diseases (*Johnston and Stroud 1977, Winkelstein et al. 1980*). In animal model of pneumococcal bacteraemia, the clearance of immunoglobulin opsonised pneumococci was shown to be completely dependent upon complement activity (*Brown et al. 1983, Hosea et al. 1980*). Therefore, the vaccine mediated protection conferred by Prevanr13 is majorly dependent on the immunogenic effect of capsular polysaccharides.

Though the results support the view that all three complement pathways play some role in Prevenar13 mediated immunity, each pathway play a distinct role. For instance, immunisation of MASP-$2^{-/-}$ mice with Prevenar13 at days 1 and 45 resulted in reduced immunogenic response compared to wildtype mice. The result suggests that the lectin pathway is important in Prevenar13 mediated immunity. Interestingly, by day 52 following the immunisation the response between MASP-$2^{-/-}$ and wildtype was normalised, suggesting that lack of lectin pathway might be delaying Prevenar13 mediated immunogenic response (*Mehlhop and Diamond 2006*). Another explanation could be a slow initial response, which could be compensated by the activity of other complement pathways (*Giebink et al. 1980*). By comparison immunisation at day 1, 8,

15 and 45 showed reduction in antibody production by day 7. In agreement with delayed immune response, by day 52 the effect of MASP-2$^{-/-}$ on immunogenic response has been normalised in the four regime experiments. Generally, the result from the different regimes of immunisation suggests that the lectin pathway plays important role in initial immunogenic response to Prevenar13. Though the four regime immunisation would increase the serum concentration of the vaccine and potentially increase immunogenic response through the activity of the other two complement pathways, concentration might not be enough to remedy the deficiency in lectin pathway in Prevenar13 mediated immunogenic response (*Welsch et al. 2008*). Therefore, the activity of other complement pathway could be enhancing the immunogenic response following initial activation of the immune response. Furthermore, deficiency in lectin pathway caused increased CD3 and CD45r/B220 cells following immunisation at day 1, 8, 15 and 45 also increased CD3 cells and reduced CD19 and CD45R/b220 cells. Four courses immunisation also increased the number of CD4 cells. Both, CD3 cells and CD4 cells are important in the protection against pneumococcal infection (*Fischer et al. 1996*). Conversely, CD19 and CD45R/B220 cell populations play an important role in signalling between innate and adaptive immunity. Combined together the results confirm the conclusion reached from the changes in antibody production.

Additionally, immunisation of C1q$^{-/-}$ mice with Prevenar13 at days 1 and 45 failed to cause any differences in immunogenic response compared to wildtype. The classical pathway impaired in C1q$^{-/-}$ mice might still be important in Prevenar13 mediated immune response because by day 52 the antibody production increased significantly compared to wildtype mice. This could be explained by either a compensatory role of other complementary pathway following initial activation of immune response (*Carroll 2004*). Furthermore, the results obtained following day 1, 8, 15 and 45 immunisation resulted in no changes in the antibody response. Therefore, the results from both immunisations suggest that the classical pathway is not important in Prevenar13 mediated immunogenic response.

Surprisingly, the result at day 52 of the two course immunisation might suggest that the classical pathway has a late negative regulation of this response (*Carroll 2004*). As such lack of C1q could be remedied by prolonged vaccination and induction of immune memory by Prevenar13 through other complement pathways (*Carroll 2004*). Interestingly, deficiency in the classical pathway failed to cause any changes in the number of immune cells infiltrating the spleen following two courses vaccination. However, four courses vaccination resulted in increased CD19 cells and decreased CD4 cells. Considering that changes in the cell types were negligible, Prevnair13 maybe eliciting immune cell infiltration to the spleen through classical pathway independent mechanisms (*Ricklin et al. 2010*). Indeed, MASP-2$^{-/-}$mice showed deficiency in more cell types compared to C1q$^{-/-}$ mice. The contribution of classical pathway seems to be more towards antibody generation than recruitment of immune cells.

The effect of fB$^{-/-}$ correlates with the results from deficiency in the other two complement activation pathways. Day 1 and 45 vaccinations with Prevenar13 reduced immunogenic response compared to wildtype. Interestingly, by day 52 the antibody production increased significantly in fB$^{-/-}$ mice. Therefore, other mechanisms could be compensating for the delayed immunogenic response caused by deficiency in the alternative pathway that needs further investigation. Furthermore, the immunisation of fB$^{-/-}$ mice four times caused reduced antibody production. This might suggest a concentration dependent mechanism of regulation, but the results showed that alternative pathway contribute to the Prevenar13 mediated antibody production. Moreover, the deficiency in the alternative pathway did not cause changes in number of immune cells in the spleen for both two and four course immunity. Though the number of CD4 cells was reduced in four courses immunisation, the effect of these groups of cells in immunity would be minor.

To confirm the contribution of individual components of Prevanr13 in the controls of the immunogenic response observed. Further experiments

were conducted on mice deficient in different components of the complement pathway. In MASP-$2^{-/-}$ mice there was reduced antibody production against polysaccharides compared to wildtype. Interestingly, the production of polysaccharides specific antibody increased with days, suggesting that the lectin pathway interacts with the capsular polysaccharides in the whole Prevanr13 vaccine. Similarly, the production of polysaccharide specific antibody was impaired in $C1q^{-/-}$ mice compared to the wildtype. Conversely, immunisation of $fB^{-/-}$ mice resulted in increased production of polysaccharide antibody. This will suggest that the response to polysaccharides is majorly through the classical and lectin pathway, not the alternative pathway. The result is in line with the fact that the alternative pathway is majorly activated through the C3 tickover. Therefore, antibody binding activity in classical and lectin pathways is important in Prevenar13 mediated response (*Carroll 2004*), which would act through the capsular polysaccharide.

Furthermore, there was differential CRM_{197} specific antibody production before day 45 in MASP-$2^{-/-}$, $C1q^{-/-}$ and $fB^{-/-}$ mice. Interestingly, CRM_{197} specific antibody production in wildtype increased by day 52 compared to all three deficiencies, and suggest that CRM_{197} might be inducing immune memory. Correspondingly, the result would signify a prolonged immunity against pneumococcal infection mediated through the activity of CRM_{197} on immune memory (*Spoulou et al. 2005*). Prolonged immunisation with whole Prevanar13 vaccine did not cause differential levels of polysaccharide or CRM_{197} specific antibodies in $C1q^{-/-}$ mice compared to two dose exposure. However, prolonged exposure caused increased polysaccharide or CRM_{197} antibodies in MASP-$2^{-/-}$ mice at days 21 and 52. This suggests that increased concentration of CRM_{197} conjugate in increased whole vaccine could facilitate the effectiveness of immunogenic response. Similarly, vaccination of $fB^{-/-}$ mice resulted in slow CRM_{197} antibody production at initial days that increased by day 21 and 45. However, by day 52 the production of antibody stabilised, which suggest that fB and potentially the alternative pathway is not associated with immune memory.

