Abstract

Complement is part of the innate immune defence. It can be activated via three pathways, the classical pathway, the lectin pathway, and the alternative pathway. It comprises soluble factors and receptors. Properdin (Factor P) is a soluble component of the alternative complement pathway which acts as an important positive regulator of complement activation that stabilises the alternative pathway convertases (C3bBb) and C3bBb5b in the feedback loop of the alternative pathway, protecting them from rapid inactivation. The thesis is interested to see the role of properdin in cellular and humoral immune responses by *in vitro* and *in vivo* analyses.

Firstly, properdin was examined for its global activity by characterisation of promoter activity of the human gene for properdin which involved using bioinformatics and molecular biology. The promoter activity was measured by dual-luciferase reporter system. The findings appeared to have activity in the 670bp properdin plasmid construct in U937 non-LPS transfection but the transfection upon LPS stimulation was not successful.

Next, by using a properdin-deficient mouse line as a tool, it is interested to investigate the role of properdin in immunity by using properdin-deficient mice as model in pneumococcal vaccination studies, *in vitro* characterisation of dendritic cells and mycobacterium infectious studies in bone marrow-derived dendritic cells culture.

In vaccination studies demonstrated that vaccination proved efficacious as both properdin-deficient and WT had increased in total IgM level and specific IgM level after the vaccination as measured by commercial ELISA for total IgM and specific ELISA for PPS2 IgM. In the absence of properdin, specific anti-polysaccharide antibodies of the IgM type are made. Vaccinated properdin-deficient mice do not differ from wild type in their immunoglobulin response to the pneumococcal polysaccharide vaccine. Meanwhile properdin-deficiency had a benefit in survival, independent of vaccination.

For the *in vitro* characterisation of dendritic cells, dendritic cells are derived from bone marrow and spleen culture, and flow cytometry measured dendritic cells phenotype surface markers. Both bone marrow and spleen dendritic cells derived from properdin deficient mice are impaired to be activated and mature as dendritic cells compared to wild type mice. The study presently concludes that the presence of properdin is essential to allow dendritic cells to develop their activated phenotype and properdin is a relevant player in dendritic cell mediated immune response.

Further investigation of function of generated BM-derived DC of WT and properdin-deficient towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

In overall findings of mycobacterial viability, secretion of TNF-α and intracellular containment of mycobacterium in BMDC, it is concluded that properdin has no role of in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

In conclusion, having properdin is essential to help the complement system as part of defence mechanism against infection. Additionally, properdin could play a 'double-edged' role.

Acknowledgement

Thank to God for all His mercy and bless.
Firstly I am writing to express my sincere appreciation both of my supervisors Dr. Cordula Stover and Prof. Peter Andrew (my highest appreciation would not be enough to be expressed in here by words for both of them). Many thanks and my highest gratitude to Dr. Cordula Stover for her best support and help during my study in Leicester. Not forgotten to Prof. Peter Andrew for all the valuable advise, critical thinking and in helping with the animal experiments.

I am gratefully to thank my family especially to my husband Mr. Shahrizan Sabtu and my parents; Roosnani Saad and Salehen Baba for all their loves and support.
I would like to extend my big thanks for my family members; sisters, uncle- Muhd Yusuf Saad and ibu for being helpful and supportive. Not forgotten to my special friends that always support me, Rabia and Ayesha, Kak min and family.

I would like to thank to all the members in department of Infection, Immunity and Inflammation and also individual:
Aline Dupont, Eddy Cheah, Dr. Rana El-Rachkidy-Lonnen, Irina Elliot, Sarah Glenn, Sara Sandrini, Fatima, Hind, Areej, Noura. Many thanks for being a good friend and helping me a lot in any of my difficulties.
Also not forgotten particularly to: Dr. Caroline Beardsmore, Dr. Galina Mukomolova, Dr. Mike Browning, Prof. W. Schwaeble and group, Dr. Primrose Freestone, Elizabeth, Sheila Hewitt, and Lynn Flower and to others too, thank you very much.

Lastly I would like to thank University Malaya (staffs: Ayu, Siti Balqis, Dato Prof. Amin, and Dept Molecular Medicine) and Ministry of Higher Education, Malaysia for the scholarship.

Please forgive me in case I missed out some names but deep in my heart I appreciated everyone around me.

Table of Contents

Abstract i
Acknowledgement ii
Abbreviations vii
Chapter 1: General introduction 1
1.1 The immune system 1
1.2 The complement system 4
1.3 The alternative pathway 8
1.4 Properdin 11
1.4.1 Deficiency and Properdin 14
1.4.2 Properdin-deficient animal model 17
1.5 Aims and objective of the study 20
Chapter 2: General Materials and methods 21
2.1 Cell culture techniques 21
2.1.1 General handling 21
2.1.2 Thawing the cells 21
2.1.3 Freezing the cells 22
2.1.4 Passaging Cells 22
2.1.5 Cell counting 23
2.1.6 Seeding the cells 23
2.2 Bacteriological techniques 24
2.2.1 Preparation of blood agar plates 24
2.2.2 Growth of S. *pneumoniae* 24
2.3 DNA techniques 25
2.4 Protein techniques 26
2.5 Statistical analysis 26
Chapter 3: Study of promoter activity of human properdin gene 27
3.1 Introduction 27
3.1.1 Overview of regulatory elements in Eukaryotic Gene 27
3.1.2 Promoter studies of complement genes 28
3.1.3 Background to the study 29
3.2 Materials and methods 32
3.2.1 Materials 32
3.2.2 DNA preparation 32
3.2.2.1 Peripheral blood mononuclear cells (PBMC) preparation 33
3.2.3 Primers 33
3.2.4 Plasmid constructs 34
3.2.5 Polymerase chain reaction (PCR) 37
3.2.6 Agarose gel electrophoresis 38
3.2.7 Gel extraction (DNA purification from agarose gels) 39
3.2.8 Estimation of the DNA concentration 39
3.2.9 DNA ligation 40
3.2.10 Transformation 40
3.2.11 Mini prep-small scale plasmid preparation 41

3.2.11.1 Experimental procedure 41
3.2.12 Maxi prep-Large scale plasmid preparation 43
3.2.13 Restriction digest 43
3.2.14 Sequencing 44
3.2.15 Dual-luciferase transfection 45
3.2.15.1 Transient transfection for U937 suspension cells 45
3.2.15.2 Evaluation of transfection efficiency by Dual Luciferase assay 46
3.3 Results 48
3.3.1 Extraction of genomic DNA 48
3.3.2 Analysis of promoter activity in the subfragment of the properdin gene 48
3.3.4 Analysis of expression of promoter activity 52
3.4 Discussion 55
3.4.1 Promoter activity in the subfragment of properdin gene 55
3.4.2 Limitations of the study 57
Chapter 4: Analysis of the immune response of properdin-deficient and wild type mice in an intraperitoneal model of polysaccharide vaccination 61
4. 1 Introduction 61
4.12 Polysaccharide antigens 62
4.13 The importance of complement activation towards polysaccharide antigens 65
4.14 Background of the study 66
4.2 Materials & methods 68
4.2.1 Mice 68
4.2.2 Pneumococcal strains and preparation of the challenge dose 68
4.2.3 Experimental procedure 69
4.2.3.1 Serum preparation 71
4.2.3.2 Disease severity score 71
4.2.4 CFU count 72
4.2.5 Archive organs for paraffin embedding 73
4.2.6 Histopathologic evaluation of lungs 74
4.2.6.1 Polymorphonuclear leukocytes (PMN) count 74
4.2.7 Peritoneal cells count 75
4.2.8 ELISA 76
4.2.8.1 Commercial ELISA for total serum IgM and IgG 76
4.2.8.2 Polysaccharide specific ELISA 78
4.2.8.3 C3 ELISA 81
4.2.9 Statistical analysis 82
4.3 Results 83
4.3.1 Analysis of immune responses following intraperitoneal polysaccharide vaccination for properdin-deficient and wild type mice 84
4.3.1.1 Determination of antibody response during vaccination stage 84
4.3.1.2 Determination of serotype specific antibody responses 88
4.3.1.3 Differential cell count of peritoneal cell population 100
4.3.2 Analysis of immune responses following intranasal pneumococcal infection (in continuation of vaccination study) for properdin-deficient and wild type mice 102
4.3.2.1 Viable counts of blood at 24 hours post-infection of streptococcal infection 102

4.3.2.2 Survival of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae* 105
4.3.2.3 Disease severity of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae* 108
4.3.2.4 Determination of antibody response following intranasal infection with *S. pneumoniae* D39 110
4.3.2.5 Serum concentration of C3 of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae* 119
4.3.2.6 PMN recruitment in the lungs 120
4.3.2.7 Haematoxylin and eosin staining 122
4.4 Discussion 125
4.4.1 Role of properdin in immune responses following intraperitoneal polysaccharide vaccination 125
4.4.2 Role of properdin in immune responses following intranasal challenge of vaccinated mice 130
4.4.3 Unexpected antibodies raised in absence of properdin following intraperitoneal PBS 135
4.4.4 Role of properdin in immune responses following intranasal challenge of non-vaccinated mice 136
4.4.5 Role of properdin in this study 138
Chapter 5: The role of properdin in maturation of dendritic cells in vitro 139
5.1 Introduction 139
5.1.1 Dendritic cell 139
5.1.1.1 Development and functional properties of dendritic cells 140
5.1.1.2 Immature dendritic cells 140
5.1.1.3 Mature dendritic cells 141
5.1.1.4 Migration, maturation and function dendritic cells 142
5.1.2 What's known about dendritic cells and complement 144
5.2 Materials and Methods 146
5.2.1 Materials 146
5.2.2 Dendritic cell culture 147
5.2.2.1 Bone marrow preparation 148
5.2.2.2 Spleen 149
5.2.3 Flow cytometry analysis of cell surface markers 153
5.2.3.1 Monoclonal antibody staining and flow cytometry 154
5.2.4 Determination of TNF-alpha production 156
5.2.5 Statistical analysis 157
5.3 Results 158
5.3.1 Generation of dendritic cells 158
5.3.1.1 Phenotypic analysis of dendritic cells by FACS histogram 158
5.3.2 Maturation of dendritic cells by lipopolysaccharides (LPS). 166
5.3.3 Morphology assessment of the characterised dendritic cells. 169
5.3.4 Measurement of cytokine TNF-α secretion by characterised dendritic cells. 172
5.4 Discussion 175
5.4.1 Phenotypic analysis of characterised dendritic cells 176
5.4.2 The findings on the bone marrow-derived dendritic cells 177

5.4.3 The findings on the splenic dendritic cells 178
5.4.4 Cytokines TNF-α production 180
5.4.5 Limitations of the study 182
5.4.6 Properdin essential to allow dendritic cells to develop their activated phenotype 183
Chapter 6: The role of properdin in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG 184
6.1 Introduction 184
6.1.1 Mycobacterium tuberculosis 184
6.1.2 Modulation of immune response: Mtb and the dendritic cells 185
6.1.3 Relationship of complement pathway in mycobacterial infection 187
6.2 Materials and methods 189
6.2.1 Infection of Dendritic cells with Mycobacterium tuberculosis H37Rv and Mycobacterium bovis 189
Mycobacteria used for infection 189
6.2.2 Bone marrow-derived Dendritic Cell (BMDC) Culture 190
6.2.3 Infection of dendritic cells 190
6.2.4 Intracellular load of mycobacterium within bone marrow-derived-dendritic cells 191
Colony-forming unit count 191
6.2.5 Determination of TNF-alpha production 192
6.2.6 Ultrastructural analysis of infected murine dendritic cells 193
Procedure for processing Mycobacteria for electron microscopy: 193
6.2.7 Statistical analysis 194
6.3 Results 195
6.3.1 Determination of mycobacterial viability within murine bone marrow-derived dendritic cells during the course of in vitro infection. 195
6.3.1.1 Infection of dendritic cells with *Mycobacterium tuberculosis* 195
6.3.1.2 Infection of dendritic cells with Mycobacterium bovis BCG 198
6.3.2 Level of cytokine TNF-α by BMDC infected with mycobacteria. 200
6.3.3 Ultrastructural analysis of Mycobacterium bovis (BCG) infected BMDC. .. 203
6.4 Discussion 208
6.4.1 The role of properdin in mycobacterial viability within murine bone marrow-derived dendritic cells during the course of in vitro infection. 208
6.4.2 The role of properdin in secretion of TNF-α by BMDC infected with mycobacteria. 210
6.4.3 The role of properdin in intracellular location and containment of mycobacterium in BMDC. 213
6.4.4 The role of properdin in the immune response of BM-derived DC towards Mycobacterium tuberculosis and Mycobacterium bovis BCG 215
Chapter 7: Conclusion 216
References 220
Appendix 232

Abbreviations

µg	microgram
µl	microlitre
ACD	Acid citrate dextrose
ADP	Adenosin tri-phosphate
AP	Alternative pathway
APC	Antigen presenting cells
APP	Acute-phase proteins
BHI	Brain heart infusion
BMDC	Bone marrow-derived Dendritic Cell
bp	Base pairs
BSA	Bovine serum albumin
C	Complement component
CD	Cluster of differentiation
CFU	Colony forming unit
CLP	Caecum ligation and puncture
CMV	Cytomegalovirus
CPS	Capsular polysaccharide
CR	Complement receptor
CRP	Collagen related protein
CWPS	Cell wall polysaccharide
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cells
dH_2O	distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide tri-phosphate
E.	*Escherichia*
ECL	Enhanced chemiluminescence
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBL	European molecular biology laboratory
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDC	Follicular dendritic cells
fig.	figure
FITC	Fluorescein isothiocyanate
HB	Hypertrophy of bronchiole
HCI	Heavy cellular infiltration
HRP	Horse radish peroxidase
i.p	intraperitoneally
IFN	Interferon
Ig	Immunoglobulin
IHC	immunohistochemistry

IL	Interleukin
IMS	Industrial methylated spirit
IPTG	Isopropyl-β-D-thiogalactopyranoside
KD	Kilodaltons
L	litre
LB	Luria broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP	MBL-asssociated serine protese
MBL	Mannose-binding lectin
MCI	Medium cellular infiltration
MFI	Mean fluorescence intensity
mg	milligram
ml	millilitre
MOI	multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MW	molecular weight
N.	*Neisseria*
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
SDS	sodium dodecyl sulfate
PO	Propylene oxide
PPS	Pneumococcal polysaccharide
PROP	Properdin
Prop-def	Properdin-deficient
rmGM-CSF	recombinant murine Granulocyte-macrophage colony-stimulating factor
RT	room temperature
RLU	Relative luciferase activity
TAE	Tris-Acetate-EDTA
TBS	Tris buffer saline
TEM	Transmission electron microscopy
TPA	Tetradecanoyl phorbol acetate
TSP	thrombospondin
TSR	type sequence repeat (type 1 repeats)
UVP	UV transluminator
v/v	volume per volume
w/v	weight per volume
WT	Wild type

Chapter 1: General introduction

1.1 The immune system

The immune response is made of innate and the adaptive mechanisms. Both systems usually work together to eliminate pathogens, thus, ensuring an effective immune system. The immune system must be continuously under strict control to avoid underactivity, which can result in severe infections and tumours of immunodeficiency, or overactivity in allergic and autoimmune disease (Delves and Roitt, 2000, Parkin and Cohen, 2001).

The innate immune system is the body's first line of defence against invasion of pathogens or foreign substances. The innate immunity capable to recognise microbial molecules such are called pathogen-associated molecular patterns (PAMPS) which include LPS (from the gram-negative cell wall), peptidoglycan and lipoteichoic acids (from the gram-positive cell wall), the sugar mannose (a terminal sugar common in microbial glycolipids and glycoproteins), bacterial and viral unmethylated CpG DNA, bacterial flagellin, the amino acid N-formylmethionine (found in bacterial proteins), double-stranded and single-stranded RNA of viruses, and glucans of fungal cell walls. The innate immunity consists of anatomical barriers and physiological responses such as skin and mucus, defense mechanisms such mechanical removal, pattern-recognition, antigen-nonspecific defense, the complement pathways, phagocytosis, inflammation, and fever together with cells of immune essentials as phagocytic cells i.e. macrophages, granulocytes, monocytes or dendritic cells which acts in immediately as a barrier to infection.

Whereas the adaptive immunity (acquired immunity) is more specialised as it is develops a specific immunity by generating antibodies and T-cells to recognise and eliminate specific pathogens. The response takes longer to develop but it is effective to prevent subsequent infection and long-lasting infections as it generates the memory cells.

There are two types of adaptive immunity which are: cellular and humoral immunity.
The cellular immunity is mainly involved T cells against pathogens upon interaction with infected cells or cancer cells. There are two types of T cells that involved; T cytotoxic cell (Tc, CD8+) and T helper cells (Th, CD4+) that recognising foreign antigens presented MHC class I and MHC class II molecule respectively on the surface of infected cells. The Tc cell is works destroying the cells by releasing cytolytic proteins whereas Th is helping B cells and result in the increasing production of antibodies by B cells.

Then the humoral immunity with function of immunisation is mediated by antibodies secreted by B lymphocytes and acts mainly against antigen in body fluids.
The humoral immunity involves naïve B cells that will activate and differentiate into memory B cells and effector B cells.

In linking between the innate and the adaptive immunity, complement system plays a part in the linkage even though the complement is an innate immunity but also acts enhance the adaptive immunity. Part of the mechanism is by the co-operation of complement activation

products and cell surface receptor in which leads for the regulation of B and T cell responses.

In cellular immunity, complement involves in T cell priming and helping the T cells in identifying the pathogens as foreign by specific receptors. Added to it, products of complement activation also alter T cell responses as releases stimulation of the chemoattractant receptors (Carroll, 2004).

Meanwhile complement enhances humoral immune response via (Carroll, 2004):

1) Complement receptors CD21 and CD35 (expressed on B cells and follicular dendritic cells (FDC)).

2) Highly maintenance of C3-coated immune complexes in lymphoid area as antigen localisation to FDC in lymphoid follicles.

The interaction of cell surface receptors, specific protein recognition or secreted antibodies releases cleavage product activates the complement to enhances humoral immune i.e. having CD21 and CD35, C3 cleavage product of complement attaches to antigen provides a specific ligand for uptake of antigen (Carroll, 2004). In a cycle, attachment to antigen leads to B cell activation. Then with help of T cell helper, the B cell binds to the antigen and elicits B cell response as memory immunity.

Additionally, natural antibodies (repertoire of natural IgM) and complement are co-operating with each other in induction of T-independent antibody responses (Ochsenbein and Zinkernagel, 2000) as a part of linking the innate and the adaptive immunity.

1.2 The complement system

The complement system is a major player in innate immunity plays an important role as defense against foreign invaders by direct lysis of microbes and infected cells, clearance of immune complexes, chemotaxis of phagocytic cells, and activation of the complement cascade leads to opsonisation of foreign microbes, promoting their elimination and ultimate destruction (Delves and Roitt, 2000, Parkin and Cohen, 2001). It has been shown to be an important player also in reperfusion injury, transplantation rejection and immunological memory (Carroll, 2004, Walport, 2001).

The complement system is a system that comprises soluble factors and receptors of more than 30 different serum proteins.

The proteins circulate in an inactive form, but in response to the recognition of molecular components of microorganism, they become sequentially activated, working in a cascade where the binding of one protein promotes the binding of the next protein in the cascade. The hierarchically connected cascade of enzymatic reactions then generates, as a central outcome, C3 cleavage and degradation products that act together to attack extracellular forms of pathogens which are essential in tagging of pathogens, chemo-attraction of inflammatory cells, and triggering of B-cells. Host cells are protected against complement activation by the presence of membrane bound and soluble regulators

The complement system is activated via: the classical pathway, the lectin pathway (MBL), the alternative pathway (AP) and latest the terminal pathway (Figure 1.1) (Carroll, 2004, Dunkelberger and Song, Walport, 2001).

The pathways differ in the manner in which they are activated but all this pathways merge at the end, the terminal pathway or lytic pathway, at the level of C3 (formation of a key enzyme called C3 convertase), the crucial enzymatic effector functions of complement leading to the activation of common terminal sequence and generation of C5b-C9 membrane attack complex (MAC). Formed at endpoint of the complement system, this membrane attack complex is then fully inserts into membranes of bacteria or pathogens to create a pore and causes osmotic lysis that kill the attacked bacteria (Walport, 2001, Wurzner, 2003). Complement fragments called opsonins direct the phagocytosis by adhering to microorganisms and anaphylatoxins promote leukocyte chemoattraction.

The classical pathway was the first pathway that had been discovered and involves individual proteins termed C1, C4, C2, C3, C5, C6, C7, C8 and C9. The important C1, in active form C1 complex with subunits: one molecule of C1q, two molecules of each C1r and C1s. The classical pathway is activated by C1q binding to the complement activator either directly or via recognition of immune complexes.

The MBL pathway is activated via recognition of carbohydrate structures on micro-organisms by the binding of mannose-binding lectin (MBL) or ficolins to the complement activator. MBL is associated with serine proteases termed MASP-1, MASP-2, and MASP-

3, of which MASP-2 upon activation of C4 cleaves (Matsushita et al., 2000, Matsushita et al., 1992).

The terminal pathway or Lytic pathway is activated as the C1 complex initiates the classical pathway, which cleaves C2 and C4, followed by convergence at the vital point of the complement component C3 to initiate the terminal pathway. This terminal pathway leads to the assembly of C5 to C9, which forms a membrane attack complex (MAC) on targets and results in cell lysis (Walport, 2001).

Of all of these, the alternative pathway from the complement system is the one interested in.

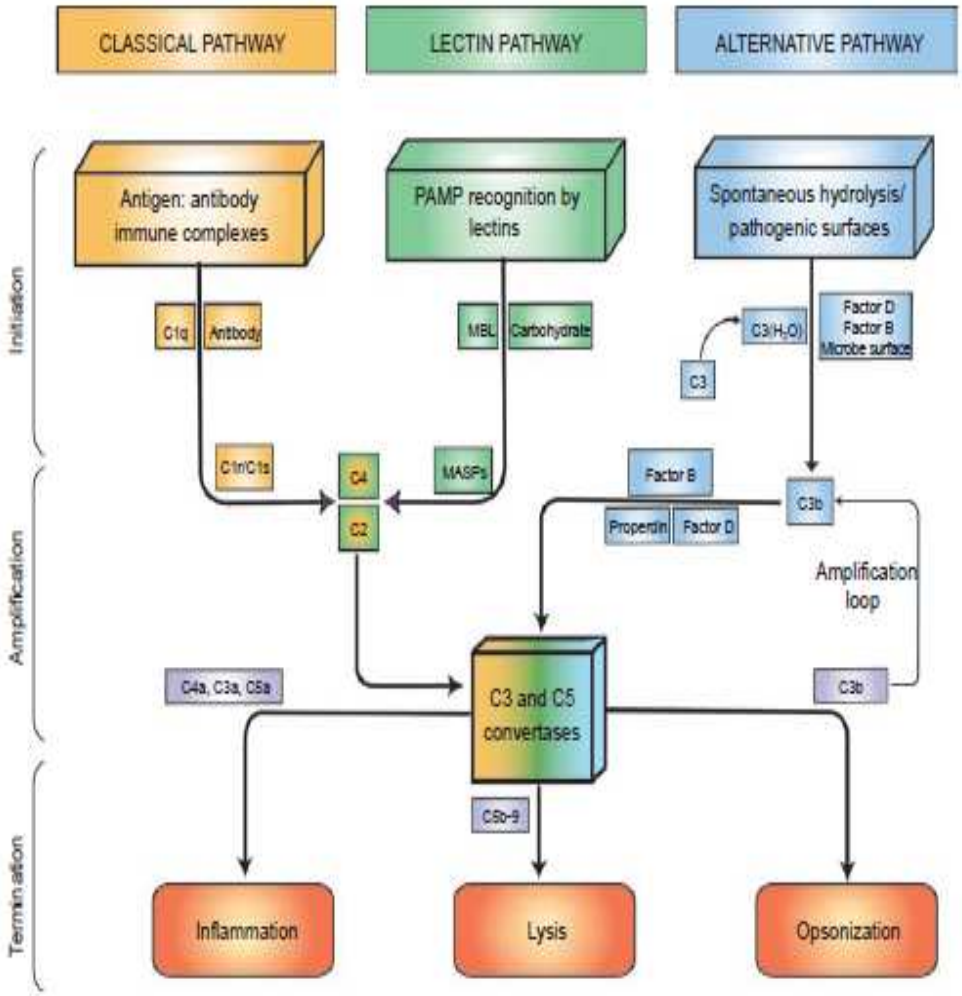


Figure 1.1: The complement pathway. Complement can be activated through: Classical pathway (which is antibody-dependent, initiated by Ab:Ag complexes); Lectin pathway (triggered by mannan-binding lectin bound to pathogen surface) and Alternative pathway (antibody-independent, initiated by C3b on microbial surfaces). All this pathways merge at the end leading to the activation of common terminal sequence and generation of C5b-9 membrane attack complex (Figure taken from Dunkelberger and Song, 2010 (Dunkelberger and Song)).

1.3 The alternative pathway

The alternative pathway involves components such as: C3b (or C3i), factor B, factor D, properdin and shared the same late proteins by the classical pathway, C3, C5, C6, C7, C8 and C9.

The activation of this pathway requires presence of C3b together with factor B and D.

The alternative pathway that can be initiated by a spontaneously activated complement component that binds on the surface of pathogen.

The alternative pathway is activated via C3b, produced either by the classical or lectin pathways or hydrolysed C3 (C3-H_2O), which continuous spontaneous activated as C3 contains an internal thioester bond can hydrolysed by water can and form C3i, a molecule similarly to C3b (Pangburn and Muller-Eberhard, 1980, Pangburn et al., 1981).

Briefly activation of the alternative complement pathway begins when C3b (or C3i or C3-H_2O) acts as a binding site binds to the cell wall and other surface components of microbes. The generated C3b molecule is quickly hydrolysed by water since is working in the fluid phase and will rapidly bind on cell surface as C3b comes into contact with pathogen surface. In the presence of Mg2+, complement protein Factor B combines with the cell-bound C3b to form C3bB.

Then, Factor B is cleaved by Factor D into Bb and Ba, subsequently Bb remains bound to C3b while Ba is released into the surrounding fluid phase and thus forming the fluid phase C3bBb (C3 convertase).

The C3 convertase enzymatically cleaves substantially molecules of C3 into the fluid phase product C3a and membrane bound C3b to deposits many C3b molecules on the target pathogen surface. The amplification loop of the alternative pathway is initiated. Here, a serum protein called properdin (also known as Factor P) then binds to the Bb to form C3bBbP to stabilise the complex with longer lasting enzyme activity essential for effective alternative pathway amplification (Fearon and Austen, 1975a, Fearon and Austen, 1975b). At same time, C3bBb3b (C5 convertase) is formed as some of the C3b also binds to some of the C3bBb. The C5 convertase then is capable of splitting C5 molecules into C5a and C5b. The pathogen surface is then opsonised, ready for terminal complement components and other immunoregulatory functions (Harboe and Mollnes, 2008). Interestingly, properdin is the only known regulator of complement which enhances activation.

The remaining complex C3bBb is the initial fluid phase C3 convertase of the alternative pathway (Pangburn and Muller-Eberhard, 1980, Pangburn et al., 1981) is parallel to the C3 convertase C4bC2b of the classical pathway (Fearon et al., 1975).

In order protecting the host, there are several inhibitory proteins in regulating the complement against tissue damage (Harboe and Mollnes, 2008).

As on host cell, the alternative pathway is inhibited by complement regulatory proteins which present in the plasma or on host cell membranes. The inhibition happen by replacing Bb from the formed convertase, when the membrane attached protein decay-accelerating

factor (DAF) and the complement receptor 1 (CR1) won over Factor B for binding C3b on the cell surface (Song et al., 1996).

In addition, formation of C3 convertase is also inhibited by cofactors, such as membrane cofactor of proteolysis (MCP), Factor H or CR1 are bind to C3b, then the plasma protease Factor I cleave C3b to its inactive derivate iC3b (Pangburn, 1983, Pangburn et al., 1983, Sim et al., 1993).

However pathogen surfaces lack these regulatory proteins thus the C3bBb convertase can form and persist.

The alternative pathway is also called the properdin pathway or amplification loop, since it gives a feedback amplification loop to generate more C3b. Properdin which may bind to many microbial surfaces and stabilises the convertase (Fearon et al., 1975, Harboe and Mollnes, 2008, Pangburn et al., 1983) and as to date, the C3 convertase of alternative pathway are found to form following direct binding of properdin to the surface of pathogen (Hourcade, 2006, Spitzer et al., 2007).

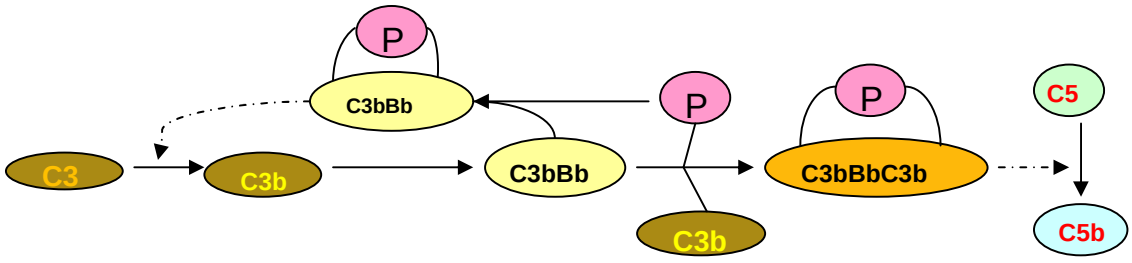


Figure 1.2: Schematic illustration of standard activation of the alternative pathway of the complement system.

1.4 Properdin

Properdin was first described by Pillemer (Nolan et al., 1992, Pillemer et al., 1954) when Pillemer and his collaborators found properdin bound to target complexes when studying properdin in interaction with zymosan (Harboe and Mollnes, 2008, Pillemer et al., 1954, Spitzer et al., 2007). After controversial discovery more than 50 years ago, the properdin system now known as the alternative pathway was reborn more than 20 years later (Lepow, 1980), as more data published point out the importance of AP as part of complement system

Properdin (Factor P), a soluble glycoprotein is a component of the alternative complement pathway which acts as a positive regulator of complement activation that stabilises the AP convertases (C3bBb) and C3bBb5b in the feedback loop of the AP protecting them from rapid inactivation (Hartmann and Hofsteenge, 2000). Properdin activates the AP has been shown to cause a 10 fold increase in the stability of the AP C3 convertase both on immune complexes and targets such as pathogens (Harboe and Mollnes, 2008, Hourcade, 2006).

Properdin, found on limited cell types has been shown in a variety of cells of immune cells with haematopoietic origin that are capable of producing or storing properdin such as macrophages (Bentley et al., 1978), monocytes (Minta, 1988), dendritic cells (Reis et al., 2006), lymphocytes (Schwaeble et al., 1993), neutrophils (Schwaeble and Reid, 1999), bronchial epithelial cells (Rosica et al., 1965), endothelial cells (Bongrazio et al., 2003),

hepatocytes (Maves and Weiler, 1994), stromal vascular fibroblasts (Choy and Spiegelman, 1996) and recently mast cells (Dupont, 2008).

In human plasma, properdin concentration is approximately 5-15ug/ml (Linton and Morgan, 1999). Properdin exists as odd structure formed by oligomerisation of a rod-like monomer into a mixture of cyclic polymers (head to tail) dimers, trimers or tetramers. Mature properdin unglycosylated monomer is a 53-kD protein (Smith et al., 1984) that occurs in plasma at a specific ratio of dimers, trimers, and tetramers of 26:54:20 (Nolan et al., 1992, Pangburn, 1989, Perdikoulis et al., 2001). Properdin oligomerisation is an early intracellular event as there are no monomer of properdin has been detected in serum and the ratio of properdin in serum is under different influence (Farries and Atkinson, 1989).
The function of properdin depends on the oligomerisation. The larger the oligomer, the greater is the activity (Pangburn, 1989). Polymeric forms of properdin are strong and stabile (Pangburn, 1989) although the interactions between monomers are non-covalent (Minta and Lepow, 1974).

Properdin is made up of thrombospondin like repeats,composed of multiple identical protein subunit called ‘thrombospondin type 1 repeats’(TSRs). The TSRs with approximately 60 amino acids each basically are six thrombospondin type 1 repeats (based on exon/intron boundaries), enclosed by N- and C-terminal part are named TSR-0 (N-terminal) to TSR-6 (C-terminal), that show no homology to other proteins (Perdikoulis et al., 2001, Sun et al., 2004).

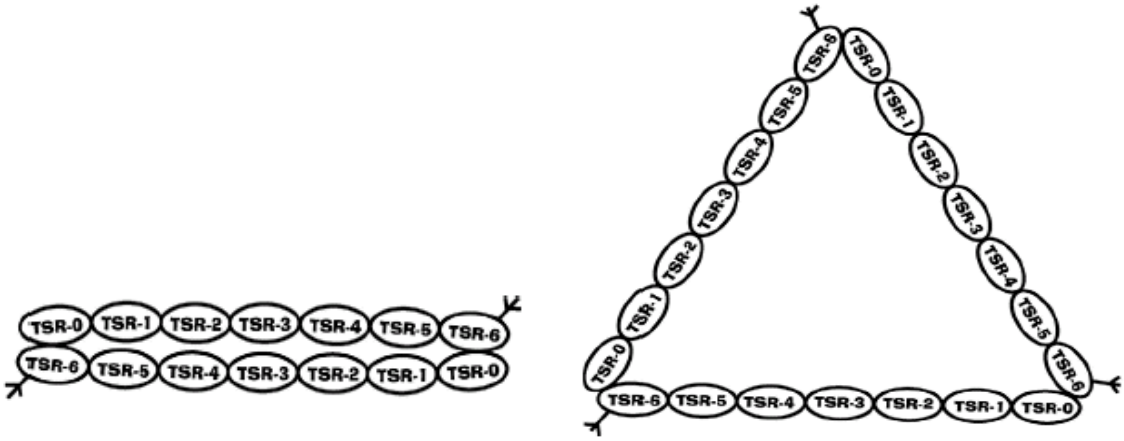


Figure 1.3: Architecture of properdin dimer and trimer. TSR arrangement in the properdin dimer and trimer. TSR-1 to TSR-6 shows the full-length TSR domains while TSR-0 show a truncated N-terminal TSR domain. ψ symbol represent the location of the potential N-linked glycosylation site on TSR-6. (Figure taken from (Sun et al., 2004))

All the TSRs are important for the functioning of properdin function as shown in table 1:

Type of TSR	Role
TSR-0 and TSR-6 (in monomer)	Important in the oligomerisation of the protein (Higgins et al., 1995).
TSR-0, TSR-1, TSR-2 and TSR-3	Act as spacers to ensure TSR-4 and TSr5 are available to others molecules (Sun et al., 2004).
TSR-4	Important in the stabilisation of the C3bBb complex by binding to low affinity C3 (Higgins et al., 1995, Perdikoulis et al., 2001).
TSR-5	Important in the stabilisation of the C3bBb complex (Higgins et al., 1995, Perdikoulis et al., 2001).
TSR-6	Important in oligomerisation as a major site of the binding to C3b and sulphatide (Higgins et al., 1995, Perdikoulis et al., 2001).

Table 1.1: Role of different TSRs that essential in properdin (Higgins et al., 1995, Perdikoulis et al., 2001, Sun et al., 2004).

Properdin is through different post-translational modifications gave beneficial to the system. Each TSR is containing the WXXW motif sequence for C-mannosylation (Hartmann and Hofsteenge, 2000). The post translational modification rise C-mannosylated trytophans in properdin then lead to expose at the surface of properdin, aided by many pathogenic microbes carry mannose-binding receptors on their surfaces therefore could influence the binding to C3b and Bb (Hartmann and Hofsteenge, 2000).

1.4.1 Deficiency and Properdin

Population studies have shown that deficiencies of the complement system, result in an increased susceptibility to infection, rheumatic disorders or angio-oedema (Fig. 1.4) (Morgan and Walport, 1991).

The importance of properdin in complement is demonstrated in properdin-deficient individuals as properdin deficiency leads to severe impairment of alternative pathway.

The properdin gene is located on the short arm of the X chromosome, at Xp11.3- p.11.23 (Coleman et al., 1991, Goundis et al., 1989) and is composed of 10 exons (Nolan et al., 1992). Therefore deficiencies inherited are as typical X-linked recessive traits.

The first case of properdin deficiency was described in a Swedish family that suffered from fulminant meningococcal disease (Sjoholm et al., 1982).

So far, there are three types of properdin deficiency have been described:

Type	Characterisation
Type 1 deficiency	a complete absence of properdin in plasma, <0.1 µg/ml immunoreactive protein (Sjoholm et al., 1982), inherited as an X-linked recessive disorder distinct mutations that demonstrated in exon 4, 5, 6, 8 and 10 of the properdin gene. (Truedsson et al., 1997).
Type 2 deficiency	less than 10% of the normal level of properdin in plasma, impairment of some but not all functional protein, and low but detectable levels of immunoreactive protein: ~2 µg/ml (Nielsen and Koch, 1987, Sjoholm et al., 1982).
Type 3 deficiency	normal levels of immunoreactive but non-functioning protein (Sjoholm et al., 1988).

Table 1.2: Three types of properdin deficiency and the characterisation of the deficient.

Individuals with properdin deficiencies are 20-fold more likely to suffer from meningococcal infections with a mortality rate of 75% in cases of rapid onset. Nevertheless although do not always suffer from meningococcal infections so there are most likely other factors affecting susceptibity.

If properdin deficient individuals survive the infection further are due to a production of antibodies as able to respond well to immunisation with a tetravalent polysaccharide meningococcal vaccine (Fijen et al., 1999, Linton and Morgan, 1999).