Here, we report that all three complement pathways contribute to Prevanir13 mediated immunogenic response. MASP-$2^{2-/-}$ mice that can still activate the classical and alternative pathways are severely compromised in their immunogenic response to Prevanir13 (*Takahashi et al. 2009*). Following immunisation, MASP-$^{2-/-}$ presented with reduction in antibody production and reduction in immune cells. The frequency of lectin pathway deficiency in the general population is estimated to be more than 10% (*Heitzeneder et al. 2012*). Indeed, dysfunctional lectin complement activation pathway is viewed as an important immunodeficiency that requires great consideration on vaccine development. Unfortunately, the decades of research into this medical condition has not been meet with a consensus on the definition of deficiency in the lectin activation pathway in relation to the threshold plasma levels of mannan-binding lectin (*Petersen et al. 2001*). Variation in the mannan-binding lectin concentration within ethic groups has played a major part in this regard (*Søborg et al. 2003*). Considering that individuals with this deficiency are healthy, complementary immunologic conditions are required for clinical manifestation (*Carlsson et al. 2005*). Interestingly, several clinical studies have shown that deficiency of the lectin pathway results in reduced serum concentrations of mannan-binding lectin. This has been attributed to increased risk, re-occurrence and increased severity of bacterial infections (*Eisen et al. 2008*). Mutations in the mannan-binding lectin are usually associated with increased infection susceptibility. Moreover, mutation in this gene is associated with about 1000-fold reduction in plasma mannan-binding lectin, and infection association is prevalent in concentration lower than 500 ng/ml. Here, reduced immunogenic response following Prevenar13 injection reflects the importance of lectin pathway in vaccine-mediated immunity against pneumococcal infection. This is especially important in patients that are prone to developing serious infections as reflected by the high percentage of congenital mannan-binding lectin deficiency in the general population. Clinically, deficiency in this pathway also poses a serious risk of complications in patients with other immune deficiencies such as cancer patients (*Beltrame et al. 2014, Holdaway et al. 2016*). In

cancer patients undergoing chemotherapy, low levels of mannan-binding lectins correlate with infection severity.

Both classical and alternative pathway deficiency caused differences in antibody production and number of immune cells in the spleen. It is well described that deficiency of the early proteins in the classical pathway of complement activation is strongly correlated with the difficulty in treating pneumococcal infection, whose predominate feature is the deposition of C4 and opsonisation of circulating pneumococci (*Agarwal and Blom 2015*). Indeed, such deficiency remains the strongest infection susceptibility gene for the development of pneumococcal disease in humans. This association has been shown to exhibit a hierarchy of disease severity according to the location of the protein in the classical pathway. The most severe infections are associated with dysfunctional C1 complex and depletion of the C4 protein. More than 75% of individuals diagnosed with deficiency of C1 complex and C4 protein have recurrent infections, which is commonly associated with severe pneumococcal infection (*Søborg et al. 2003*). By contrast, deficiency in C2 protein results in lower infection severity, which is estimated at about 9% (*Agarwal and Blom 2015*). Interestingly, C3 deficiency is highly associated with the development of severe pneumococcal disease, and it is plausible that this stronger association is because C3 deficiency also causes deficiency in the activation of classical complement pathway through defects in C3a, C5a and the membrane attack complex (*Søborg et al. 2003*). The cases of increased pneumococcal disease associated with C deficiency are common and account for a significant majority of patients with complement deficiencies. Therefore, they provide an important clue to the contribution of complement pathways and to the aetiology of pneumococcal diseases (*Søborg et al. 2003*). They demonstrate that there is an important activity of the classical pathway that protects against infection with *S. pneumoniae*. Results from independent studies using animal models have demonstrated that C1q deficiency may lead to abnormal in-vivo opsonisation of invading bacteria that in the context of vaccine mediated immunity could initiate

an autoimmune response promoting immune evasion of *S. pneumoniae* (*Agostoni and Cicardi 1992*). Moreover, despite the growing evidence of a clearance defect in the progression of pneumococcal disease associated with classical pathway deficiencies, it is still unclear the precise contribution of this pathway components in Prevenar13 immunogenic response and which impact deficiency in any of the components would have on the effectiveness of Prevenar13 immunisation (*Nuorti and Whitney 2010*). An alternative concept is that deficiency in the C1q may impair vaccination, because of the importance in recognition of self B cells and the defects in the C1q might result in the failure of regulatory B-cell selection, allowing invading pneumococci to evade the immune system (*Nuorti and Whitney 2010*). However, the essential role of C1q complex is to maintain immune complex in solution as such deficiencies in C1q would also result in abnormal immune complexes (*Nuorti and Whitney 2010*). This is a phenomenon that has been shown to result in dysfunctional adaptive immune response. Therefore, the result from this study is in line with the findings of other studies that describe the importance of adequate function of the classical pathway in the development of adaptive immunity.

Additionally, the result reported here is in agreement to the reported survival and mortality rates to pneumococcal infection reported for MASP-2$^{-/-}$, C1q$^{-/-}$ and fB$^{-/-}$ mice, using matched strains, doses and route of infection (*Garcia-Laorden et al. 2008, Rupprecht et al. 2007*). However, the current study is the first to show that the three complement pathway contribute partly in the protection against pneumococcal infection through Prevenar13 induced immune response. Indeed, no combination of the three complement pathway can fully compensate for the effect of losing any of the individual pathway. However, infiltration of immune cells remained unchanged in C1q$^{-/-}$ and fB$^{-/-}$, but number of CD19 and cd45R/B220 positive cells were reduced in MASP-2$^{-/-}$ mice. This suggests that Prevenar13 induces cell infiltration to the spleen through lectin pathway dependent mechanisms. The reduced antibody secretion in fB$^{-/-}$ mice could be due to dysfunction in the alternative

pathway amplification loop. The alternative pathway can be modulated by other complement pathways through the amplification loop involving C activation. This would explain the increase in the antibody secretion by day 52 of the immunisation. The alternative pathway amplification loop serves as a positive feedback process that has been shown to argument C3 activation through the classical and lectin pathway (Brown et al 2002). Indeed, $fB^{-/-}$ mice have increased susceptibility to *S. pneumoniae*, as such the finding of this chapter correlates with the known mechanism of the amplification loop. The generation of mice with deficiencies in different aspect of the alternative pathways has helped determine the role of alternative pathway in the defence against various infectious diseases (*Rus et al. 2005*). Indeed, the role of alternative pathway in host defence received great input from the identification of individuals with deficiencies in fB, fD, properdin and C3. Furthermore, a great deal of knowledge has been gained from mice models like $fB^{-/-}$. In conclusion, this result from this chapter further supports the role of alternative pathway in vaccine mediated immunity.