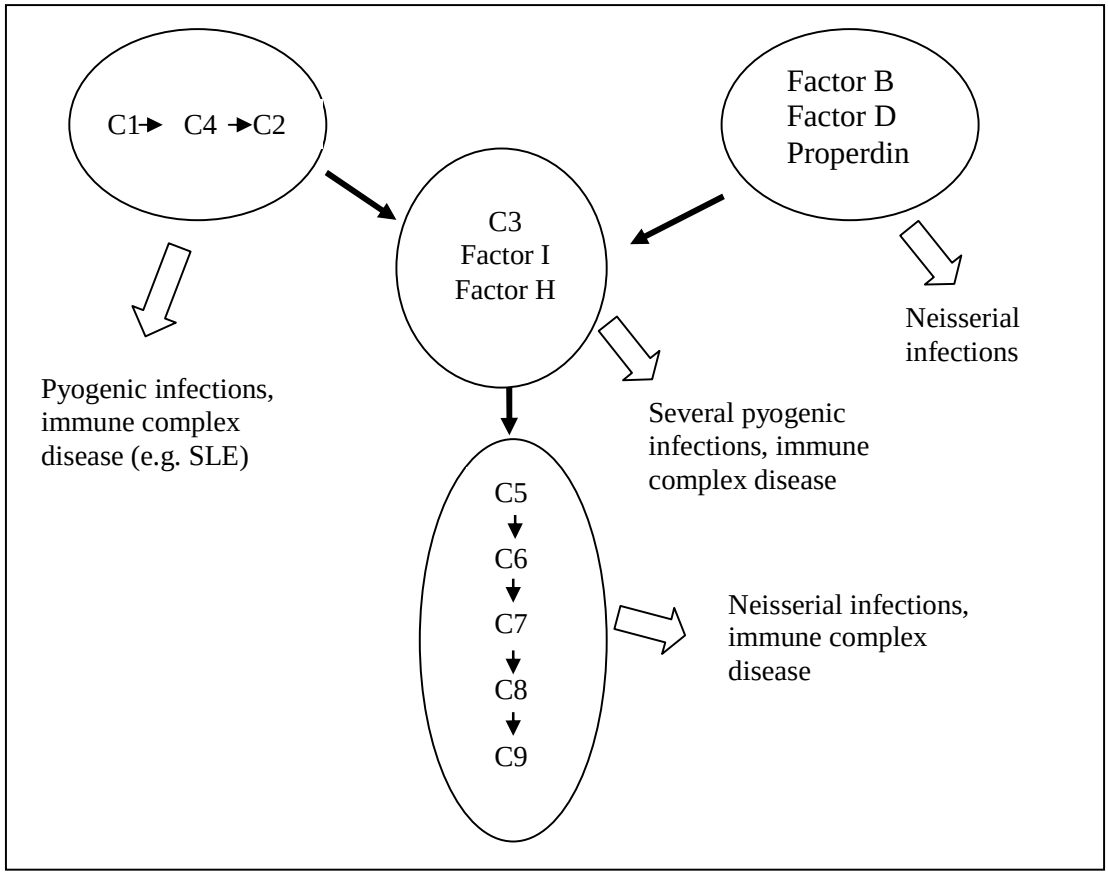


Figure 1.4: Deficiencies of components of the same pathway cause similar clinical problems (adapted from (Morgan and Walport, 1991)).

1.4.2 Properdin-deficient animal model

Mouse properdin have been sequenced and showed 72% homology with the human properdin at a nucleic acid level (Goundis and Reid, 1988, Maves et al., 1995). In relation to study properdin function in depth, generation of properdin knockout mice provided additional knowledge.

The first properdin-deficient of mouse model was generated by Dr. Cordula Stover (University of Leicester) by gene-specific targeting (Stover et al., 2008).

The model is deficient in properdin gene as disruption of the murine properdin gene, which is located on chromosome X by deletion exons coding for thrombospondin repeats (TSR).

For the deletion, a construct which contain the promoter region, exon for TSR1 (5 part of the properdin gene) and the exons for TSR 5, 6a, and 6b (comprise 3 part of the gene) flanking a positive selection was generated and introduced in embryonic stem cells. After identification of target-specific recombination, the characterised stem cell clone was microinjected in C57BL/6 blastocysts. Two chimeric mice were derived and intercrossing of their offsprings and then backcrossed with background C57BL/6 male resulted in obtaining properdin-deficient males, heterozygous females and wild type littermates. The mice used in properdin-deficient model experiments were backcrossed onto the C57BL/6 for 9 and 10 generations. WT littermates produced are used as controls in properdin-deficient model experiments.

Studies of this mouse line have shown these mice effectively lack properdin in their serum no detectable properdin protein in serum and have an impaired alternative pathway activity did not lyse rabbit red cells in the standard test for AP activity. However, they do not differ in their serum levels of C3 and IgM (Stover et al., 2008). Unchallenged these properdin-deficient mice are not immunocompromised, however they have been shown to be impaired in their survival compared to their wild type littermates distinctly decreased survival following *in vivo* sublethal caecal ligation and puncture (CLP) and in non-septic shock models (LPS) (Ivanovska et al., 2008, Stover et al., 2008).

To date, study has showed that properdin is important in the processes involved in inflammatory joint degradation as been investigated in zymosan-induced arthritis study. The findings showed both properdin-deficient and wild-type mice had bone erosion, proteoglycan loss and cell infiltration, initial phase of zymosan induced inflammation but compared to wild-type, properdin-deficient mice had reduced C5a and IL-6. The properdin-deficient mice had significantly less circulating zymosan-specific IgG antibodies, elevated IFN-γ production and STAT1 signaling in splenocytes and a shift to Th1 response in popliteal lymph nodes. In the chronic phase, the lack of properdin resulted in significant proteoglycan loss in the joints and lower cartilageneous STAT1 expression (Dimitrova et al.).

In addition to current model, the generation of another genetically engineered properdin-deficient mouse line (on mixed background 129/C57BL/6) was reported (Kimura et al., 2008) by targeted exon 3-5 of the properdin gene for disruption. The mouse studies have

shown that properdin is needed for the activation of the AP by lipopolysacharides (LPS) and lipooligosaccharides (LOS) and demonstrated in properdin null serum and mice LPS and LOS no longer activated AP.

All the study gave better understanding to answer properdin function and important role in immune system.

1.5 Aims and objective of the study

The aim of this study was to expand the knowledge on an important regulator of complement activation, the properdin.

In order to do so, using molecular biology, promoter activity of the human properdin gene was characterised and analysed (Chapter 3)**.** With knowledge from published review which reviewed the evidence that complement influences differentially the response to unconjugated vs. conjugated polysaccharide antigen and microanatomic site of splenic immune reaction towards polysaccharide antigens. In relation, then the role played by properdin in immune response was investigated *in vivo* upon vaccination during pneumococcal infection using a mouse model of pneumococcal pneumonia and the properdin deficient mouse line (Chapter 4). Next further characterised was *in vitro* generation of dendritic cells derived of spleen and bone marrow of properdin deficient mouse line and wild type littermate (Chapter 5).

Finally, functional investigation of generated bone marrow-derived dendritic cells of wild type and properdin-deficient was analysed towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG (Chapter 6).

The specific aims and background of each study will be discussed further in the specific chapter.

Chapter 2: General Materials and methods

Stated in this chapter is this the general material and method that commonly used in the study. Otherwise the material and method were stated in individual chapter accordingly.

2.1 Cell culture techniques

2.1.1 General handling

All work with cell culture was handled within sterile conditions under the hood. All cells were maintained at 37°C in the tissue culture humidified incubator containing 5% CO_2.

2.1.2 Thawing the cells

Frozen aliquots of cells were taken from –80 °C and thawed on ice. As soon as they become completely defrosted they were pelleted by using a microcentrifuge for 5 min at 500 rpm. The supernatant was decanted and pellet was washed once in appropriate medium to remove the toxic freezing media. The pellet was re-suspended in 1 ml media and replaced into a small tissue culture flask. 5 ml medium was added. Viability was checked using an inverted microscope.

2.1.3 Freezing the cells

Cells were counted under the microscope, using a Neubauer counting chamber. They were diluted to 1 x 106 cells / ml and centrifuged for 15 min at 500 rpm. The supernatant was discarded and 1 ml freezing medium (10% Dimethyl Sulfoxide (DMSO) (Sigma, Gillingham, UK) in FCS) was added. Cells were kept in liquid nitrogen.

2.1.4 Passaging Cells

Cells were split when their density reached about 80 % confluence. For suspension cells, these were split by transferring 1/3 of cells into a new labeled flask and topped up with approximately 15ml of medium.

Meanwhile for adherent cells, medium was removed into a waste pot using a sterile pipette. The culture flask was washed with several ml of sterile PBS. 3-4 ml 1x Trypsin-EDTA was added enough to cover the surface of the flask. Then, flask was placed in incubator afterwards for 1 min. Trypsin interacts with the cell adhesion molecules and releases the cells from the surface. Cells that floated off were observed under the inverted microscope. Using sterile pipette, cells were pipetted and washed around the flask so that cells that still attached will float off. Approximately 6-10 ml of cell culture medium was added to the flask to neutralise the trypsin and all the cells were transferred into 50 ml tube. Cells were centrifuged for 2 minutes at 1000rpm. The media supernatant were removed and cell pellets were re-suspended in 10ml fresh medium. About half of the cells were transferred to a new labelled flask and topped up with 15 ml of medium.

2.1.5 Cell counting

Cell numbers were determined using a haemocytometer (Neubauer counting chamber, Weber, England). A cover slip was placed onto the counting chamber. About 10 µl of cell suspension was put next to the gap between the chamber and the cover slip. The cell suspension was allowed to drift into this gap following the capillary force. Under the microscope, the chamber was seen as 4 squares consisting of 16 small squares. All cells within these 4 squares were counted. The number was divided by 4 to give the cell number average and the cells number was expressed as $x10^4$/ml.

No. of cells (4 field) / 4 = Average of cell number ($x10^4$/ml)

Cells counting by trypan blue dye-exclusion method

100 µl of the cell suspension was suspended in 100 µl 0.9 % (w/v) trypan blue solution. Dead cells appeared in blue since the dye invaded the cell membrane meanwhile live cells did not pick up the stain. The cells were counted in the chamber (see above). However by using this method, dilution factor was taken into consideration as follows:

$$\frac{\text{No. of cells (4 field)}}{4} \times 2 \text{ (dye dilution factor)} = \text{Cell number } (x10^4/\text{ml})$$

2.1.6 Seeding the cells

About 10 ml of cells were aspirated into 50mls tube. Then the cells were counted as outlined in 2.1.1.5. Cell number ($x10^4$/ml) that was obtained then was multiplied by the

cells volume. After that the volume of cells that are to be seeded can be determined as follows:

$$\frac{\text{No. of cells wanted (per ml)}}{\text{No. of cells counted (per ml)}} = \text{Volume of cells to seed}$$

2.2 Bacteriological techniques

All work with bacteria occurred under sterile conditions.

2.2.1 Preparation of blood agar plates

4 % (w/v) blood agar base from (Oxoid, Basingstoke UK) was resolved in distilled water, autoclaved and heated in the microwave (serum free blood agar plates have been prepared with 3.7 % (w/v) vegetable infusion powder and 1.5 % (w/v) agar biological (Oxoid).
The agar was allowed to cool down until the bottle could be touched by hand. 5 % (v/v) horse blood defibrinated (Oxoid) was added and mixed gently by pipetting up and down. Approximately 20 ml agar base was filled per plate without bubbles and allowed dry. Plates were stored upside at 4 degree up to two weeks.

2.2.2 Growth of S. *pneumoniae*

Frozen aliquots of *S. pneumoniae* strains D39, were kindly provided by Dr. Rana Al-Rachkidy-Lonnen. Briefly, single colonies were plated out on blood agar and incubated at 37 °C over night, within a dense jar containing a burning candle to keep the environment anaerobic. A single colony was picked up the next day and grew over night in 10 ml Brain

Heart Infusion (BHI) medium anaerobically. The next day the bacteria were spun down at 3000 g for 5 min. the supernatant was discarded carefully and the pellet was gently resuspended in a mixture of 80 % BHI medium and 20 % fetal calf serum from (FCS) (Sigma) .15 ml of BHI and 3 ml of FCS were added to the bacteria, which were allowed to grow for 5 hours under anaerobic conditions. Bacteria were aliquoted into 500 µl and frozen at –80°C.

2.3 DNA techniques

Determination of the concentration and purity DNA by measurement of the optical densesity (OD). 1 µl of the DNA sample solution of the miniprep was diluted in 999 µl of autoclaved dH_2O. OD_{260nm} and OD_{280nm} were measured using UltraSpec300 Spectrometer (Pharmacia Biotech) in a Quartz cuvette. The following equations were used for obtaining the purity and the DNA conentration.

OD_{260} (1:1000 diluted DNA) x 50 = concentration (µg/µl)

$\frac{OD_{260}\text{ (1:1000 diluted DNA)}}{OD_{280}\text{ (1:1000 diluted DNA)}}$ = purity of DNA

The samples were diluted accordingly to the concentration required for the various techniques adopted.

2.4 Protein techniques

Bradford assay

Protein concentration was determined using commercial Protein assay (adapted Bradford assay) following the manufacturer´s instructions (Bio-Rad, Hemel Hemstead, UK) .

A protein standard curve was constructed by diluting 2, 4, 6, 8 and 10µg of BSA into 800µl of H_20. Each sample was appropriately diluted as well into H_20 to obtain a final volume of 800µl. About 200µl of the dye reagent (Coomassie brilliant blue G-250 dye, Bio-Rad) were added to each sample and standard which prepared in eppendorf tube. Then it is mixed by vortexing and transferred into a 96 well plate. The dye changed colour from red to blue when binding to protein occurred and this was visualised after 15 minutes of incubation at room temperature by measuring the absorbance at 595nm using a microplate reader (BioRad). A standard curve was established using the microplate manager 5.2.1 software (BioRad) and the total protein concentration of each sample was determined by plotting the O.D value against the standard curve.

2.5 Statistical analysis

All statistical analyses were performed using Prism (v.4.02 for Windows; GraphPad Software, San Diego, CA). A p value of <0.05 was considered statistically significant. Specific analyses were stated on specified chapter.

Chapter 3: Study of promoter activity of human properdin gene

3.1 Introduction

3.1.1 Overview of regulatory elements in Eukaryotic Gene

Gene expression as part of machinery of life, encompasses distinct processes such as transcription, RNA splicing, translation, and post-translational alteration.

Transcription processes take place as an initial process. Transcription occurs when DNA copied into messenger RNA (mRNA) by DNA dependent RNA polymerase. The transcription result later can be encodes as protein by translation process. The translational products usually are proteins (although alternatively in non-protein coding genes, ribosomal (rRNA), transfer RNA (tRNA) or other functional RNA) can be produced as a functional gene product which leads as gene expression (Tjian and Maniatis, 1994).

Eukaryotic genes at the transcriptional level requires the core promoter which contains the binding site for RNA polymerase together with transcriptional factor, protein that binds to specific DNA sequences and formed transcriptional complex, also working together are other proteins known as activators and repressors as regulatory elements, thereby regulating the transcription activity.

Promoters usually located upstream of the regulated gene, relatively to the transcriptional start site (TSS), where the transcription begins. Amongst all eukaryotes, mammalian gene promoters are difficult to characterise, and often having the core promoter known type as TATA box, which a short DNA sequence binding site for a transcription factor at -25-30 bp upstream of TSS.

Promoter is important as transcription control element as involved in initiation of mRNA synthesis by RNA polymerase II, initiates transcription and controls location of site of transcription initiation.

3.1.2 Promoter studies of complement genes

So far, only a few complement genes promoters of have been characterised and studied. Structural analysis of complement gene structures has shown that most complement gene promoters are TATA-less promoters (Volanakis, 1995). Instead, complement gene promoters may have similarities of consensus regulatory elements with other genes, particularly Acute-phase proteins (APP) genes (Volanakis, 1995).

Some of the findings in the literature are:

Complement gene	Description	Reference
mouse C4	gene promoter lacks TATA box at the -30 region	(Miyagoe et al., 1994)
human C2	partially characterized, contained in a 228 nucleotide sequence which lacked a TATA box	(Sullivan et al., 1994)
mouse C3 human C3	promoters contain a TATA box located at position -30	(Vik et al., 1991)
human factor H	The promoter lacks canonical TATA and CCAAT boxes	(Williams and Vik, 1997)

3.1.3 Background to the study

Properdin, positive regulator in alternative pathway has been shown to be expressed in a variety of immune cells with haematopoietic origin; macrophages (Bentley et al., 1978), monocytes (Minta, 1988), dendritic cells (Reis et al., 2006), lymphocytes (Schwaeble et al., 1993), neutrophils (Schwaeble and Reid, 1999)) at the time of this study. More recently, properdin expression has been documented for mast cells (Dupont, 2008).

U937 monocytic cell line (human leukemic monocyte lymphoma cells) is used to contribute in this study since they are susceptible to manipulations and have been shown positive expression of Properdin (Minta, 1988, Salehen, 2006).

Previous study in the lab, immunofluorescence was used to detect and visualise properdin on U937 grown on coverslips. Briefly, U937 cells after 72 hours induction with PMA (Phorbol myristate acetate) were labelled with primary antibody for properdin HYB039-04 (anti-human properdin monoclonal) and secondary antibody, anti mouse IgG (Fab specific) Fitc conjugate).

Properdin reactivity was detected using PMA-treated U937 (Figure 3.1). Properdin diffuse through both channels has discrete expression. Negative control in figures shows that no non-specific binding found from secondary antibody. High levels of properdin were constantly detected during the differentiation of U937 with PMA (Minta, 1988, Yamauchi et al., 2002).

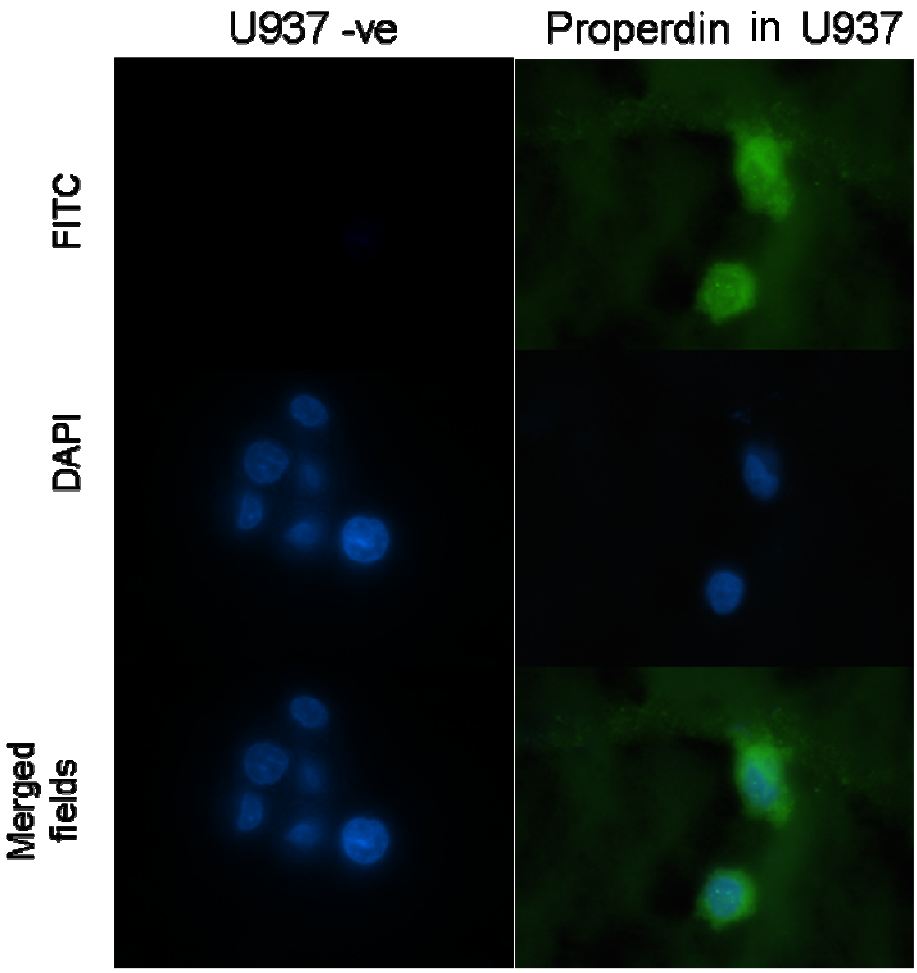


Figure 3.1: Properdin expression in U937-PMA treated on coated coverslip and visualised through Fitc channel, DAPI channel and merged fields of Fitc and DAPI channel. Cells have been treated with Triton-X for permeabilised and labelled with anti-human properdin (monoclonal antibody) as primary antibody and anti mouse IgG (Fab specific) Fitc conjugate (Fab fragment) as secondary antibody (Salehen, 2006).

To date, regulatory elements and expression of properdin have not been characterised and been studied.

Bioinformatics studies done in the lab (Yue, 2005) had searched to predict properdin regulatory elements in promoters of mammalian properdin gene sequences available at the time in public databases. The computer-based study retrieved properdin promoter

sequences for the 6 species (Human, chimpanzee, mouse, rat, cow and dog) from online genome database and obtained about ten probable transcription factors that might be functional during the expression of properdin. The multiple alignments in the study did not show any highly conserved regions that were common to all species and led to finding that the properdin gene control regions are varied across the species. The analysis of properdin gene control elements managed to characterise a bimodular properdin promoter model encompassing NFAT/AP1 and Atp1a1/GATA/MyoD.

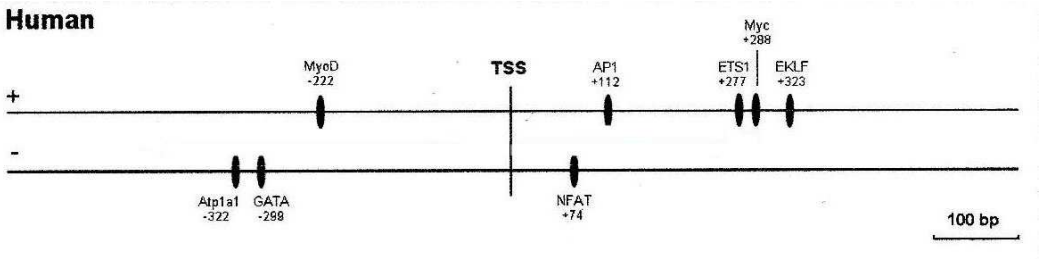


Figure 3.2: Bimodular properdin promoter model encompassing Transcription factor binding site NFAT/AP1 and Atp1a1/GATA/MyoD with its start position below
TSS: Transcription binding site (Source: (Yue, 2005))

Therefore, data of predicted structure is used in this thesis to verify which promoter elements could be essential for properdin expression which may lead to understanding properdin expression in term of cell lineage specificity.

3.2 Materials and methods

3.2.1 Materials

All the chemicals used were purchased from Sigma-Aldrich Ltd (Gillingham, UK), BDH VWR International (Lutterworth, UK) and Fisher Scientific (Loughborough, UK), all the primers were ordered from MWG Biotech (Ebersberg, Germany) and the restriction enzymes from Roche (Lewes, UK), unless otherwise stated in the text.

The media were prepared:

- Luria broth (LB): 4g tryptone, 2 g yeast extract, 2 g NaCl, 400 ml distilled H_2O.
- Luria Agar (LA): 1.5 % (w/v) Agar was added to Luria broth

Vectors used: pGEMT Easy and pGL4

Restriction enzyme: *HindII* (Roche*), XhoI* (Roche), SURE/Cut Buffer B (Roche)

ECoRI (Promega, Southampton UK)

3.2.2 DNA preparation

Human gDNA was prepared from peripheral blood mononuclear cells from venous blood from normal, healthy donor by Lymphopreparation (separation technique). Then human DNA was diluted 1:100 with dH_2O and subjected for PCR.

3.2.2.1 Peripheral blood mononuclear cells (PBMC) preparation

Peripheral blood mononuclear cells (PBMC) were separated from whole blood by their sedimentation rate. Heparinised venous blood was kindly donated by Dr. Cordula Stover and mononuclear cells were isolated from the blood by centrifuging the blood on a Lymphoprep^{TM} layer (Axis-Shield, Oslo, Norway). Heparinised blood was diluted by addition of an equal volume of PBS. Carefully the diluted blood was layered over Lymphoprep^{TM} in a 50ml tube. Mixing of blood and separation fluid needed to be avoided. The tube was centrifuged at 800 x g for 20 minutes at 20ºC in a swing rotor centrifuge without break. After centrifugation, the mononuclear cells form a distinct band at the middle layer between the sample and medium border. The cells were removed using a pipette without removing the plasma layer. The harvest fraction was diluted with PBS and the cells were pelleted by centrifugation at 250 x g for 10 minutes. Lastly the supernatant was decanted and media or PBS was added to the cells pellet for counting.

3.2.3 Primers

Primers were designed to amplify the region of interest. A set of forward and reverse complementary primers was designed at similar melting temperatures (Tm). All the primers were HPLC purified and supplied lyophilised. They were made up to 1µg/ml stock in PCR-

grade nano pure water and were diluted to be used in the PCR reaction at a final concentration of 200ng/ml.

The primers used for Polymerase chain reaction (PCR) and their respective restriction sites are listed in Table 3.1:

Primer	**Primer sequence**	**R. E***
XhoHumPropF1	5' GG CTC GAG AAC TCG CAA TCT TTG CAA CAC ACC 3'	*XhoI*
XhoHumPropF2	5' GGG CTC GAG GAG CAC CGC ACA CTC ACT TCA C 3'	*XhoI*
HindHumPropR1	5' GGG AAG CTT GAG GTT CCC TGT GGT CAG CTT G 3'	*HindIII*
HindHumPropR2	5' GGG AAG CTT CCC AGC ACC TCG CGC TCC TC 3'	*HindIII*

Table 3.1: The sequence of primers used in this study

***** Restriction enzyme recognition sequence

3.2.4 Plasmid constructs

Activity of potential promoter sequences can be analysed using luciferase reporter assays. For this work, previous in silico characterisation of properdin promoter sequences across several species was used to design primers that encompass the relevant regions. Properdin gene of interest: ATG in exon 2 position at +467 counted from the position that the longest

known DNA clone maps to at its 5'end. (NCBI sequence ID: NM_002621, accession number of human properdin: M83652).

In bioinformatics analyses (comparative across many species), characterised relevant transcription factor binding sites for the stretches: +1 to +250 and -420 to -120.

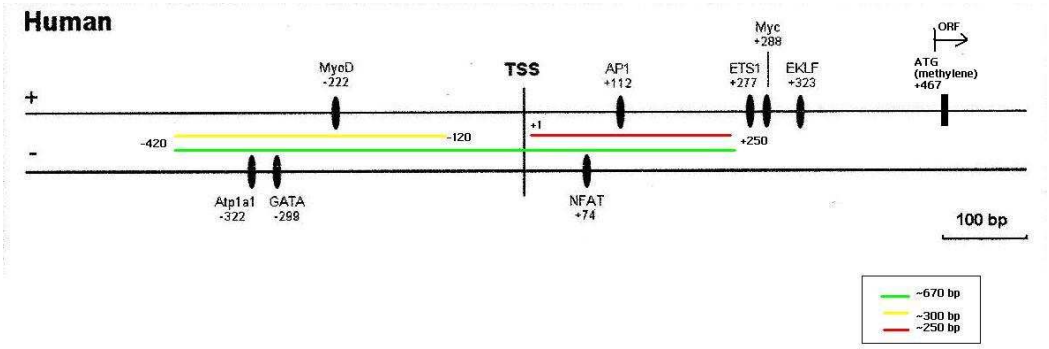


Figure 3.3: Illustration of human properdin gene promoter model for PCR product construct strategy TSS: transcription start site (defined as position 1 in human properdin mRNA, accession number NM_002621).

Construct	Primers	Location	Product size (bp)
1	*Xho* F1 + *Hind* R1	(-420) – (-120)	~300
2	*Xho* F2 + *Hind* R2	(+1) – (+250)	~250
3	*Xho* F1 + *Hind* R2	(-420) – (+250)	~670

Table 3.2: The sequence of primers used in this study

These forward and reverse primers were modified with *XhoI*- linker and *HindIII*-linker at the 5' end respectively. The products were designated as: fragment 1, 2 and 3. The PCR products were cloned into pGEMT-easy first as a PCR rescue vector. Then the fragments

were excised and ligate to the *XhoI* and *HindIII* sites of a promoterless pGL4 luciferase expression vector (Promega) and termed pPROP-Luc (Properdin-Luc reporter plasmid). The work yielded two DNA fragments of 670 and 300bp, upstream of the luciferase gene, denoted as pPROP-Luc$_{670}$ and pPROP-Luc$_{300}$, respectively.

To prepare various reporter plasmids, three fragments derived form genomic DNA were initially prepared by PCR

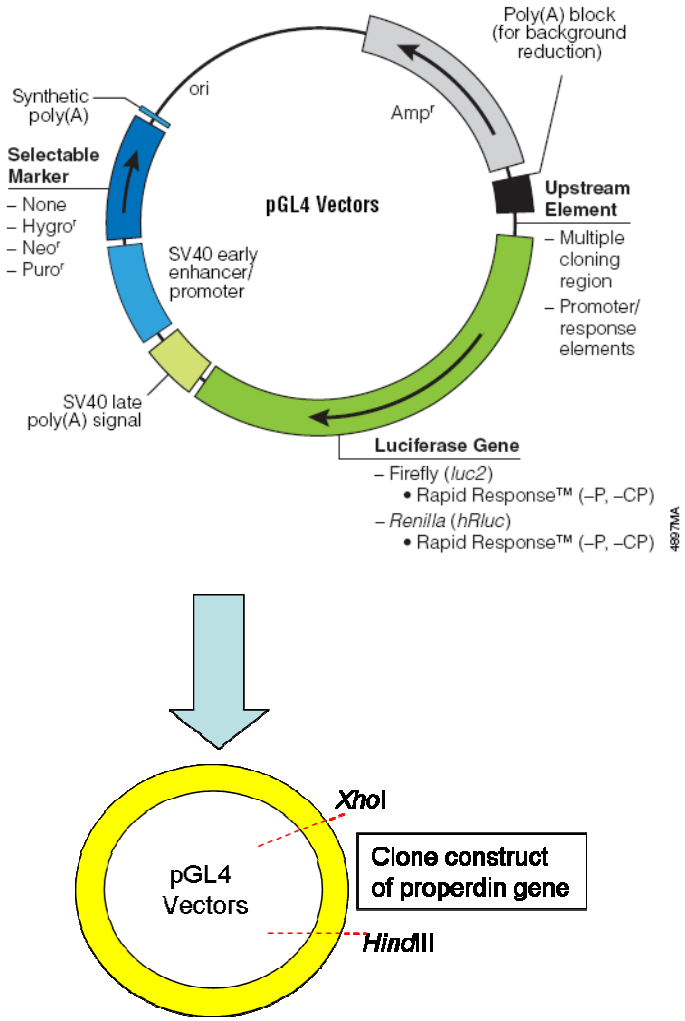


Figure 3.4: Schematic diagram of pGL4 vectors (figure taken from PROMEGA pGL4 notes (Promega)) and illustration of plasmid construct strategy.

3.2.5 Polymerase chain reaction (PCR)

The reaction was performed by mixing the following reagents in a 0.5ml PCR reaction tube with a total volume of 25µl.

Template DNA	1µl.
Primers (forward and reverse each)	0.5µl.
10x Reaction Buffer IV (ABgene)	2.5µl.
$MgCl_2$ (25mM, ABgene)	2µl.
dNTPs (dCTP,dATP, dGTP, dTTP)	
(1.25mM each, Nucleotide mix, Promega)	4µl.
Thermoprime Plus DNA Polymerase (ABgene)2U/ µl	0.2µl.
DEPC-treated distilled water	top up to make up volume of 25µl.

The tube was then pulsed-centrifuged and placed in a thermocycler. PCR was conducted under the following conditions: denaturation at 95°C for 60s, annealing at 63°C for 60s and extension at 72°C for 150s. Taq polymerase adds 3'terminal dATP overhangs, which are used for the cloning step section 3.2.9. Next, PCR products were subjected for agarose gel electrophoresis.

3.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA is used to separate different sizes of DNA fragments. The percentage of the gel depended on the size of DNA fragments to be separated; the higher the percentage, the better smaller the fragments separate. Agarose gel 0.8% was made by dissolving 0.4g of agarose into 50ml of 1xTAE (Tris-Acetate-EDTA) buffer (40mM Tris, 20mM glacial acetic acid, 1mM EDTA, pH8.0) and heating it up for 90seconds in a microwave oven. The dissolved agarose was let cooled down slightly before the addition of 0.5 μg/ml ethidium bromide (SIGMA).The gel was poured in the assembled gel mould until it was set. Then the gel was transferred to the running tank containing 1xTAE buffer. Each sample of DNA was mixed to 3 μl of loading dye [6x bromophenol blue loading dye (30% (v/v) glycerol, 70% (v/v) TAE, 0.3% (w/v) bromophenol blue)] and loaded into the wells of the gel, along with 1 kb DNA ladder **(**Eurogentec, Aylesbury, UK**)** to estimate the size of the DNA samples.

Electrophoresis was performed by applying a constant voltage of 100V for 45 minutes or the electrophoresis was stopped when the dye had at least migrated through ¾ of the gel.

DNA fragments on the gel were visualised by UV transluminator (UVP) and recorded using a Olympus camera connected with Image Quant 100 Capture software (GE Healthcare, Little Chalfont, UK).

3.2.7 Gel extraction (DNA purification from agarose gels)

DNA extraction from agarose gel was done using Sephaglas BandPrep kit (Amersham Biosciences, Chalfont St Giles, UK) following manufacturer's instructions.

Briefly, the band of interest was cut from the agarose gel using a clean scalpel and put into a pre-weighted eppendorf tube. The gel was solubilised completely by vigorously mixing together with an appropriate volume 250 µl gel solubiliser and heated up at 60°C for 5-10 minutes. Sephaglas BP beads were then added to the solubilised mixture and incubated 5 minutes at RT to allow the DNA to bind to the beads. The DNA bound to the beads was then washed 3 times with a wash buffer, followed by air drying. Finally the purified DNA was eluted from the beads and transferred to a new tube. Purified DNA was stored at -20°C until further use.

3.2.8 Estimation of the DNA concentration

The concentration of each DNA sample purified from the gel was estimated by mixing 3µl of each eluted DNA with 2 µl of loading dye. These and 5 µl of the molecular weight marker SmartLadder (Eurogentec) were run into an agarose gel. In the dark room, the DNA concentration could be estimated by comparing the fluorescence intensity of the DNA bands with the fluorescence intensity of the known standard DNA ladder.

3.2.9 DNA ligation

Ligation was performed using the pGEM-T Easy vector system I (Promega). The amount of PCR product to add to the ligation reaction was calculated using the following formula:

$$\frac{\text{PCR product size (bp)}}{\text{vector size (bp)}} = \frac{\text{PCR product (ng)}}{\text{vector (ng)}}$$

To improve efficiency of the ligation, a 1:3 molar ratio vector: insert was used. Then, the ligation reaction mixture with a final volume of 10µl was done by adding to the vector/insert mix, 1 µl T4 DNA ligase, 2x rapid ligation buffer appropriately diluted in distilled water. Lastly, the ligation mixture was incubated at room temperature for 1 hour.
The same principle was followed to ligate *XhoIHindIII* excised pGEMT-Easy insert with *XhoI*/*HindIII* cut pGL4 vector.

3.2.10 Transformation

For preparation, an empty universal tube was pre-cooled on ice and a universal tube containing LB broth was pre-warmed at 37°C.
An aliquot of Top10F' competent cells (100µl, laboratory's stock) was thawed on ice and transferred into the pre-cooled universal tube, where 10µl of the ligation product were added. The mixture was mixed by stirring and was incubated for 15 minutes on ice. This

was followed by heat shock incubation at 37°C for 5 minutes. Then 900µl of the pre-warmed LB broth were added to the mixture and the tube incubated by shaking at 37°C for 45-60 minutes.

The culture was then plated out onto LB agar plates supplemented with ampicillin (100µg/ml), X-Gal (80µg/ml; Promega) and IPTG (0.5mM; Promega) under sterile conditions. Agar media were cooled down to 50°C before antibiotic and selecting markers were added. The plates were dried and placed inverted in a 37°C incubator for more than 12 hours.

3.2.11 Mini prep-small scale plasmid preparation

Small scale plasmid preparation was performed using the following solution:

PI buffer: 50mM TrisHCl, 10mM EDTA, 50mM Glucose, 100µg/ml RNaseA, pH8.0

P2 buffer: 0.2M NaOH, 1% SDS

P3 buffer: 60ml of 5M potassium acetate, 28ml of acetic acid, 12ml of H2O, pH 4.8

TE buffer: 10mM TrisHCl pH7.5, 1mM EDTA pH8.0

In preparation, solutions P1 and P3 were chilled on ice and P2 buffer was freshly prepared.

3.2.11.1 Experimental procedure

A single white colony from the agar plate after transformation was inoculated and grown overnight at 37 °C with shaking at 200 rpm in 3ml of LB supplemented with ampicillin (100µg/ml) in a universal tube.

The next day, 1ml from the culture was transferred in an eppendorf tube and pelleted at using a Micro Centaur bench-top microfuge, 15,000g for 1 min.

The supernatant was discarded and the pellet was completely resuspended into 100µl of ice-cold PI buffer (resuspension solution) by vortexing the eppendorf.

Then, 200µl of the P2 solution (lysis solution) were added by inverting 5 times and the mixture was let for 5 minutes on ice to allow bacterial lysis.

To precipitate bacterial proteins, genomic DNA, carbohydrates and SDS, 150µl of the ice-cold P3 buffer (precipitation buffer) were added, mixed thoroughly by vortexing and incubated on ice for 5 min. The precipitate was centrifuged for 5 min at 13000rpm and the clear supernatant was carefully transferred into a new eppendorf tube which contained 800µl 100% ethanol. This step could be repeated if the supernatant was not clear. The tube was inverted several times and incubated at RT for 2 min.

The plasmid was pelleted afterwards by centrifugation at 15,000g for 15 min. The supernatant was discarded and the pellet was washed with 70 % (v/v) ethanol. The tube was centrifuged at 13000 rpm for 5 minutes. The ethanol was carefully removed and the pellet was left to dry completely at room temperature. Finally the DNA pellet was resuspended in 50 µl of TE buffer. The plasmid was analysed later by restriction digest.

3.2.12 Maxi prep-Large scale plasmid preparation

To reduce toxicity and increase efficiency for maxiprep, Endotoxin-Free Plasmid Maxiprep Kit (Qiagen, Crawley, UK) was used and following the manufacturer´s instructions. In this method, plasmid purification was based on a modified alkaline lysis procedure, followed by plasmid preparation by column chromatography. This method has the advantage of generating large amounts of very pure plasmid DNA.

Briefly, a single white colony was inoculated from transformation culture was grown overnight 37°C with vigorous shaking in LB medium under ampicilin selection.