In conclusion, studies on animals with deficiencies in different components of classical complement activation pathways including deficiencies in C1q, C4 and C3 demonstrated that classical pathway deficiencies increase susceptibility to infection. Indeed, in the case of defence against pneumococcal infections experimental results suggest that the activation of the classical pathway is the key mechanism in complement mediated opsonophagocytosis of *S. pneumoniae*. By contrast, the lectin pathway was originally thought to play a negligible role in the opsonophagocytosis of *S. pneumoniae*. However, the results presented in this chapter suggest that deficiency in lectin pathway has significant implication for vaccine mediated immunity. Additionally, the role of the alternative pathway and the mechanisms of vaccine mediated protection as it relates to complement mechanisms remains poorly described. Here we investigated the contribution of the classical, lectin and alternative pathway in the immunogenic response to Prevenar13 and potential role in vaccine induced immunity. The study reached the

conclusion that all three pathways play unique roles in Prevenar13 mediated immunogenic response. Therefore, Prevenar13 mediated protection against *S. pneumoniae* would be dependent on the contribution of the three pathways and individuals with deficiency in the complement pathway requires further research effort to help reduce invasive pneumococcal diseases.

4.6 Summary Discussion: (all the results together)

The complement system functions by a complex interaction between several proteins and surface receptors that promote host defence against pathogens. Indeed, the complement system has been shown to be activated through mechanisms that are based on pattern recognition, ligands and immune complexes. The activation through the classical, lectin and alternative pathways is associated with several protective functions, including pathogen destruction and modulation of immune responses. By modulating both the innate and adaptive immune response the complement system plays important role in host immunity against pneumococcal infections. Several strains of pneumococcal infections have been shown to trigger distinct complement activation profiles in humans. The carbohydrates on the capsule of Pneumococcus can interact with the complement pathways in different ways.

First, it has been shown that the classical pathway is activated following infection with Pneumococcus through the activation of structural carbohydrates. Second, they can activate the lectin pathway by binding to the mannose binding lectins. Similarly, these structural carbohydrates can activate the alternative pathway. However, the contribution of each of the three pathways to the development of immunity against pneumococcus following vaccination remains elusive. In this study, we report that the three complement pathways contribute independently to the immunogenic response to two commonly used commercial vaccines against pneumococcal infections. Both PneumovaxII and Prevanr13 interact with the complement pathways, and to some extent the activation of all the three complement pathways is required in their immune activity. However, differences in aspect of immunogenic response were observed in mice deficient in classical, lectin and alternative activation pathways. The study expands the understanding of the immune mechanisms underlying PneumovaxII mediated protection against pneumococcal infections, and specifically highlights the importance of the complement activation pathways. This is particularly interesting in

the research into effective treatment of pneumococcal infections in patients with impairment in the activation of the complement system. Indeed, the expression and production of the components of the three complement pathways are crucial in the adequate induction of the innate immune response (*Bruyn et al. 1992, Hyman et al. 1975*). Therefore, it is not surprising that deficiency in the activation of the classical, lectin and alternative pathways of the complement system modulated immunogenic response, considering the complex crosstalk between the innate and the adaptive immune system. Generally, the results from PneumovaxII immunisation are analogous to other studies that have shown that some of the components of the classical, lectin and alternative complement activation pathways are expressed following human and mice exposure to polysaccharides.

Furthermore, the Prevanir13 mediated protection against the 13 serotypes of pneumococcal infections covered by the vaccine is partly dependent on its interaction with the three activation pathways of the complement system. Though mice with individual defects in one of these pathways can still activate the other two pathways, they presented with compromised immunogenic response to Prevanir13. Irrespective of the fact that full mechanisms of action involved in this important interaction remain to be fully characterised, the results could serve as the basis of further investigation into this important immune function. Moreover, the observation that all three complement pathways positively contribute to Prevanir13 mediated immunogenic response further highlights the importance of conjugate vaccines in the vaccination against pneumococcal infection. It is now established that conjugation of polysaccharides to carrier proteins increases the induction of humeral/mucosal immunity, which facilitates the clearance of serotypes covered by the conjugate vaccine. However, a disadvantage of these classes of vaccines is the low coverage of pneumococcal serotypes. The development of more conjugate pneumococcal vaccines with expanded serotypes will help overcome this problem. In contrast, polysaccharide vaccines do not induce mucosal immunity and hence has little impact on

carrier rates. However, the large number of included pneumococcal serotypes presents some advantages. Therefore, future therapeutic approaches in complement deficient individuals could aim to combine both classes of vaccines.

Combined with previous studies, the current study shows that development of vaccine mediated immunity against pneumococcal infections, in part requires the unique contribution of each activation pathway. Although further studies are needed to identify the exact mechanisms by which these complement pathways contribute differentially to the activity of both PneumovaxII and Prevanr13, this study is novel as it describes the role of the classical, lectin, and alternative pathways in immune function after vaccination with both vaccines. Independent of the differences in serotypes in both vaccines, the study shows that conjugation of vaccines to carrier proteins increases positive modulation of the complement activation pathways. Indeed, the experiments on PneumovaxII showed that classical pathway might be playing a negative regulatory role, whilst the lectin and the alternative pathways are involved in positive regulation and minor positive regulation of immunogenic response, respectively. In the case of Prevenar13 all three complement activation pathways play a varying levels of positive role, which could be explained by the differences in the mechanisms of the pathways. Interestingly, optimisation experiments confirmed that the immunogenic activity of CRM_{197} is independent of the three complement activation pathways. This equally confirmed the polysaccharide specific effect of conjugate vaccines, which could be exploited in the development of new vaccination approaches. Particularly, this observation can be used in the development of new approaches for the treatment of pneumococcal infections in complement deficient individuals. The observation that the complement system does not interact with the carrier protein was expected because CRM_{197} elicited response has less immune activity (*Del Giudice and Rappuoli 1999*). In agreement with other studies, CRM_{197} carrier protein may modulate immunologic response at high doses (*Del Giudice and*

Rappuoli 1999), and the full understanding of the mechanisms underlying this potential dose dependent effect would promote its use in delivery of vaccines. Generally, the conjugate vaccine interacts more with the complement pathways, compared to the free polysaccharides. Though surface polysaccharides from bacteria are essential virulence factor that are exploited in many vaccines, they are poorly immunogenic because of their T-independent activity. Our result is in line with the fact that saccharides lack immunological memory, which makes re-vaccination necessary. Interestingly, conjugate to carrier proteins enhances T-dependent activity to these polysaccharide vaccines which enhances protection against bacterial infections.