The culture was pelleted and lysed by the Buffer P1-alkaline / SDS buffer. The denatured lysate was then neutralised and precipitated using Buffer P2 and Buffer P3. The plasmids lysate were then captured by filter Cartridge, while endotoxins were prevented from absorbing to the membrane. The filtered lysate were allowed to enter the resin by gravity flow. Two washings were applied to remove contaminants

The plasmid was finally eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. DNA pellet were washed lastly with endotoxin-free 70% ethanol, air-dried and redissolve the DNA in a suitable volume of endotoxin-free Buffer TE and stored at -20°C until further used.

3.2.13 Restriction digest

DNA restriction digests were done by using restriction endonucleases that cut double-stranded DNA at specific recognition sequences within the DNA. In this study, the pGEMT-Easy constructs were analysed for the presence of inserts by restricting with *EcoRI*. Plasmid DNA from the positive recombinants was than restricted with *HindIII* and *XhoI* to excise fragments.

The digestion reactions were performed following manufacturer's instructions. The digest reaction mixture with final volume of 20µl was done by having 2µl of the plasmid (extracted as previously described), the appropriate 10x buffer correctly diluted in distilled water, 10 to 12U of the appropriate restriction enzymes (usually 1 unit of enzyme is needed to digest 1 µg of DNA). The reaction mixture was incubated for 1 hour at 37°C. The digestion reaction was stopped by adding loading dye and was analysed by agarose gel electrophoresis.

3.2.14 Sequencing

A few microlitres of plasmid preparation (pGEMT-Easy and pGL4 constructs) were appropriately diluted to obtain a final concentration of 50ng plasmid/µl TE buffer and were sent to PNACL (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester) for sequencing. The DNA sequences were compared with the Gene bank databases using the BLAST program available at NCBI website and aligned using Gene Tool or Chromas (Technelysium Pty Ltd).(EMBL accession number of human properdin gDNA: X70872).

3.2.15 Dual-luciferase transfection

The dual luciferase assay is for quantifying gene expression by transcriptional regulation, coupled to the expression of a luciferase reporter gene. The dual luciferase assay is performed by sequentially measuring the firefly and Renilla luciferase activities of the same sample, expressed as the ratio of firefly to renilla luciferase activity. Normalising the experimental reporter gene to the activity of internal control is necessary because of variability caused by differences in cell viability and transfection efficiency.

3.2.15.1 Transient transfection for U937 suspension cells

U937 monocytic cell line were ideal to employ in this experiment since U937 have been shown to be positive for Properdin (Minta 1988; N.Salehen MSc 2006). U937 cells were split the day before transfection and were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100U/ml penicillin and 100U/ml streptomycin. Cells were maintained at 37ºC with 5% CO_2.

On the day of transfection, cells were plated at a density of 2 x 10^6 per ml, using 3ml of cells per well on a 6-well cell culture plate and were incubated approximately for an hour. For the following experiments, transfection assay for U937 suspension cells were done using Effectene Transfection kit from Qiagen. 1µg DNA of reporter plasmid diluted in TE buffer (pPROP-Luc), co-transfected with Renilla-Luc (dilution 1:40 to make up maximum DNA concentration: 1µg/µl) with the DNA-condensation buffer, Buffer EC, to a total

volume of 150µl in an eppendorf tube. Another reporter plasmid pCMV-luc (Cytomegalovirus plasmid)(kindly prepared by Dr. B.Burke, Leicester) DNA with known activity also was added as a positive control in the assay. Then 8µl Enhancer was added and mixed by flicking gently the eppendorf tube. Complex mixture was incubated for 2-5 minutes at room temperature. Next, followed by addition of 25µl Effectene Transfection reagent into the complex mixture and again was incubated for 5-10 minutes at room temperature. After incubation, diluted mixture complexes were applied to cells and gently swirled the plate to ensure uniform distribution of the complexes. The transfected cells were divided into 24-well cell culture plate with 1ml of cells for each well to obtained triplicate reading for harvesting. Cells were incubated at 37ºC with 5% CO_2 for 24 hours before stimulating or harvesting for reporter assay. In one series of experiments, transfected cells were stimulated with LPS (1µg/ml) for 24 hours after 24 hours of transfection.

3.2.15.2 Evaluation of transfection efficiency by Dual Luciferase assay

For measurement of luciferase activity, all protocols were followed from Promega Dual Luciferase System kit protocol. Cells were harvested and centrifuged at 4000g for a few minutes. Supernatant was discarded and cells pellet was washed with 1x PBS. After washing, cells pellet was resuspended in 1x reporter lysis buffer. Cell lysates then were frozen at -80ºC at least for half an hour or kept frozen until luciferase evaluation. Luciferase activity in cell lysates was determined using a Sirius model luminometer (Berthold Technologies) and Dual Luciferase Assay System from Promega.

Promoter activity is expressed as relative luciferase activity (RLU/s) of the level of pPROP-Luc which normalised against Renilla control activity. Relative luciferase activity is represented as the mean ± SD of at least five independent experiments, and each experiment was performed in triplicate. Significant differences between samples were assessed using the Student T-test. P values< 0.05 were considered statistically significant.

3.3 Results

3.3.1 Extraction of genomic DNA

DNA samples were successfully extracted from human blood mononuclear cells from venous blood as explained in section 3.2.2 and this DNA was used as a template to do PCR. The DNA stock samples were aliquoted into microfuge tubes to prevent nucleotide degradation caused by repeated freeze/thaw cycles and kept in freezer for routine experiments as genomic working samples.

3.3.2 Analysis of promoter activity in the subfragment of the properdin gene

A set of forward and reverse complementary primers called “HumProp” were designed and the melting temperature (Tm) of each primer was calculated to obtain a PCR product that included upstream and downstream of the transcription start site of the properdin gene.

In an attempt to characterise the regulatory elements upstream and downstream of this transcription start site of properdin gene that may be important for properdin expression in human, three constructs from human genomic DNA were initially prepared by PCR. The products were designated as: construct 1, 2 and 3.

Successful amplification by PCR is shown on figure 3.5, PCR products with the expected size of 670bp, 300bp and 250bp were visualised on the agarose gel. These PCR products obtained were all excised from the gel and the DNA was extracted from the gel using Sephaglas BandPrep kit (see section 3.2.6).

From these extracted fragments, the DNA concentration was measured (section 3.2.7) as the fragments then were cloned into pGEMT easy vector (Promega) as decribed in section 3.2.8. The PCR products of the constructs were subcloned into pGEMT Easy basic vector as PCR rescue vector. The plasmid obtained was used for transformation of Top 10F' competent cells and transformed bacteria were plated onto LB agar plates (ampicillin, X-Gal and IPTG supplemented). White and blue colonies were grown the next day and cells contained the inserted fragment construct in vector insert region appeared as white colony. White single colonies were inoculated and the selected individual single colonies were then prepared for plasmid extraction. The plasmids obtained were screened by restriction digest.

Only construct 1 (Prop300) and construct 3 (Prop670) were successful rescued from pGEMT-Easy, then were ligated to pGL4 luciferase expression vector (Promega) and assayed for promoter activity.

As seen in Figure 3.6 successful band subjected for double digestion using *HindIII* and *XhoI* for plasmid constructed of pGEMT-easy and pGL4 vector with PCR products of HumProp and human DNA. The vector was cut twice for all the samples as seen with the 670bp band observed on the gel for corresponding to the size of the linearised vector (Figure 3.6)

This plasmid was sent for sequencing to PNACL to confirm the identity of its insert. The sequences obtained were analysed for identity with human properdin promoter area by comparing sequences obtained with NCBI deposited gene sequence for human properdin the Gene bank databases using the BLAST program available at NCBI website and then aligned with the known sequence of the human complement factor properdin gDNA (Gene bank accession number of human properdin gDNA: X70872) and overlap was found.

The products were cloned again into the *XhoI* and *HindIII* sites of a promoterless pGL4 and termed pPROP-Luc (Properdin-Luc reporter plasmid) yielding two DNA fragments comprised of 670 and 300bp, upstream of the luciferase gene, denoted as pPROP-Luc_{670} and pPROP-Luc_{300}, respectively.

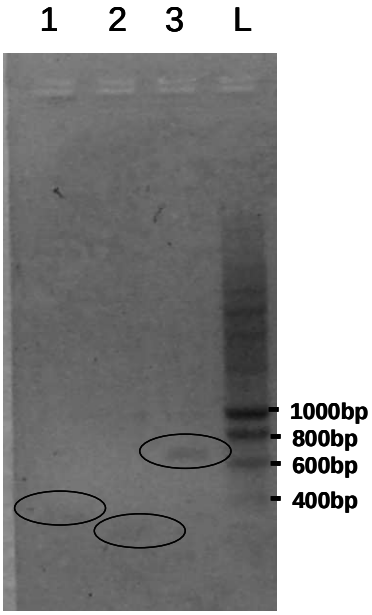


Figure 3.5: Representation of gel image of PCR product using human DNA and HumProp primers after subjected on 0.8% agarose gel. L : 1kb DNA Ladder, 1: construct 1- 300bp, 2: construct 2- 250bp, 3: construct 3: 670bp.

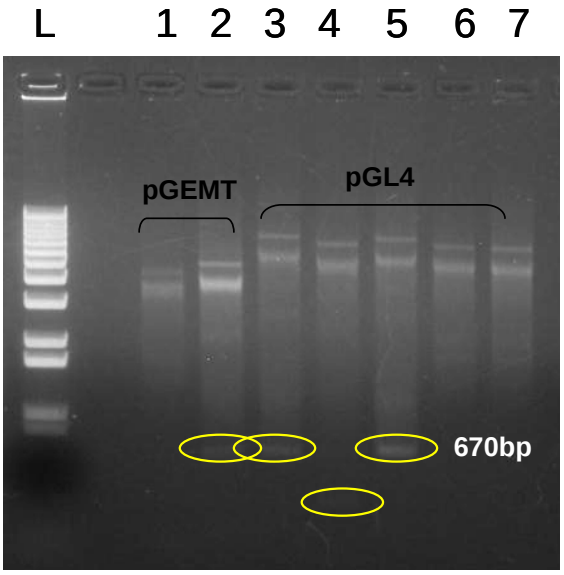


Figure 3.6: Representation of gel image of double digest using *HindIII* and *XhoI* for plasmid constructed of pGEMT-easy and pGL4 vector with PCR products of HumProp and human DNA. L : 1kb DNA Ladder, 1: pGEMT300bp, 2: pGEMT670bp 3: pGL4 670bp 4: pGL4 300bp 5: pGL4 670bp 6: pGL4300bp 7: pGL4 300bp

3.3.4 Analysis of expression of promoter activity

Successful clone constructs were inserted into luciferase reporter plasmid. Reporter plasmids (pPROP-Luc) co-transfected with Renilla-Luc then were transiently transfected into U937 cells using the transfection kit. Another reporter plasmid pCMV-luc (Cytomegalovirus plasmid DNA) with known activity also was added as a positive control in the assay.

Transfected cells were incubated at 37ºC with 5% CO_2 for 24 hours and the transfected cells were also stimulated with LPS (1µg/ml) for the next 24 hours. Cell lysates of transfection assay then were obtained for measurement of luciferase activity. The dual luciferase assay was used to quantify promoter gene expression coupled to the expression of a luciferase reporter gene. Promoter activity is expressed as relative luciferase activity (RLU/s) of the level of pPROP-Luc which normalised against Renilla control activity.

Figure 3.7 shows relative luciferase activity of properdin promoter-driven luciferase pPROP-Luc_{670} and pPROP-Luc_{300}, pCMV positive control and co-transfected U937-driven renilla luciferase expression represented the mean ± SD of at least five independent experiments.

Negative control containing untransfected cells were added in the experiment as a background reading showing no luciferase expression.

pCMVluc is a strong promoter thats act as a positive control to evaluate success and efficiency of transfection. High luciferase reading for pCMV positive control indicates that

the transfection was successful. As expected significantly higher luciferase activity of control CMV driven vector compared to low renilla activity is shown in figure 3.5A.

For transfection efficiency promoter activity of pCMV is normalised by renilla luciferase expression showing 6 fold ratios.

However upon stimulation with LPS, promoter activity of pCMV as well the renilla activity was markedly decreased by at least by 9 fold and 8 fold respectively.

In figure 3.5B, pPROP-Luc$_{670}$ and pPROP-Luc$_{300}$ transfection were measured for 24 hours of transfection and after LPS added.

Promoter activity of pPROP-Luc$_{670}$ at 24hours of transfection was higher than renilla control activity by at least 1.6 fold increased. Nevertheless there is no significantly different between them both. The promoter activity of pPROP-Luc$_{670}$ was massively decreased by 16 fold upon stimulation of LPS. In parallel appeared pPROP-Luc$_{670}$ promoter activity was higher than pPROP-Luc$_{300.}$ Construct pProp-Luc300 did not express any luciferase activity with and without LPS stimulation.

The control renilla activity for both pPROP-Luc$_{670}$ and pPROP-Luc$_{300}$ increased consistently after LPS added in the transfection assay.

It is concluded that transfection of the properdin promoter plasmid construct properdin plasmid upon LPS induction was not successful.

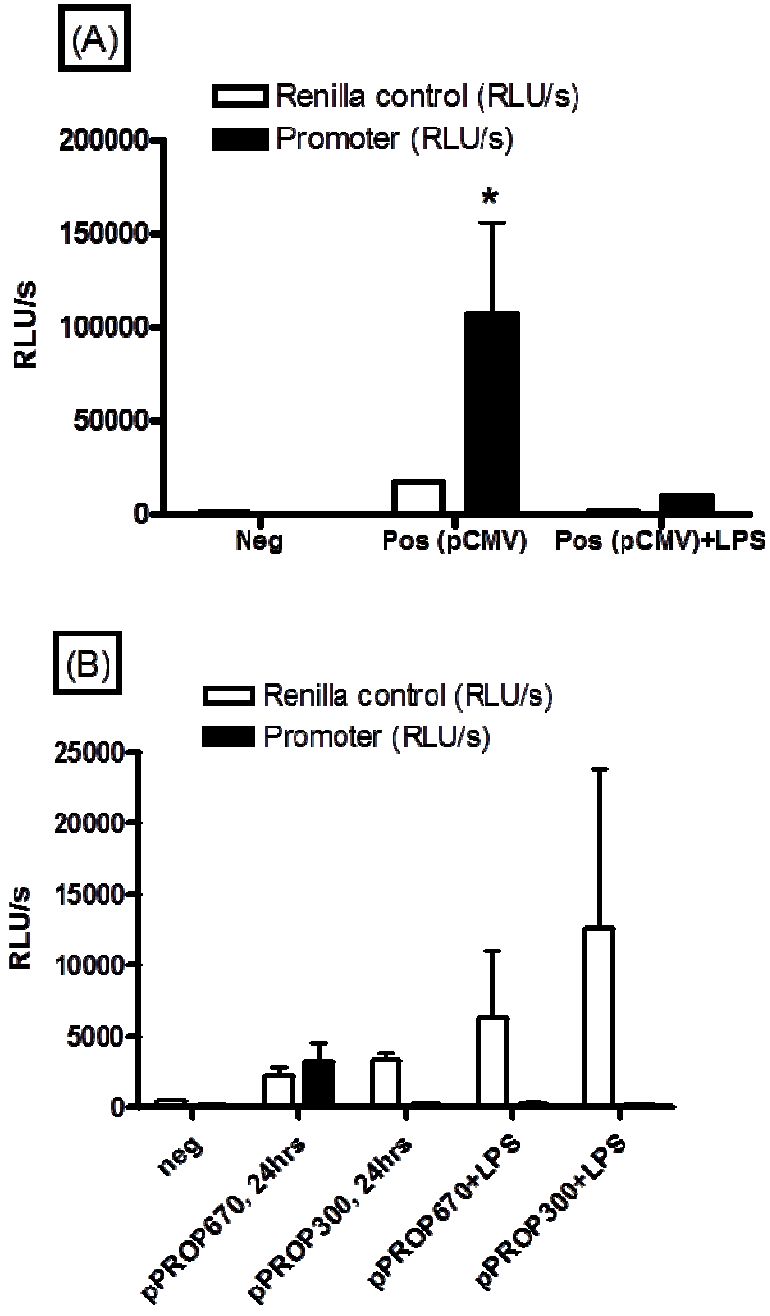


Figure 3.7: Promoter activity in plasmid construct of Properdin promoter-driven luciferase (pPROP-Luc_{670} and pPROP-Luc_{300}) is express as relative luciferase level normalised by that of co-transfected U937-driven renilla luciferase expression for transfection efficiency expression. Negative control as a background reading and pCMV as positive control is added in the experiment. Data represent the mean ± SD of at least five independent experiments, and each experiment was performed in triplicate (*$P < 0.05$ vs. control, Student T-test analysis)

3.4 Discussion

3.4.1 Promoter activity in the subfragment of properdin gene

There are numerous sources of properdin DNA as properdin is expressed in a variety of cells of haematopoietic origin such as macrophages, monocytes, dendritic cells or lymphocytes. Hence, blood is an excellent source of large amounts of genomic DNA of human properdin DNA. The genomic DNA was successfully isolated from peripheral blood mononuclear cells samples based on leukocytes preparation by sedimentation rate.

A reporter gene assay of dual luciferase system was used in this study to study the regulation of a gene of interest, the properdin promoter by quantifying the gene expression. The dual luciferase assay was a perfect reporter gene assay because there was no endogenous luciferase activity in mammalian cells and the functional enzyme was created upon translation. The dual luciferase assay system contained two different reporter genes, Renilla and firefly luciferase and performed by measuring the firefly and Renilla luciferase activities of the same sample. The upstream regulatory elements for gene of interest were cloned and integrated at upstream of the firefly luciferase gene. Expression of gene of interest coupled to the expression of a luciferase reporter gene was quantified by transcriptional regulation correlation with the effect of specificity of gene of interest i.e. experimental promoter activity. The Renilla luciferase was co-transfected together act as an internal control which serves as the baseline response.

In present study, 3 constructs of the human genomic properdin were prepared and 2 constructs, Prop300 and Prop670 with location of (-420 ~ -120) and (-420 ~ +250) respectively were successfully inserted into luciferase reporter plasmid and transiently transfected to U937 cell denoted as pPROP-Luc_{670} and pPROP-Luc_{300}, respectively. Another reporter plasmid pCMV-luc (Cytomegalovirus plasmid DNA) with known activity also was added as a positive control in the assay.

The study was involved observation of transfected cells with unstimulated and stimulated with LPS of U937.

Upon the transient transfection, negative control contains untransfected cells of the experiment shown with no reading of luciferase activity indicated there were no background reading from the untransfected cell (Fig. 3.7). Preliminary experiment data indicated, pCMVluc is a strong promoter that acts as positive control to evaluate success and efficiency of transfection. High results were obtained during the experiments indicated success of transfection (Fig.3.7A). Any other reading lower than positive control indicates as a weak promoter.

Conversely the pPROP-Luc_{670} and pPROP-Luc_{300} have lower luciferase level (Fig.3.7B) compare to positive control. This could because the insert construct have a weak promoter as positive results for positive control would indicate that the lack of promoter activity for the interested artificial insert constructs is not due to the fact that the cells lack essential transcription factor drive for this expression.