5 Appendices

5.1 Impact of ALUM in WT mice

WT mice were injected with ALUM [containing an aqueous solution of aluminium hydroxide (40mg/mL) and magnesium hydroxide (40mg/mL) plus inactive stabilizers] after the ALUM was diluted 1:3 in a PBS buffer. Mice were divided to three groups and 20µl, 100µl and 200µl of alum buffer were used in the final dose. All these groups were immunised i.p. weekly for three weeks. The organs were affected by the doses of alum buffer. As can be seen from pictures of the results rebating alum doses higher than 100µl is not recommended for mice.

Table 49: the number of mice, which have necrosis in their organs.

Mice groups	Spleen	Kidney	Liver	Lung
20 µl	Null	Null	Null	Null
100 µl	2 out of 3	Null	1 out of 3	Null
200 µl	3 out of 3	2 out of 3	3 out of 3	Null

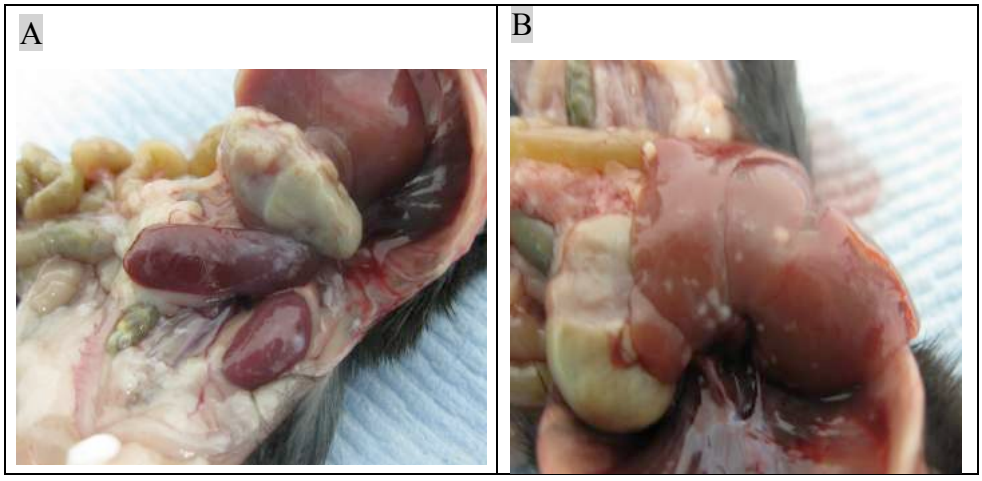


Figure 79: WT mice immunised weekly with Alum for three weeks. A: showing necrosis in spleen and kidney. B: showing Liver necrosis.

5.2 The impact of CWPS incubated in sera

NHS from humans immunised with PneumovaxII and mice immunised with PneumovaxII were used (Figure 80). Sera were incubated with 10µg/ml of CWPS in BBS/30 minutes. Heated inactivated serum at 56°C/30 minutes was used as a negative control. CWPS affects the immune response to PneumovaxII on humans sera. However, CWPS has no effect on mice sera. The effect comes about by giving non-specific binding, which has an effect on the Ab titre. In this case the incubation of CWPS in the sera removes CWPS contamination.

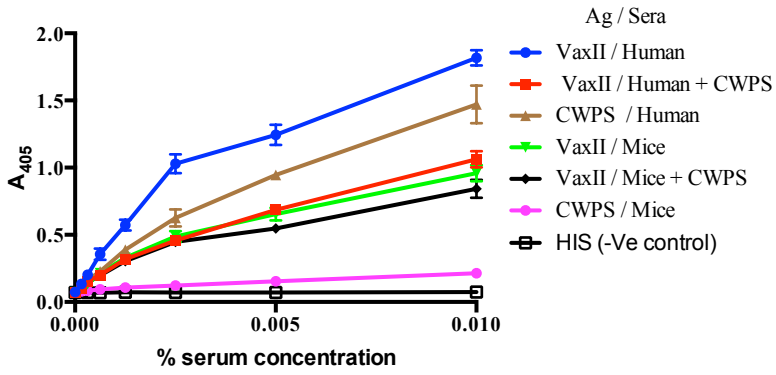


Figure 80: ELISA plate coated with 1µg of PneumovaxII and with 1µg of CWPS. Human serum and mice sera after being immunised with PneumvaxII, serum serial diluted starting with 1:100 in BBS buffer. Sera + CWPS: sera incubated in 10µg /ml of CWPS in BBS/30 minutes. Using heated inactivated serum (HIS) at 56°C/30 minutes (-ve control).

5.3 Some of the clinical Symptoms in the experiments

During the CRM197 experiments, mice were immunised with CRM197 on day 1 and boosted at day 45. Two mice out of ten had liver necrosis in $C1q^{-/-}$group. Moreover, the mice were multiply immunised with CRM_{197}. 3 or 2 mice in each of the WT and $C1q^{-/-}$groups had liver necrosis.

In the PneumovaxII experiments, when the mice received multiple immunisation with PneumovaxII, one of the 5 in the MASP-$2^{-/-}$ mice group had symptoms that look like dermatitis on its hind limbs.

During the Prevenar13 experiments, mice were immunised at days 1and 45; one mouse in the WT group and another one in the $C1q^{-/-}$group had liver necrosis. Mice were immunised with Prevenar13 at days1, 8, 15 and 45. One mouse in the WT group had liver necrosis as had two mice in the $C1q^{-/-}$group.

5.4 Immunised mice with C1q protein and PneumovaxII

WT mice were immunised i.p. with 100µg of C1q Protein. Then, 12 days after this immunisation, the experiment started with WT mice being divided to two groups (group-1: 100µg C1q Protein mixed with 1µg PneumovaxII; group-2:1µg PneumovaxII). They were immunised three times on days 1, 8 and 15, and blood samples were collected in days 0, 7, 14 and 21. The graph below shows that for a higher immune response to the PneumovaxII vaccine in WT mice, the mice should be given C1q Protein to produce anti-C1q Protein 12 days before the start of the PneumovaxII vaccine course.

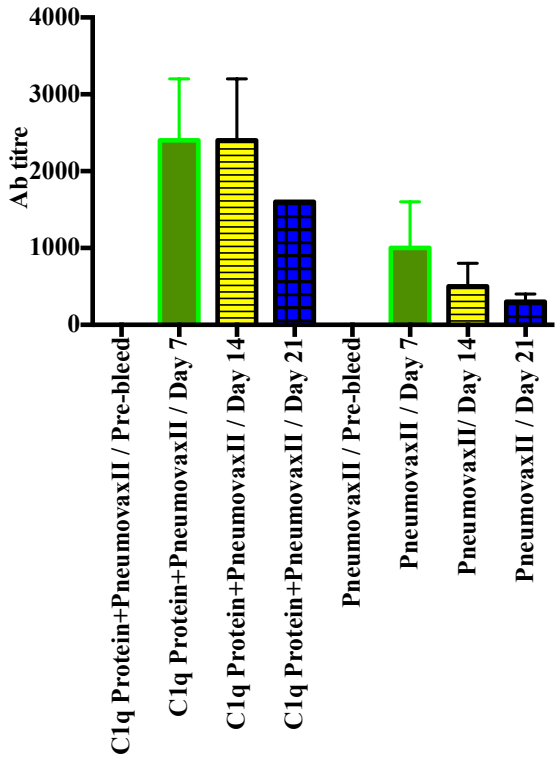
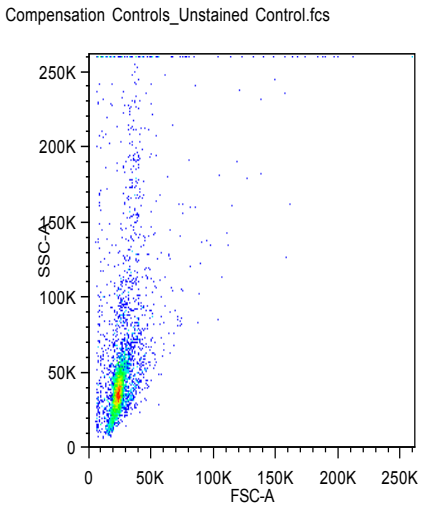
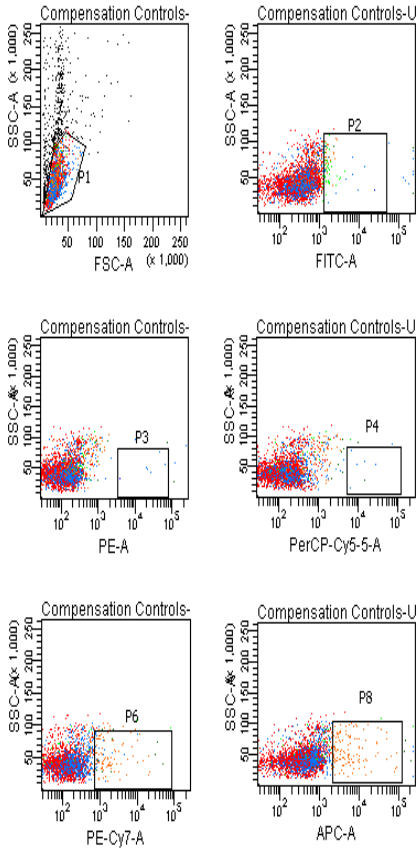
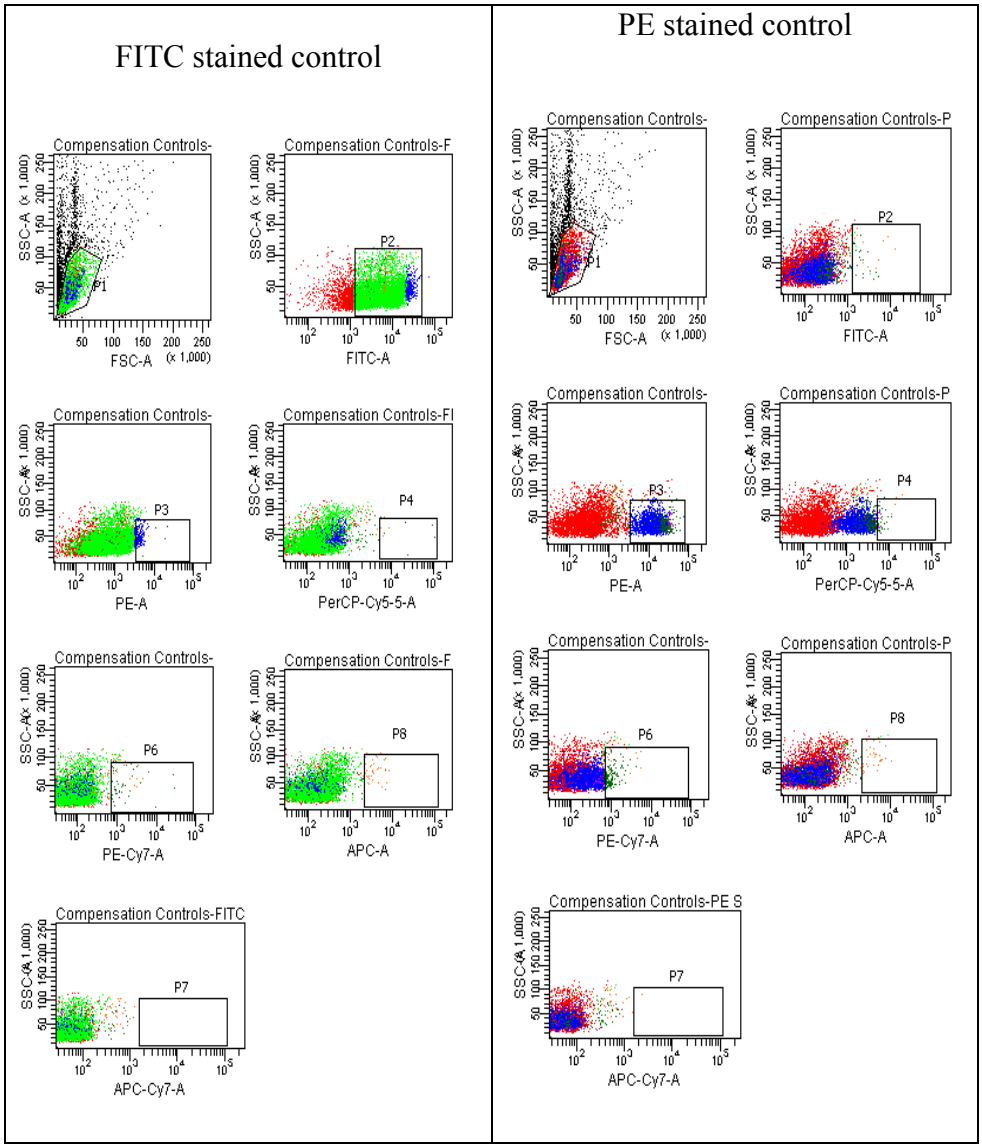


Figure 81: WT mice start immunised with C1q protein. Then after 12 days, the experiment start as mice were immunised with C1q protein mixed with PneumovaxII vaccine and blood samples were collected as pointed in the graph.