Construct pPROP-Luc_{670} at 24hours of U937-transfection had an increase in expression but a decrease upon stimulation of LPS. Although weak, there seems to be more activity in 670bp fragment (-420~+250) in unstimulated U937. However there is no significantly different between the promoter activities with internal control. Whereas construct pPROP-Luc_{300} had consistently decreased luciferase activity with and without LPS stimulation. The control renilla activity for both pPROP-Luc_{670} and pPROP-Luc_{300} increased consistently after LPS added in the transfection assay. Again this indicates a weak specific promoter activity compared with variably strong renilla control promoter activity. Normalising or comparing the experimental reporter gene to the activity of internal control is necessary because of variability caused by differences in cell viability and transfection efficiency.

3.4.2 Limitations of the study

To the best of our knowledge, this thesis only covers a preliminary analysis of properdin promoter. Several attempts have been made to optimise the experiments. Although the PCR that has specificity of reaction, flexibility, simplicity and reliability but theoretically feasible, the technique was not easy and required extensive optimisation due to the reaction such as primer having varying melting temperatures and other parameters like $MgCl_2$, dNTPs and Taq DNA polymerase concentration.

Success of transfection assay depends on insertion and correct orientation of a functional promoter upstream luciferase gene. Not only depend on the DNA plasmid but also cell's

condition (healthy without contamination) must be included as a factor of expression of luciferase activity.

Previously in first attempt, U937 cell contamination and too diluted DNA probably as major problem in this experiment. Optimisation with new DNA ratio, new plasmid preparation with endo-free reporter plasmid DNA, and also obtained healthy maintained new batch of U937 cells to encounter the possible problems. In addition, as Prop300 and Prop670 construct was found to be as a weak promoter (as they low/not expressing promoter activity), so in attempt to produce bigger fragments. However due to time constraints, the experiment was unable to progress further.

In relation to the characterised properdin promoter model that encompassing NFAT/AP1 and Atp1a1/GATA/MyoD site, it is worth to say that in Liang's thesis (Yue, 2005) mentioned about GATA site that may direct the expression of properdin in monocytes. GATA presents in many properdin-producing cell types, such as mast cell, T cells and can be down-regulated by tetradecanoyl phorbol acetate (TPA)(Duvoix et al., 2004). In relation to the finding, raised an idea that perhaps due to functioning of GATA to down-regulate that may direct the expression of properdin promoter activity as both Prop300 and Prop670 fragment with location of (-420 ~ -120) and (-420 ~ +250) respectively comprises GATA site.

In a search of literature managed to find about Thrombospondin-1 (TSP-1) that maybe comparable to properdin as properdin consist of six thrombospondin type 1 repeat (TSR1-

6) (Kang et al., 2004) showed their study of luciferase reporter assay of human genomic TSP-1 promoter and transiently transfected the Hep3B human hepatocarcinoma cell. The finding was their construct pTSP-Luc-4 (-767~+756) had consistently decreased luciferase activity with or without PMA stimulation of Hep3B cells. They found that, repressor Yin Yang-1 (YY-1) at nucleotide -440 and the suppression induced by this side.

In addition TSP-1 is thought as specific cell-type regulated and the levels are tightly regulated at the transcriptional level (Kang et al., 2004). These as shown by TSP-1 upregulation upon PMA stimulation in the human hepatocarcinoma cell line Hep3B (Kim et al., 2001), but TSP-1 is down-regulated in PAE cells (Kim and Hong, 2000). Again, perhaps can relate with the current finding as maybe presence of repressor that may suppress the expression of promoter activity and maybe properdin activity is cell-type specifically regulated. However further studies have to be done.

U937 monocytic cell line was ideal to employ in this study since U937 have been shown to secrete and synthesise Properdin (Minta, 1988). Alterations in the profile of protein expressed by U937 cells vary according to their differentiation.

Although PMA and LPS is known to induce U937 to differentiate into monocyte-macrophage like cells (Kahler et al., 1999). Perhaps need to change inducer as PMA is best to be used. U937 following stimulation with PMA able to synthesise a precursor properdin subunit polypeptide chain which undergoes post-synthetic glycosylation and polymerization to give rise to the oligomers characteristics of properdin (Minta, 1988). Minta (Minta, 1988) have shown non-stimulated U937 secrete properdin in low level compared to stimulated U937 cells.

The experiment for transfection with LPS induction was setup for 24 hours low expression of promoter activity; probably it was not sufficient time for induction as this could have been the time frame the cells needed for differentiation and to begin to change morphology. In addition there could possibly be toxicity with LPS. In the future perhaps the experiment can be repeated with longer incubation period for LPS stimulation and change strategy by stimulate cells first then transfection step.

The status has yet to be elucidated. The methods used in this study can be improved in the future. Therefore it is concluded that the transfection of the properdin plasmid upon LPS induction was not successful.

Chapter 4: Analysis of the immune response of properdin-deficient and wild type mice in an intraperitoneal model of polysaccharide vaccination

4. 1 Introduction

Humans with deficiency in complement and total splenectomy or congenital asplenia are known to have a significant risk of infections caused by encapsulated organisms.

Administration of vaccine plays a role to help produce immunity against a disease. Throughout the world, effective immunisation procedures take a lead as the primary prevention method to prevent infectious diseases such as influenza, meningitis and others.

Bacterial infectious diseases still remain threat to the world and among all are two commonest causes of bacterial species, the *Neisseria meningitidis* and *Streptococcus pneumoniae*. These pathogens are characterised by their ability to colonise the nasopharynx and invade the host and cause bacteraemia and infections, such as otitis media, pneumonia, or meningitis (Klein et al., 2007). As found in cell walls of encapsulated bacteria such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria meningitidis* which having a cell surface polysaccharide capsule (capsular polysaccharide, CPS), these bacteria are able to prevent activation of the complement by cell surface bacterial proteins that inhibits phagocytosis and bacterial killing.

Complex polysaccharides are poorly immunogenic compared to peptide antigens however due to the chemical characteristic by negatively charged, phagocytic uptake can be difficult (Lee et al., 2001).

4.12 Polysaccharide antigens

N.meningitidis that has been defined on the basis of the immunochemistry of CPS consists of 13 serogroups. Based on high molecular weight of CPS, groups A, C, Y and W135 infections of *N.meningitidis* can be prevented by vaccines. Unfortunately, these vaccines do not induce T-cell-dependent immunity as poorly effective in young children and infants and do not induce long-term immune memory (Salehen and Stover, 2008).

Composed of 90 serogroups of distinct polysaccharides of *S. pneumoniae* and among the serogroups, the twenty three capsular polysaccharides (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F) are used in the currently available vaccine (Pneumovax).

Pneumococcal polysaccharide (PPS) vaccines are T-cell independent antigens and induce IgM responses by cross-linking, in the absence of T-cell help. There is in efficient processing and presentation of polysaccharides and no upregulation of co-stimulatory molecules triggered by CR2 mediated B-cell (Brown et al., 2006). Here comes in important binding of C1q-tagged complexes to the BCR of follicular B-cells as B-cell provides a surface for complement activation and C3 ligands that can bind of the BCR, to CR1 or CR2

(Rossbacher and Shlomchik, 2003). In relation, polysaccharide interacts with complement and formed polysaccharide-complement complexes which localise in the marginal zone of the spleen as a main location of initiation of the antipolysaccharide response (Breukels et al., 2005).

Marginal zone B-cells are a non-activated B-cell subset which function within the innate immunity (Viau and Zouali, 2005) as they are different from follicular B-cells (of the germinal centers). However on activation, the marginal zone B-cells migrate to the follicular zone where CD21 (CR2) is proteolytically cleaved and antigen transferred to follicular dendritic cells (Timens et al., 1989).

Polysaccharide antigens are bound by CD21hi marginal zone B-cells and are, in the mouse, additionally captured by marginal zone macrophages expressing complement receptors CR3 and CR4 (Ochsenbein and Zinkernagel, 2000). Polysaccharide antigens crosslink polyreactive B-cell receptors and lead to their activation.

Immunoglobulins IgM and IgG are antibodies detected after immunisation with T independent antigens. It is usually generated simultaneously within 3 days, along with lesser opsonophagocytic and bacteriolytic activities, compared to antibodies elicited by immunisation with T-dependent antigens (Harris et al., 2003, Hu and Test, 2004, Lee et al., 2001). Although it is reflecting absent affinity maturation of these antibodies, but the polyreactive and persistent stimulation of B-cells by polysaccharide structures can provide long lasting antibody levels. The antigen persistence and continuous stimulation are important in maintaining memory B-cells (Macallan et al., 2005). However, to date,

polysaccharides were thought unable to elicit a memory B-cell response (Kelly et al., 2006) in conjunction of the fact that polysaccharide antigens are poorly presented by MHCII which lead to no recruitment of T cell help, may induce tolerance or generation of plasma cells by crosslinking BCR and might be poorly retained in the germinal center (Toellner et al., 2004). Yet, importantly, there are findings demonstrate that IgM memory B-cells are indeed generated, independent of T-cell help (Obukhanych and Nussenzweig, 2006).

Currently improvement in vaccine development by coupling of vaccines with attenuated toxins, such as diphtheria or tetanus toxoid, are shown to enhance the delivery of low immunogenic vaccines, such as polysaccharides, to antigen presenting cells, and recognition by T-cells.

In the mouse, the T-dependent immune response involves metallophilic macrophages of the marginal zone, marginal zone B-cells and follicular B-cells of the germinal centre in the spleen. Antibodies in circulation, initially of the IgM-, subsequently of the IgG-type, are specific for the protein carrier as well as the polysaccharides (McCool et al., 1999).

Importantly, the finding of subcutaneous administration of T-independent or T-dependent antigens in mice, shown that the spleen significantly reacts with formation of antibody forming cells before the draining lymph nodes, especially in response to T-independent antigen (Delemarre et al., 1989).

4.13 The importance of complement activation towards polysaccharide antigens

The complement system is an innate first line host defence mechanism against microbes. The complement system interacts with the adaptive immune response that is important in, stimulation and maintenance of B-cells by which particularly to the generation of antibody response and immunological memory (Carroll, 2004, Walport, 2001).

The alternative pathway of complement activation is activated by bacterial capsular polysaccharides. Properdin stabilises the activity of the alternative pathway, and enhances the availability of C3 products, which are important in the B-cell immunoglobulin response.

Complement activation and B cell immunity are linked. Complement enhances adaptive immunity by several ways: (i) Binding of C3d coated antigen by B cells results in enhanced signaling via the B-cell receptor and increase of secreted antibodies production, (ii) C3d is necessary to localise Ag to follicular dendritic cells (FDC) in lymphoid follicles. FDC express high levels of CD21 and CD35. Follicular dendritic cells retain complement tagged antigen and promote antigen selection of high affinity germinal centre B cells leads to maintenance of memory B-cells (Salehen and Stover, 2008).

The fact that complement deficiency susceptible to infection with bacteria with a complex polysaccharide capsule emphasising that complement important to raise an appropriate antibody response to polysaccharide antigens. Among the deficiency, C3 deficiency is the most serious complement defect as C3-deficiency predisposes to infections with not only encapsulated, but a variety of microorganisms (Reis et al., 2006), its activation and degradation products reflecting the central role of C3 and in the complement activation cascades, in addition link the adaptive and innate immune response on the surface of the B-cell (Rickert, 2005). Some other complement-deficient individuals may have sufficient immunoglobulin responses to polysaccharide antigens. Therefore, other functional polymorphisms, immaturity or age may contribute as factors if individuals with a complement deficiency other than C3 deficient have an inability to mount a protective titre of antibodies to polysaccharide antigens.

4.14 Background of the study

A few studies of complement deficiencies investigating the impact of these deficiencies on vaccine success have shown that most of the complement deficient patients are able to mount specific antibody response. Of all, the largest of these studies involved 53 patients with history of meningococcal disease with background of a variety of genetic complement deficiencies (properdin, Factor H, late components) were immunised with the tetravalent meningococcal polysaccharide vaccine ACYW135 (Fijen et al., 1998). These patients showed not only vaccination-induced, but also infection-induced. Important findings of the

study shown there were no vaccine failures although there was wide variation in the specific antibody response between individuals. In the study, 19 properdin deficient people that been vaccinated shown impaired IgG response but there was a wide variation of specific antibody (Fijen et al., 1999). Comparable other study, vaccination of three properdin-deficient patients with meningococcal vaccine was protective against meningococcal disease (Densen et al., 1987, Soderstrom et al., 1989). As properdin-deficient is not tested routinely, detection of properdin-deficient in individuals seems to be a concern. However, not every properdin-deficient individual succumbs to fatal meningococcal disease and there involvement of certain immunoglobulin allotypes which are specifically efficient towards polysaccharide antigens that may be compensate of this deficient (Spath et al., 1999).

Based on these observations current study hypothesis is that Properdin is required for optimal response to immunisation with capsular polysaccharide. In addition, this is the first study to assess relevance of complement activation towards polysaccharide antigen and protection. A properdin deficient mouse strain has been generated (University of Leicester, (Stover et al., 2008)) which will be instrumental in answering the following hypotheses.

4.2 Materials & methods

4.2.1 Mice

Experiments were performed using properdin-deficient mice ((Stover et al., 2008), University of Leicester) that were generated and backcrossed for 9–12 generations onto C57BL/6 background and WT littermates (all males) with 8 to 16 weeks of age.

4.2.2 Pneumococcal strains and preparation of the challenge dose

The wild-type *S. pneumoniae* strain used was serotype 2 strain D39 (kindly prepared by Dr. R. El-Rachkidy Lonnen, University of Leicester). Briefly, pneumococci were cultured on blood agar base containing 5% (v/v) horse blood or in brain heart infusion broth containing 20% (v/v) fetal bovine serum**.** *S. pneumoniae* was passaged through mice, and aliquots were stored at −70°C. When required, the suspension was thawed at room temperature and bacteria were harvested by centrifugation before being resuspended in sterile phosphate-buffered saline (PBS).

4.2.3 Experimental procedure

Experimental procedure was adapted from Sen *et al* (Sen et al., 2005). Study consisted of two sets of experiments.

First experiment:

12 properdin knockout mice and 12 wild type mice were used at 8 to 16 weeks of age in this experiment. Animal experiment was kindly performed by Prof. PW Andrew. The experiment procedure can be illustrated by using the schedule (figure 4.1).

First of all, mice were tail bled to obtain blood for day 0 or pre-vaccinated sample. On the first day of experiment, 10 Properdin knockout and 10 wild type mice were vaccinated intraperitoneally (i.p) with Pneumococcal Polysaccharide vaccine (Pneumovax-23), while control mice (2 each, Properdin deficient and wild type) were given PBS. Pneumovax-23 (Merck, Merck Biosciences, Nottingham, UK) consist of a mixture of highly purified capsular polysaccharides from the 23 most prevalent or invasive pneumococcal types of *S. pneumoniae*. In each 0.5ml dose of vaccine contained 25µg of each PS type dissolved in isotonic saline solution containing 0.25% phenol (a preservative). Pneumovax was diluted 1:5 dilution with sterile PBS. Following sera were prepared from blood obtained from the tail vein on day 0 (before vaccination) and after 4 weeks of vaccination. Mice were then been left for 4 weeks. After 4 weeks of vaccination period, lightly anaesthetised mice were challenged by intranasal infection with *S. pneumoniae* serotype 2 strain D39 (type 2 polysaccharide is contained in the vaccine) by giving each mouse 100µl of CFU dose

$1x10^6$ D39 and observed for a week for infection stage. Mice were culled if significant morbidity (lethargic ++) was observed. All mice were culled at the end stage of experiment (after a week of infection) under terminal anaethesia 2.5% (v/v) fluothane over oxygen (1.5 to 2 litres/min) and cardiac puncture was performed to recover blood from mice (kindly performed by Miss Sarah Glenn). Blood obtained were clotted on ice until further treatment. Disease severity during infection stage was recorded at least twice daily.

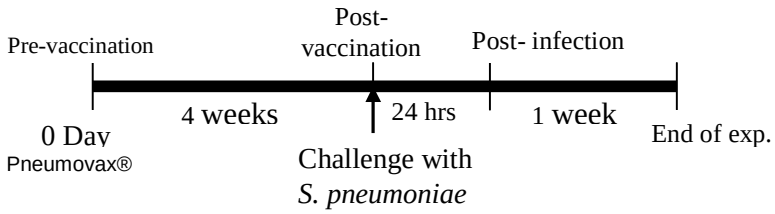


Figure 4.1: Schedule of *in vivo* experiment (first experiment) using properdin deficient and wild type mice. (n=10 prop-def i.p vaccinated, n=10 WT i.p vaccinated with (Pneumovax-23), while control given i.p PBS n=2 each, prop-def and WT).

Second experiment:

For the second experiment, similar procedure with the first experiment was used but the experiment was improved by increasing group size of experimental mice and weekly time bled were performed to obtain weekly time point post vaccination sample. The experiment procedure was performed according to illustrated schedule (figure 4.2).

For vaccination procedure, experimental properdin-deficient and wild type littermates (15 each) were vaccinated i.p with Pneumovax-23, Merck while control mice (5 each, Properdin deficient and wild type), were given i.p PBS.

Sera were obtained and aliquot from blood of tail bled of each time point-pre-immunisation, weekly post immunisation, 24hrs post infection and end of experiment. As the times shown by asterisks* in Figure 4.2

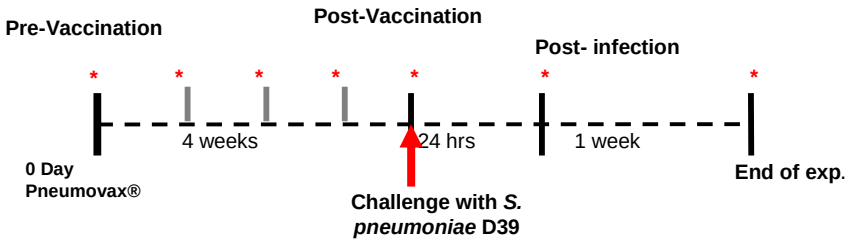


Figure 4.2: Schedule of *in vivo* experiment (second experiment) using properdin deficient and wild type mice. (* shown tail bled each time point-pre-immunisation, weekly post immunisation, 24hrs post infection and end of experiment and serum were obtained and aliquot). (n=15 of each prop-def and WT i.p vaccinated with Pneumovax-23, while control given i.p PBS n=5 each, prop-def and WT).

4.2.3.1 Serum preparation

Sera were separated by centrifugation (13,000g for 5 minutes) from blood clotted obtained from the tail bleed and cardiac puncture of the mice. Sera were aliquoted and stored at -20ºC until further analysed.

4.2.3.2 Disease severity score

Assessments were performed twice daily during infection stage and by guideline from Morton and Griffith (Morton and Griffiths, 1985) the condition or disease severity were evaluated as the table:

score	Condition/ disease severity
0	normal
1	+hunched
2	++hunched
3	+ starey/ piloerect
4	++ starey/ piloerect
5	+lethargic
6	++lethargic
7	moribund
8	dead

Table 4.1: Disease severity score table to evaluate condition of the experimental mice.

4.2.4 CFU count

The Miles-Misra technique was used to determine the number of colony forming units (CFU) to assess the number of viable and growing bacteria presence in the sample. CFU counts were calculated as an average CFU formed from triplicate 10µl drops of Miles-Misra plating onto Blood agar base (BAB) with 5% (v/v) defibrinated horse blood following 10 fold serial dilutions of sample in sterile PBS incubated overnight at 37°C incubator. After 24 hours at 37°C, colonies were counted and expressed as CFU/ml serum.

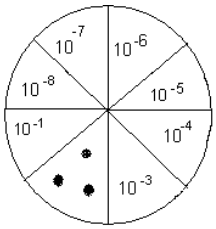


Figure 4.3: The Miles-Misra plating technique with triplicate drops of sample

4.2.5 Archive organs for paraffin embedding

At the end of the experiment, organs (lung and spleen) were dissected and immediately placed in formal saline. Archived organs were kept in the fridge until further treatments. The organs in formal saline then were transferred to a plastic cassette and immersed with 70% IMS. Later the samples were sent to histopathology department (Hodgkin building, University of Leicester) for fixation in tissue processor (Shandon Scientific, Runcorn, UK) with different concentration of ethanol and xylene. The organs then were embedded in paraffin wax (TCS Biosciences Ltd, Buckingham UK) and prepared in block. Lungs and spleen paraffin embedded blocks were cut into sections (7µm-thick) using a microtome (Leitz Wetzlar). Sections were picked up on a microscope slide (Histobond, Lamb) and slides were kept in a slide box at room temperature until further use.

4.2.6 Histopathologic evaluation of lungs

For histologic examination, at the end of experiment lungs were excised and inflated by using a small diameter catheter with 1 ml of 10% formal saline to improve resolution of anatomic structure. Then lungs were prepared for paraffin wax as described in 4.2.5. Slides section were deparaffinized with xylene and stained with H&E.

H&E staining was performed firstly by deparaffinized the paraffin-emmbedded sections of lungs with xylene for 3 changes with 3 minutes each. Then transfer to absolute alcohol for 3 changes with 3 minutes each and once in 70% alcohol for 3 minutes before bringing the section into water. The slide was washed in running tap water for 3 minutes. Then slide was stained with Mayer's hematoxylin for 15 minutes and washed in running tap water for 20 minutes after stained. Counterstained the slide with eosin from 15 seconds to 2 minutes and dehydrated in 70% and absolute alcohols, three changes of 3 minutes each. Staining was checked under microscope and slide was transferred into xylene with three changes of 3 minutes each. Lastly slides were mounted in mount solution DPX. H&E stained slides of lungs were evaluated by light microscope to examine the lung histologically.

4.2.6.1 Polymorphonuclear leukocytes (PMN) count

H&E stained slides of lungs were evaluated by light microscope to count number of PMNs in the lungs. Normal lungs for both wild type and properdin-deficient were evaluated as

well as comparison with the experimental mice. All slides were blindly read to their identification.

PMN in the lungs per mm^2 area were calculated by:

$$\frac{\text{Mean number of cells}}{} \quad X100\ (mm^2 \text{ area of graticule squares})$$

Area multiplied by the magnification factor

(E.g. if counted at x40 magnification, then it is actually x400)

4.2.7 Peritoneal cells count

The method of this experiment was adapted from (Tang et al., 1998).

A separate set of mice model with 4 of each properdin-deficient and wild type were prepared and followed the same procedure of vaccination; 2 Properdin knockout and 2 wild type mice were vaccinated intraperitoneally (i.p) with Pneumococcal Polysaccharide vaccine (Pneumovax-23), while control mice (2 each, Properdin deficient and wild type) were given PBS and were left for 4 weeks of vaccination period. Mice were culled after 4 weeks of vaccination period. Then a gentle massage of the abdomen was performed for 60 seconds and the peritoneal cavity was opened. The peritoneal cavity was washed with 50 ml ice-cold PBS and again the abdomen was massaged before harvested the cells. Cells were collected from the peritoneal washes then were spun (13,000g for 5 minutes). A red blood cell lysis step was included by suspended in lysis buffer (17mM Tris HCl- 14mM NH_4Cl, pH 7.2) and incubated at 37°C for 5-10 minutes to lyse the red blood cells. Then was washed twice with PBS before proceed for cytospin step.

Briefly, 100µl of peritoneal washes were cytospun onto a cytospin slide (Shandon) in a cytocentrifuge (Cytospin 2, Shandon) at 100g for 5 minutes. Slides were then air-dried, fixed in 100% methanol for 5 minutes and stained with Giemsa stain (BDH, Poole, UK). Microscpic differential cell counts were done for monocytes, macrophages, lymphocytes and mast cells on each slide were identified and quantified according to their morphology. At least 100 cells per total were counted on two different slides for each sample. The relative percentages of each type of cells were calculated.

4.2.8 ELISA

4.2.8.1 Commercial ELISA for total serum IgM and IgG

Total serum IgM ELISA

The capture ELISA technique is used to quantitatively measure levels of immunoglobulin in serum. All protocol was followed from commercial ELISA kit (Mouse IgM Quantitation Kit;cat no:E90-101; Bethyl Lab Universal Biology, Cambridge UK) protocol. All steps were performed at room temperature.

Briefly, captured antibody (100µl coating buffer (1.5M carbonate-bicarbonate pH9.6) containing of 1:100 dilution of the goat anti-mouse IgM antibody) was used to coat the microplate (96 Maxisorp Nunc-Immuno plates, Nunc Scientific Lab Supplies, Wilford, UK) for an hour and blocked for 30 minutes with 200 µl of blocking solution (1xTBS, 1%BSA). Then washes step were performed in between by three times in a washing

solution (1x TBS, 0.05% Tween 20). Then sample and standard were added after washes step. 100 µl of each standard and each serum diluted in 1:300 in sample diluent (1x TBS, 1% BSA, 0.05% Tween 20) were added to the plate and incubated for an hour. The plate was washed three times and incubated for another hour with 100 µl/well of the goat anti-mouse IgM HRP-conjugated antibody (diluted 1:75,000 in sample diluent). For last washes, plate was washed with five times and 100 µl of the TMB substrate (Single component TMB peroxidase EIA substrate kit, Bio-Rad, Hemel Hempstead, UK) were added to the plate. The reaction was stopped by adding stop solution (0.8M H_2SO_4) approximately after 6 minutes. The absorbance was measured at 450nm using microplate reader (Model 680, Bio-Rad) and analysed using the microplate manager software, version 5.2.1 (Bio-Rad). The standard curve was generated using five parameter logistic curve fitting (5-PL, type Rodband). Antibody concentration is expressed in ng/µl.

Total serum IgG ELISA

Measurement of total IgG levels was using a commercial mouse IgG ELISA kit (Bethyl Lab). Total serum IgG concentration were determined using method identical to measure total IgM antibodies except that plates were coated with 100µl of containing of 1:100 dilution of the goat anti-mouse IgG antibody, serum samples dilution in 1:1000 in sample diluent and detected by goat anti-mouse IgM HRP-conjugated antibody (diluted 1:10,000 in sample diluent).

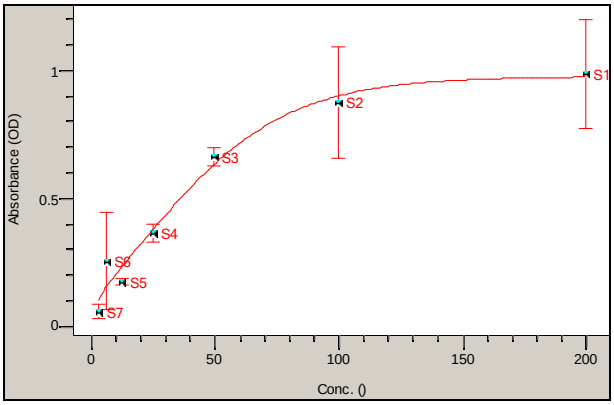


Figure 4.4 : Representative of standard curve produced by five parameter logistic (5-PL) curve-fit (using Bio-Rad Microplate Manager Software Version 5.2.1).

4.2.8.2 Polysaccharide specific ELISA

Polysaccharide specific ELISA was performed with aim to see effectiveness of the immunisation to individual pneumococcal polysaccharide serotype (PPS), PPS2, PPS3 and PPS6b by measuring anti-capsular polysaccharide immunoglobulin, IgM and IgG3.

IgM level was measured as to determine the primary immune response towards infection and immunisation meanwhile IgG3 was measured as the most abundant of IgG subclass against carbohydrate polysaccharide antigen in mouse (Harris et al., 2003, Hu and Test, 2004, McLay et al., 2002, Snapper et al., 1992).

Specific ELISA for determination of anti-PPS 2 IgM antibodies

Specific ELISA method was adapted from (Russell et al., 2000, Wernette et al., 2003).

Previously for optimisation to setup the experiment, a few ELISA had been setup to determine optimal serum dilutions. This was done by doing serial dilutions and 1:300 dilutions were chosen based on range of this dilution in standard curve.

96-well plates (Nunclon Maxisorb**,** Wilford UK) were coated with 100µl of PPS2/well (Purified Pneumococcal Polysaccharide, ATCC, Middlesex, UK) at 2µg/ml prepared in coating buffer and were incubated for overnight at 4°C. The next day, the plate was left at room temperature before washing four times with washing buffer (1xTBS, 0.05% Tween 20). After washing, the plate was blocked with 200µl blocking solution to each well (1xTBS, 1% BSA) for 1 ½ hour on shaking incubator at 37°C. Meanwhile serum samples were prepared at 1:300 in sample diluent (1xTBS, 1% BSA, 0.05% Tween 20). Serum samples were pre-absorbed with purified pneumococcal cell wall polysaccharide (CWPS Statens Serum Institute, Denmark) 5µg/ml at 37°C for 1 hour to neutralise antibodies against cell wall polysaccharide before an antigen capture ELISA was used to detect antibodies to PPS.

Then, the plate was washed four times and serum samples were added on the plate. Plate was incubated for 1 hour at 37°C. After incubation, the plate was washed four times. To detect serum IgM specific against PPS2, 100µl of goat anti-mouse IgM antibodies HRP labelled (Bethyl Laboratories) diluted 1:10,000 with conjugate diluent (1xTBS, 1% BSA, 0.05% Tween 20) was added and incubated for 1 hour at 37°C. Again after incubation, plate was washed for four times. 100µl of 3,3',5,5'-tetramethylbenzidine chromogen substrate (TMB**,** Bio-Rad Hemel Hempstead UK) was added. Reaction was stopped after

appearance optimal blue colour by adding 100µl Stop solution (0.8M H_2SO_4) and reaction with change colour from blue to yellow.

Lastly, the quantity of bound enzyme varies directly with the IgM level in the plate was read at absorbance 450nm by ELISA plate reader (model 680, Bio-Rad). Results for the experiments were calculated by average the duplicate readings of each samples and subtracting background reading; [IgM level = (O.D 1 + O.D 2)/2 - (background reading)]

As no standard was added, the results of IgM presence were expressed by absorbance (O.D) level.

In order to compare experiments, a few serum samples of normal WT (n=2) and KO (n=2) were established as internal control and used in all repeated ELISA. This internal control was having same O.D level in all repeated ELISA.

In determination of anti-PPS IgG3 antibodies, serum samples were diluted in 1:100 dilution and detected by adding 100µl of goat anti-mouse IgG3 antibodies HRP labelled (Bethyl Laboratories) diluted 1:30,000 with conjugate diluent.

Also the specific ELISA has performed with pooled serum. This is done by pooled the serum samples according to mice group i.e. control prop-def, control WT; experimental

prop-def and wild type. This was done by taking out 1µl of serum from individual mice and pooling it together in one reaction tube.

Specific ELISA for determination of Anti-PPS3 and Anti-PPS6B antibodies concentrations

Serum anti-PPS3 and anti-PPS6B IgM concentration were determined by ELISA using methods identical to those used to measure IgM antibodies against PPS2 except that plates were coated with 100µl of PPS3/well at 2µg/ml and 100µl of PPS6B/well at 2µg/ml respectively.

4.2.8.3 C3 ELISA

All protocol was followed from ELISA kit (Commercial kit-ICL E90C3, Immunology Consultants Lab, Newberg USA) protocol.

The microplate wells of precoated anti-mouse C3 antibody were loaded with 100 µl of standard and mouse serum samples that been diluted 1:50,000 in sample diluent. The plate was then incubated for 20 minutes at room temperature. After 4 washes in washing buffer, the plate was incubated for 20 minutes with HRP conjugate of the coating antibody. Again, the plate was washed 4 times with washing buffer and 100 µl of TMB substrate solution were added into the wells. The reaction was stopped with 100 µl of stop solution after 10 minutes. The absorbance was measured at 450nm of microplate reader and C3 concentrations were calculated using ELISA software (Bio-Rad Microplate Manager

Software Version 5.2.1) from standard curve produced. The antibody concentration is expressed in mg/ml and C3 concentration in normal mouse sera in a range 0.3-1.15mg/ml.

4.2.9 Statistical analysis

Normal ranges for 95% of the reference population were estimated for each group. Differences among the groups and each parameter were analysed using the Mann-Whitney non-parametric test applied with Bonferroni correction. Comparisons between mice vaccinated without pretreatment and those receiving PBS were done using Mann-Whitney test for unpaired samples. The significance level employed for all tests was set at $p= 0.05$

4.3 Results

In the study to analyse the immune response of properdin-deficient mice, the work was interested in the relevance of properdin *in vivo* by intraperitoneally polysaccharide vaccination and intranasally infection of *S.pneumoniae*.

To achieve this (as described in Chapter 4.2 -Materials and Methods section) the role played by properdin during immunisation and bacterial infection was investigated using the properdin-deficient mouse line and wild type littermate.

Briefly, properdin-deficient and wild type littermates (C57Bl/6) were immunised intraperitoneally with Pneumococcal Polysaccharide vaccine (Pneumovax-23), 4 weeks after post-infection, mice were challenged intranasally with *S. pneumoniae* serotype 2 strain D39 (type 2 polysaccharide is contained in the vaccine). Total IgM and specific anti-capsular type 2 IgM antibody levels were measured by ELISA using blood collected at various time point .The severity of pneumococcal disease and mortality were recorded.

4.3.1 Analysis of immune responses following intraperitoneal polysaccharide vaccination for properdin-deficient and wild type mice

4.3.1.1 Determination of antibody response during vaccination stage

4.3.1.1.1 Level of total serum IgM

In order to determine the primary immune response towards infection and immunisation, IgM level was measured in Properdin-deficient and wild type mice.

Total serum IgM concentrations present in both properdin-deficient and wild type serum at various time intervals (pre-vaccinated, post-vaccinated and end of experiment) were measured by using commercial ELISA. In Figure 4.5 (Table 4.2 along with the graph) summarised data for total serum IgM for the time points.

First experiment (figure 4.5 (A)) with total of 12 of each mice group, properdin-deficient and wild type demonstrated baseline level of IgM concentrations at Day 0 pre-vaccinated with at range of 50-180ng/µl. This detectable level of IgM demonstrated as natural IgM level present naturally at time point zero.

At pre-immunisation, wild type appeared to have more IgM concentration with mean concentration 120.28 ng/µl compared to properdin-deficient samples. However there was no significance difference between the groups.

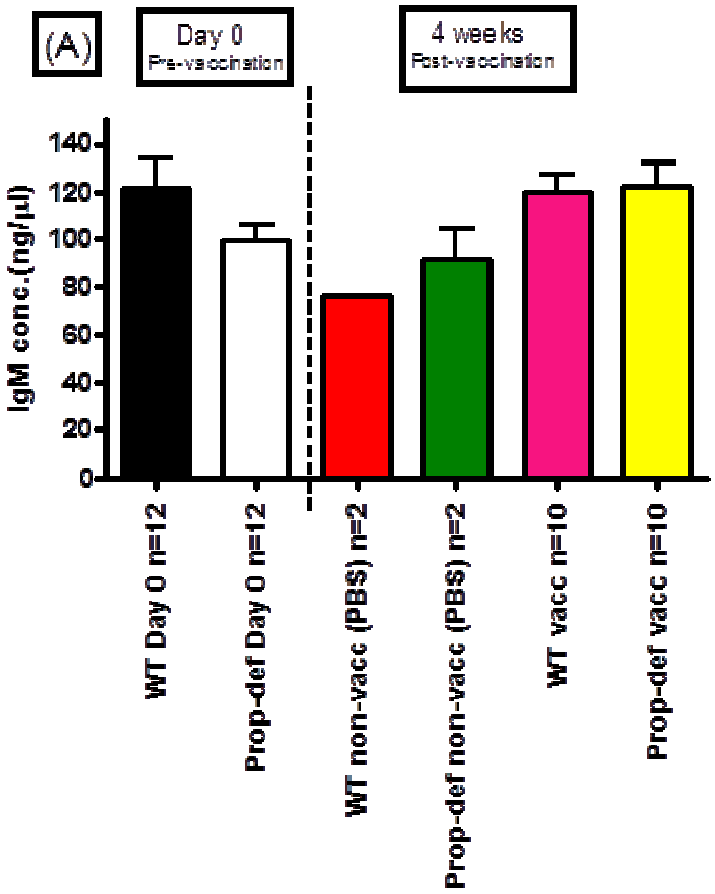
After 4 weeks of post-vaccination, both groups of control mice given PBS were still at the same range of concentration as pre-vaccinated while both vaccinated properdin-deficient and wild type had an increase in their IgM concentration with starting range at 76-154 ng/µl of wild type and properdin-deficient 80-180 ng/µl.

The experiment was repeated with total of 20 mice of each group, properdin-deficient and wild type (figure 4.5 (B)). As detectable level for both groups natural IgM level demonstrated baseline level of IgM concentrations at Day 0 pre-vaccinated with at range of 50-180ng/µl. At pre-immunisation, properdin-deficient have significantly higher IgM with mean concentration 130ng/µl compared to wild type with 100ng/µl. However there was no significance difference between the groups.

At time point 4 weeks post-vaccinated, wild type control mice given PBS were at the same range of concentration as pre-vaccinated. Unexpectedly non-vaccinated control properdin-deficient was significantly higher concentration of total serum IgM compared to wild type control.

Vaccinated wild type increased a little compared to non-vaccinated with highest range at 119ng/µl while vaccinated properdin-deficient was significantly higher concentration of total serum IgM with mean concentration 140ng/µl compared to wild type vaccinated and similarly to non-vaccinated control properdin-deficient.