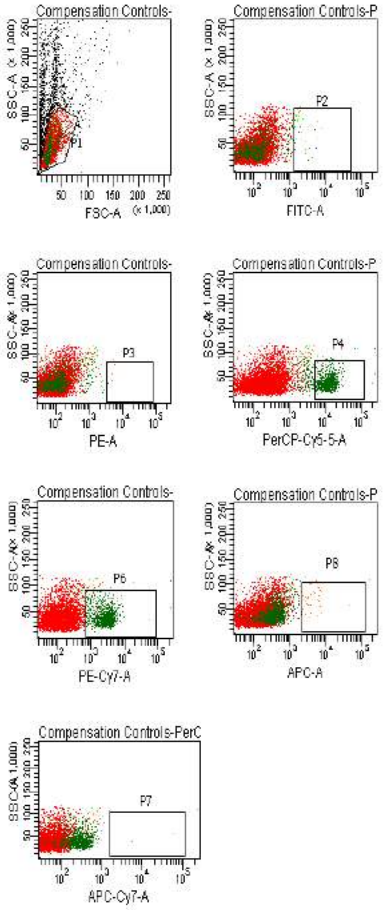
5.5 Designated the gates in the Flowcytometery technique

The splenocytes were analysed using the manufacturer's standard protocol for BD FACSCanto™II. Here, compensation and fluorescence intensity was setup for each individual spleen tissue to analyse the gating of cell populations.

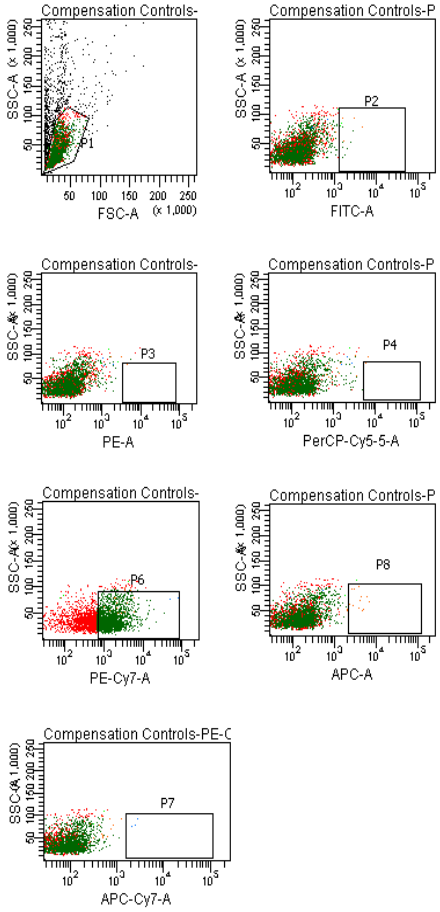
Compensation controls unstained cells	Compensation controls stained cells
	

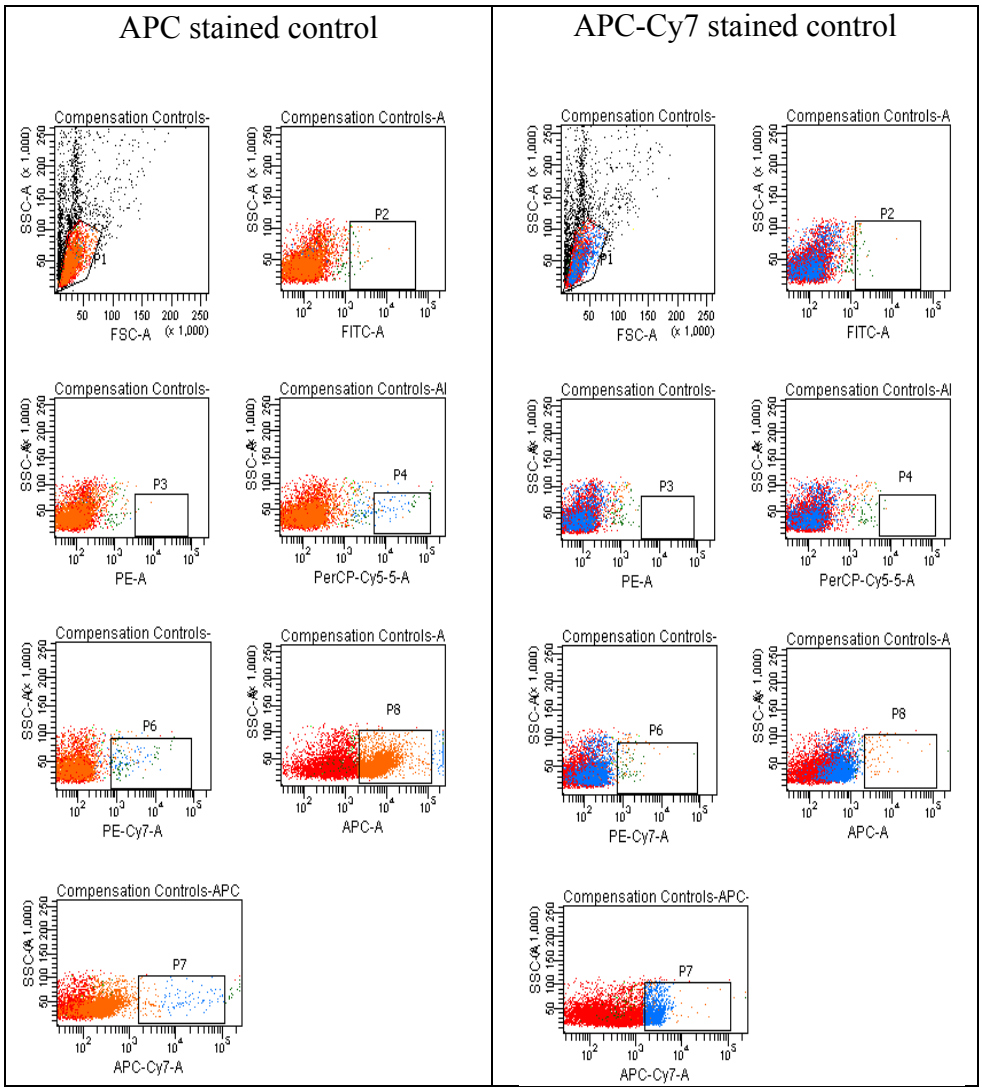

FITC stained control
PE stained control
Compensation Controls-
Compensation Controls-F
Compensation Controls-P
Compensation Controls-FI
Compensation Controls-FITC
Compensation Controls-PE S
SSC-A (x 1,000)
FSC-A (x 1,000)
FITC-A
PE-A
PerCP-Cy5-5-A
PE-Cy7-A
APC-A
APC-Cy7-A
P1
P2
P3
P4
P6
P7
P8

PerCP-Cy5-5 stained control



PE-Cy7 stained control




APC stained control
APC-Cy7 stained control
Compensation Controls-
Compensation Controls-A
Compensation Controls-Al
Compensation Controls-APC
Compensation Controls-APC-
SSC-A (x 1,000)
FSC-A (x 1,000)
FITC-A
PE-A
PerCP-Cy5-5-A
PE-Cy7-A
APC-A
APC-Cy7-A
P1
P2
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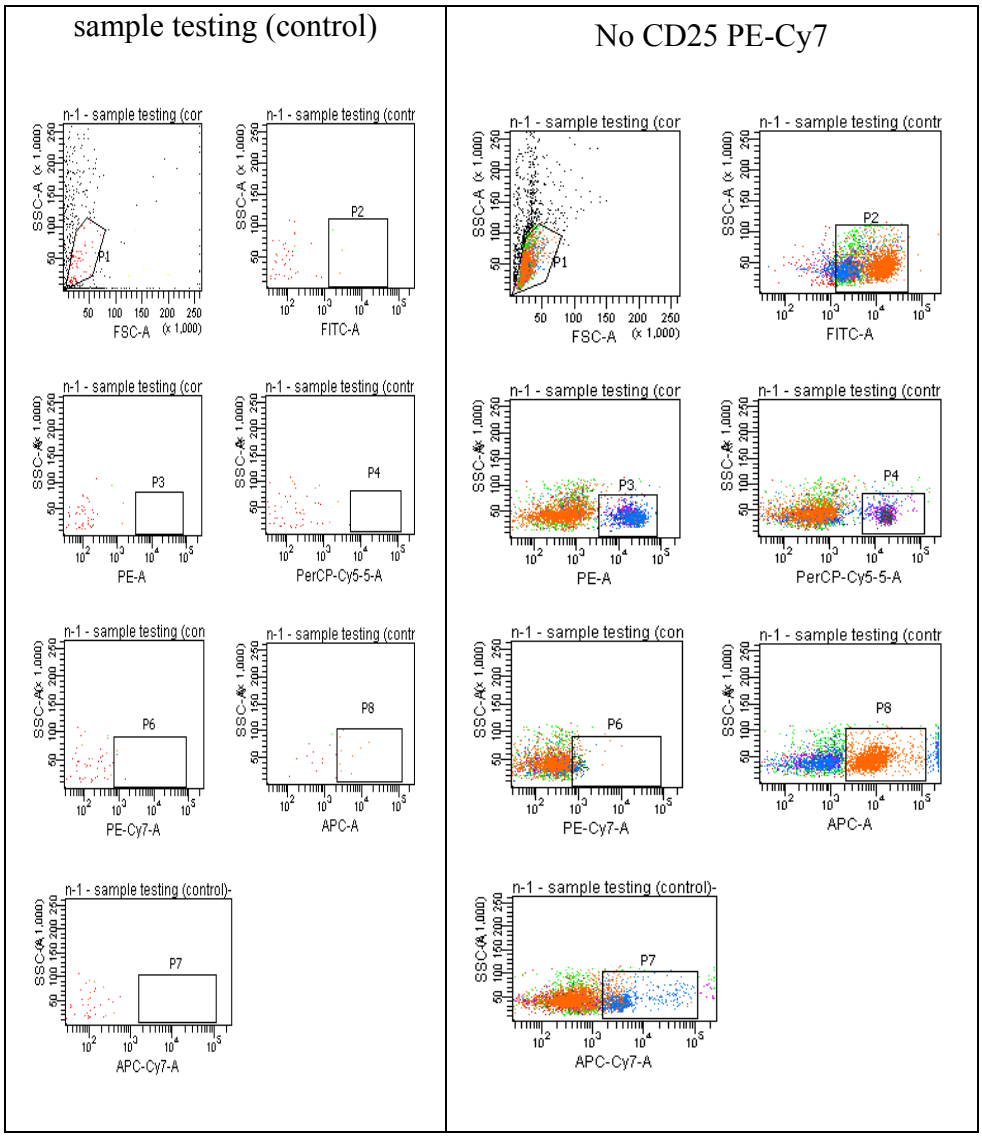
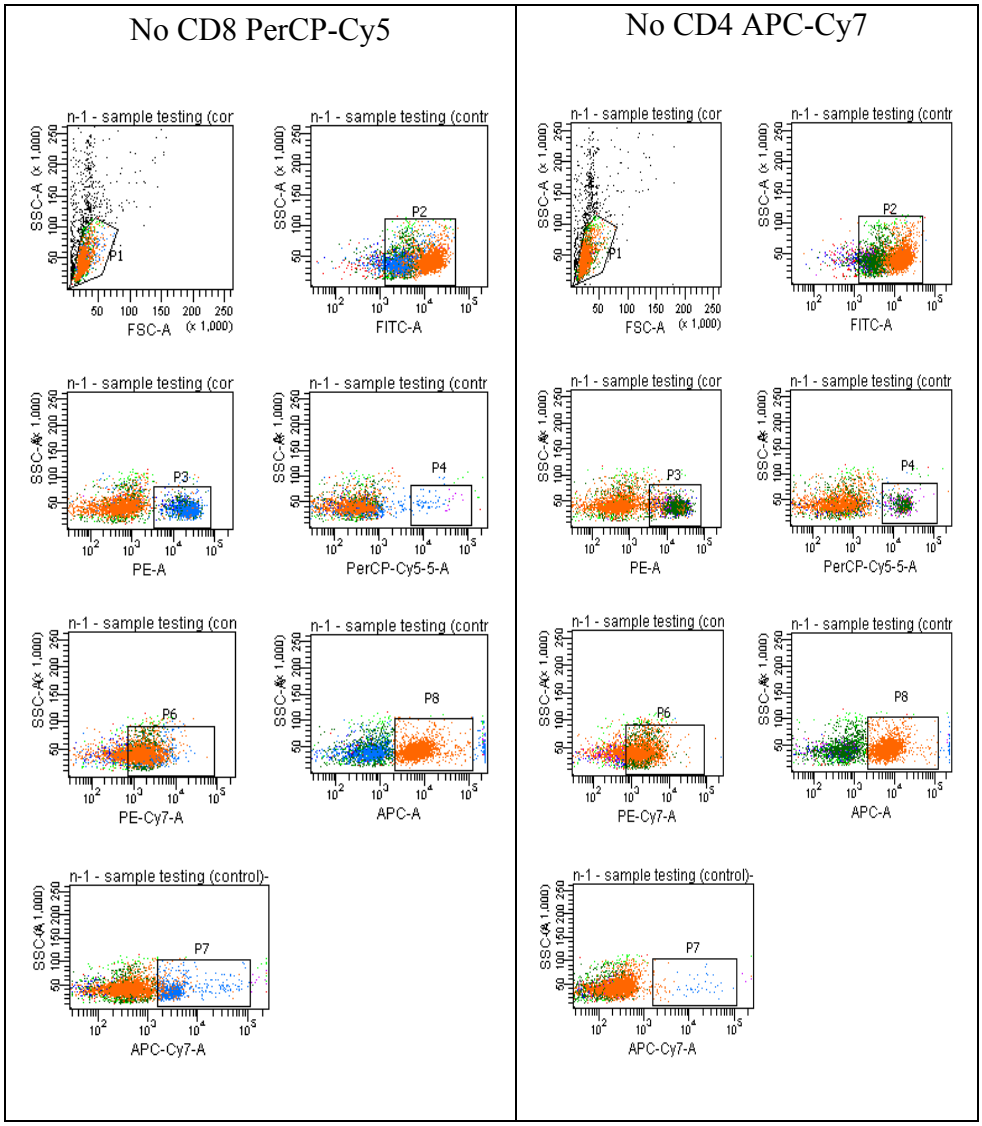
5.5.1 FMO control or all the Fluorescence -1




No CD8 PerCP-Cy5
No CD4 APC-Cy7
n-1 - sample testing (cor
n-1 - sample testing (contr
SSC-A (x 1,000)
FSC-A (x 1,000)
FITC-A
PE-A
PerCP-Cy5-5-A
PE-Cy7-A
APC-A
APC-Cy7-A
P1
P2
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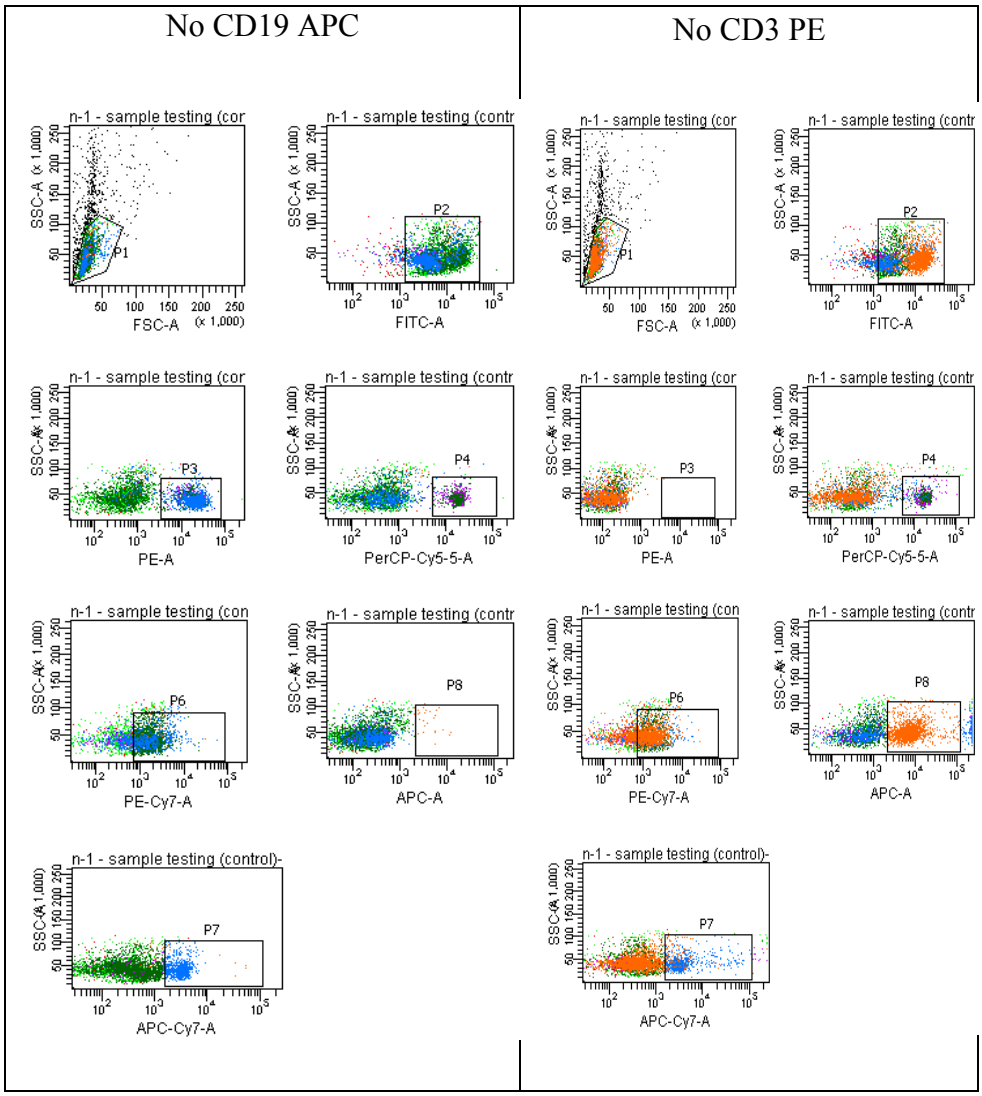
P4
P6
P7
P8
n-1 - sample testing (con
n-1 - sample testing (control)-


No CD19 APC
No CD3 PE
n-1 - sample testing (cor
n-1 - sample testing (contr
n-1 - sample testing (control)-
SSC-A (x 1,000)
FSC-A (x 1,000)
FITC-A
PE-A
PerCP-Cy5-5-A
PE-Cy7-A
APC-A
APC-Cy7-A
P1
P2
P3
P4
P6
P7
P8

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