(A)	Wild Type	Properdin-deficient
Day 0 (Pre-vacc) ng/µl	55-180 (n=12)	60-140 (n=12)
4 weeks (Post-vacc) ng/µl	Non-vacc, (n=2):70-76 Vacc, (n=10): 76-155	Non-vacc, (n=2): 78-104 Vacc, (n=8): 80-180



(B)	Wild Type	Properdin-deficient
Day 0 (Pre-vacc) ng/µl	50-152 (n=20)	70-180 (n=20)
4 weeks (Post-vacc) ng/µl	Non-vacc. (n=5): 40-79 Vacc. (n=15): 32-119	Non-vacc. (n=5): 112-215 Vacc. (n=15): 82-231

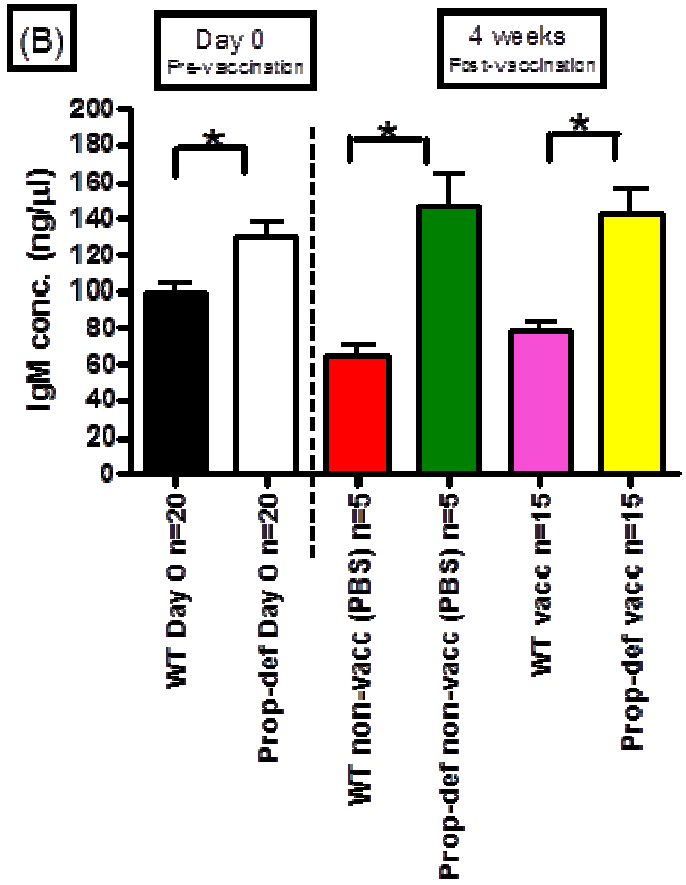


Figure 4.5: Data range of total serum IgM level (ng/µl) in properdin-deficient and wild type mice for the time point of indicated are presented in the Table 4.2 along with graph represent mean ±SD of total serum IgM level in properdin-deficient and wild type mice for the time point of indicated. The first experiment (total n=12 of each WT and prop-def) **(A)** and the second experiment (total n=12 of each WT and prop-def) in **(B).** (*$p<0.05$, Non-parametric Mann-Whitney with Bonferroni correction compared wild type and properdin-deficient)

4.3.1.2 Determination of serotype specific antibody responses

Polysaccharide specific ELISA was performed to see effectiveness of the immunisation and whether properdin-deficient mice differ from wild type mice in their specific anti-polysaccharide immunoglobulin response after vaccination. Polysaccharide specific ELISA was used to detect IgM and IgG3 class specific antibodies to individual pneumococcal polysaccharide serotype (PPS), PPS2, PPS3 and PPS6b as this polysaccharide are contained in the vaccine

Serum samples were pre-absorbed with cell wall polysaccharide (CWPS) to neutralise antibodies towards cell wall (Goldblatt et al., 1992). Previously during optimisation in setting up the specific ELISA, the findings in comparison with and without CWPS pre-absorbed serum have produced a comparable level of IgM against polysaccharide capsular type 2 and found that with CWPS pre-absorbed the serum samples, the levels showed with at least a fold lower in comparison than the one without CWPS pre-absorbed (data not shown). This has indicated a successful of neutralising the antibodies against cell wall thus to reduce non specific binding activity.

4.3.1.2.1 Determination of serotype specific anti-PPS 2 IgM level

Polysaccharide specific ELISA was used to detect IgM class specific antibodies to individual PPS type, PPS2 determined because of D39 which is polysaccharide antigen type 2 (type 2 polysaccharide is contained in the vaccine).

The polysaccharide specific ELISA was setup by coating overnight the 96-well Maxisorp plate with PPS2 and proceeded for ELISA.

In figure 4.6 (A) showed level of specific IgM antibodies responses to capsular polysaccharide type 2 (mean±SD) in first set of experiment with total of 12 of each mice group, properdin-deficient and wild type.

At day 0 day experiment before vaccination procedure, the mice showed a similar level of IgM with similar mean among the groups. IgM levels at Day 0 of pre-vaccination time point were at baseline with optical density reading (O.D) ranges 0.10 to 0.30. These showed a natural IgM repertoire produced by the mice.

However, after 4 weeks vaccinated with pneumovax, both properdin-deficient and wild type demonstrated a significantly increased anti-capsular polysaccharide type 2 IgM antibodies (at least about 3 fold increased, $P<0.05$) compared to their control that been given with PBS. These differences between vaccinated and non-vaccinated mice may represent the successful of the vaccination. Nevertheless, there was no significance difference between properdin-deficient and wild type vaccinated mice as well between the controls.

Non-vaccinated wild type and properdin-deficient appeared to have higher anti-capsular type 2 IgM level at the end of the experiment compared to post-vaccinated time point. In contrast, both vaccinated wild type and properdin-deficient were decreased in their specific PPS2 IgM level compared to post-vaccination time point. Again, there was no significance difference between properdin-deficient and wild type vaccinated mice as well between the controls.

Figure 4.6 (B) showed the mean±SD of specific IgM antibodies responses to capsular polysaccharide type 2 after Pneumovax administration for experimental mice and PBS given for control mice in the second set of experiment with total of 20 mice of each properdin-deficient and wild type respectively. Weekly bled time points were done to observe kinetic response during post-vaccination. At Day 0, Pre-vaccination sera at baseline level for both group wild type and properdin-deficient mice.

However following vaccination, experimental mice developed comparable overall IgM antibodies responses for every week results a protective response towards infection stage. After vaccination with Pneumovax, properdin-deficient and WT (n=15, each) showed an increased of anti-capsular type 2 IgM antibodies ($P<0.05$) compared to non-vaccinated (PBS injected) mice (n=5, each). There was no significant difference between the vaccinated properdin-deficient and wild type mice. Unexpectedly, PBS injection of properdin-deficient mice lead to a consistent increased over the time of 4 weeks of post-immunisation of anti-capsular type 2 IgM ($P<0.05$) compared to the wild type. Surprisingly

increased of specific anti-capsular type 2 IgM levels in properdin-deficient mice with PBS given have to be investigated further.

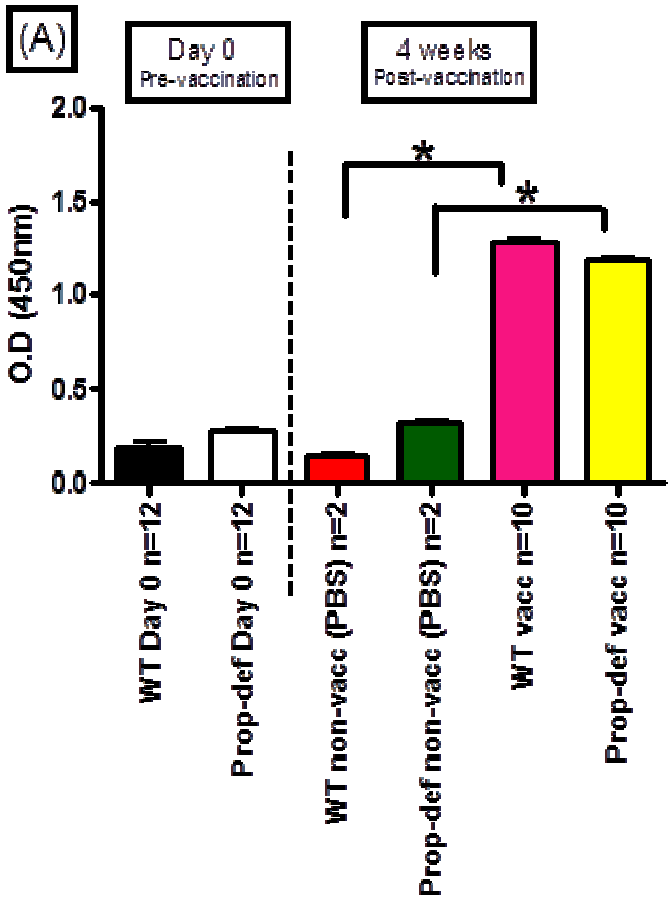


Figure 4.6 (A): Anti-capsular polysaccharide type 2 (PPS2) IgM in experimental mice.
Levels of IgM specific antibodies responses to capsular polysaccharide type 2 (mean±SD) at time points, the pre-vaccination and post-vaccination. After vaccination with Pneumovax, properdin-deficient and WT show an increase of anti-capsular type 2 IgM antibodies ($P<0.05$) compared to non-vaccinated (PBS injected) mice. There is no significant difference between the vaccinated properdin-deficient and wild type mice. (*$p<0.05$, Non-parametric analysis Mann-Whitney with Bonferroni correction, compared non-vaccinated and vaccinated group).

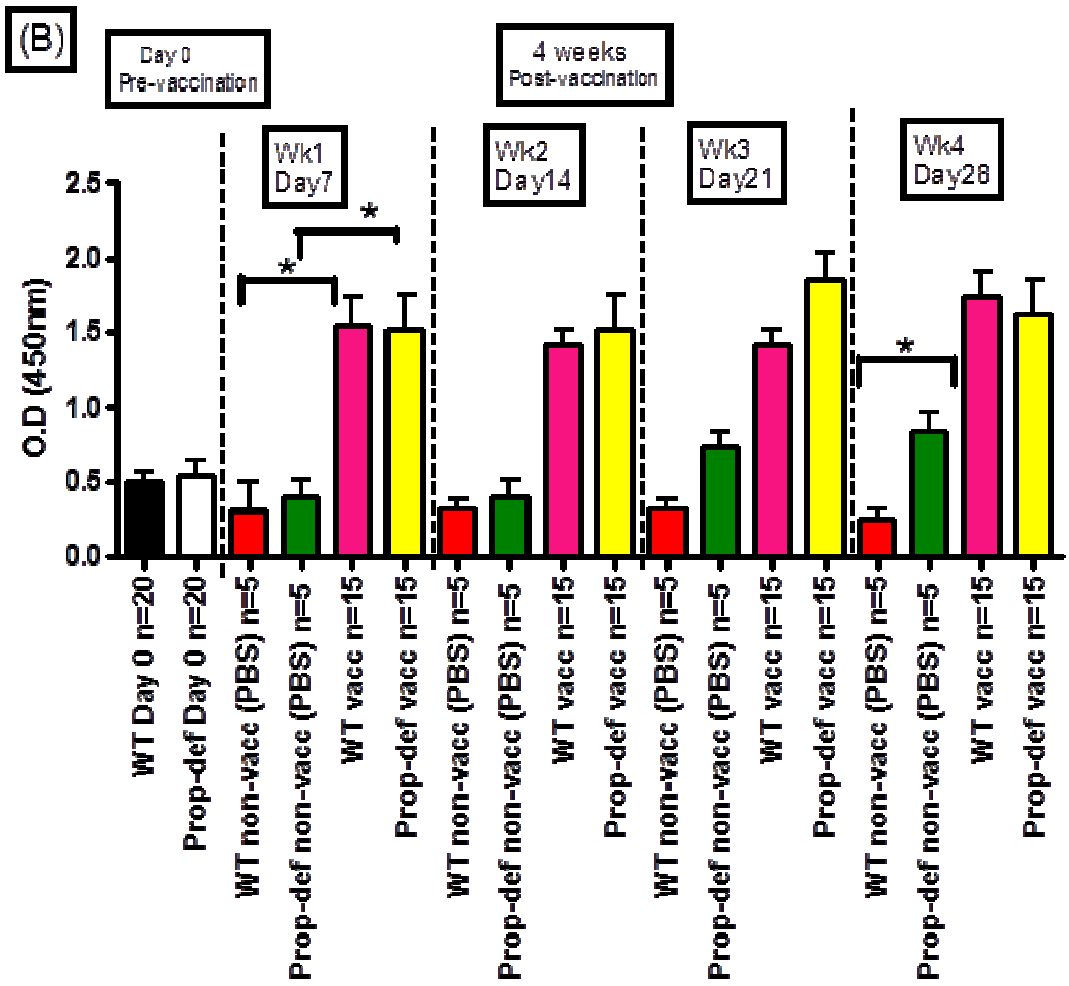


Figure 4.6 (B): Anti-capsular polysaccharide type 2 (PPS2) IgM in experimental mice
The mean±SD level of specific IgM antibodies responses to capsular polysaccharide type 2 after Pneumovax administration for experimental mice and PBS given for control mice at various time points, the pre-vaccination and weekly post-vaccination. (*$p<0.05$, Non-parametric Mann-Whitney with Bonferroni correction, compared vaccinated group with PBS control group)(*$p<0.05$, Unpaired T-test with Welch's correction compared wild type and properdin-deficient).

4.3.1.2.2 Determination of serotype specific anti-PPS 3 IgM level

A similar specific ELISA protocol was repeated with PPS3 coated plate to determine level of IgM against specific capsular pneumococcal polysaccharide type 3. PPS3 was determined as being an immunogenic among the polysaccharide that contained in Pneumovax.

The pattern of level produced for specific IgM against PPS2 (Figure 4.6) were observed similarly to the levels of IgM specific antibodies responses to PPS 3 (Figure 4.7).

IgM levels against PPS3 at baseline level of pre-vaccination were at ranges of 0.10 to 0.035 (O.D reading) for the wild type and properdin-deficient. After 4 weeks post-vaccination, both vaccinated properdin-deficient and wild type demonstrated a significantly increased anti-capsular polysaccharide type 3 IgM antibodies (at least 3 fold increased, $P<0.05$) compared to their control (PBS given).

However there was no significance difference between the groups, wild type and properdin-deficient during the experiment.

.

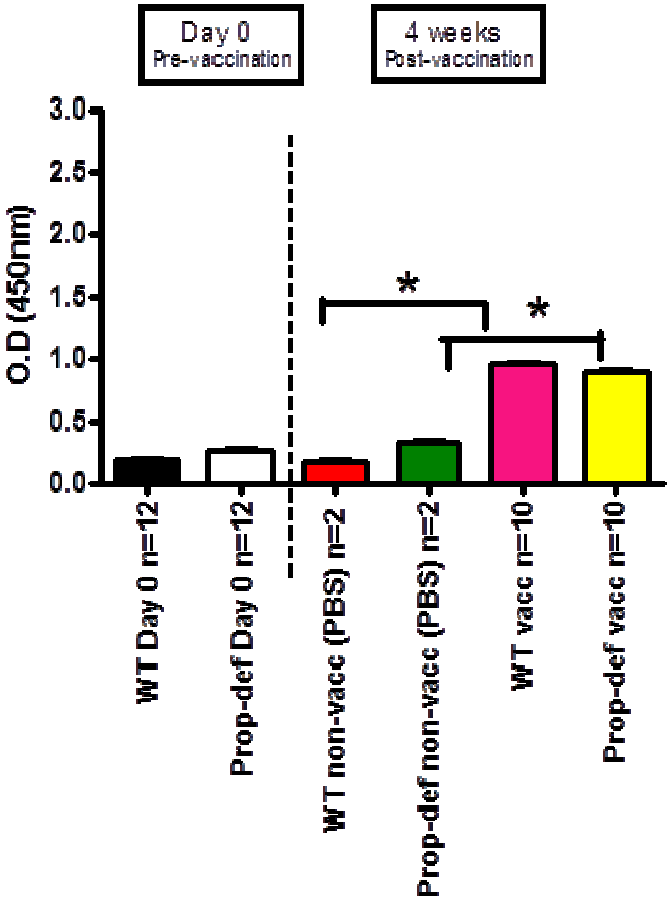


Figure 4.7: Anti-capsular polysaccharide type 3 (PPS3) IgM in experimental mice
Levels of IgM specific antibodies responses to capsular polysaccharide type 3 (mean±SD) at time points, the pre-vaccination and post-vaccination. After vaccination with Pneumovax, both properdin-deficient and WT show an increase of anti-capsular type 3 IgM antibodies ($P<0.05$) compared to non-vaccinated (PBS injected) mice. There is no significant difference between the vaccinated properdin-deficient and wild type mice. (*$p<0.05$, Mann- Whitney non-parametric analysis compared non-vaccinated and vaccinated group).

4.3.1.2.3 Determination of IgM level anti-PPS6B IgM level

The same specific ELISA protocol was repeated with PPS6B coated plate to determined level of IgM specific pneumococcal polysaccharide serotype 6B.

PPS6B was determined because it is the most immunogenic among the polysaccharide that contained in Pneumovax and most frequently cause drug-resistant pneumococcal infections

Levels of IgM specific antibodies responses to capsular polysaccharide type 6B (mean±SEM) showed no difference at time points, the pre-vaccination and post-vaccination (Figure 4.8) as after vaccination with Pneumovax, both properdin-deficient and WT show no increase of anti-capsular type 6B IgM antibodies similarly to non-vaccinated (PBS injected) mice.

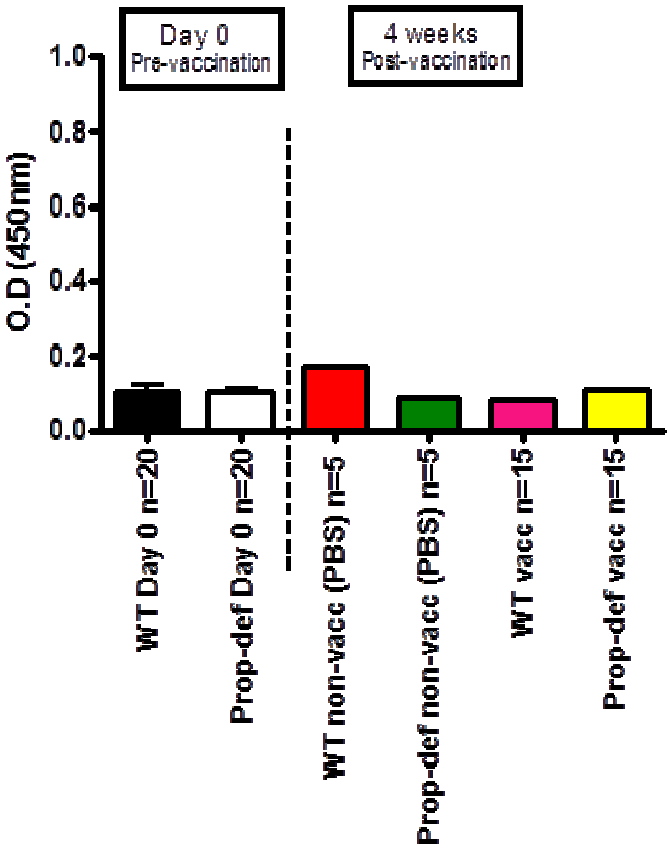


Figure 4.8: Anti-capsular polysaccharide type 6B (PPS6B) IgM in experimental mice
Levels of IgM specific antibodies responses to capsular polysaccharide type 6B (mean±SD) at time points, the pre-vaccination and post-vaccination.

4.3.1.2.4 Determination of serotype specific anti-PPS 2 IgG3 level

In determination of capsular polysaccharide type IgG3 antibody, the PPS2 serotype specific ELISA were performed with PPS2 coated plate and detected with goat anti-mouse IgG3.

IgG3 level against Specific PPS2 ELISA were measure because IgG3 is the most abundant of IgG subclass against carbohydrate polysaccharide antigen in mice experiment.

The experiment (figure 4.9 (A)) with total of 12 of each mice group, properdin-deficient and wild type demonstrated baseline level of IgG3 at Day 0 pre-vaccinated with ranges 0.09-012 (O.D reading). This detectable level of IgG3 demonstrated as natural IgG3 level present naturally at time point zero. However in overall of the experiment demonstrated a consistent level of IgG3. There was no significance difference between the mice groups, properdin deficient and wild type.

The experiment was repeated with total of 20 mice of each group, properdin-deficient and wild type (figure 4.9(B)). The detectable level at Day 0 for natural IgG3 was quite high with range O.D reading at 0.1-0.35. At 4 weeks post-vaccination, both of control mice were at same level while vaccinated wild type IgG3 level was significantly higher ($p<0.05$) compared to the properdin-deficient. Nevertheless there were no significance difference between properdin-deficient and wild type.

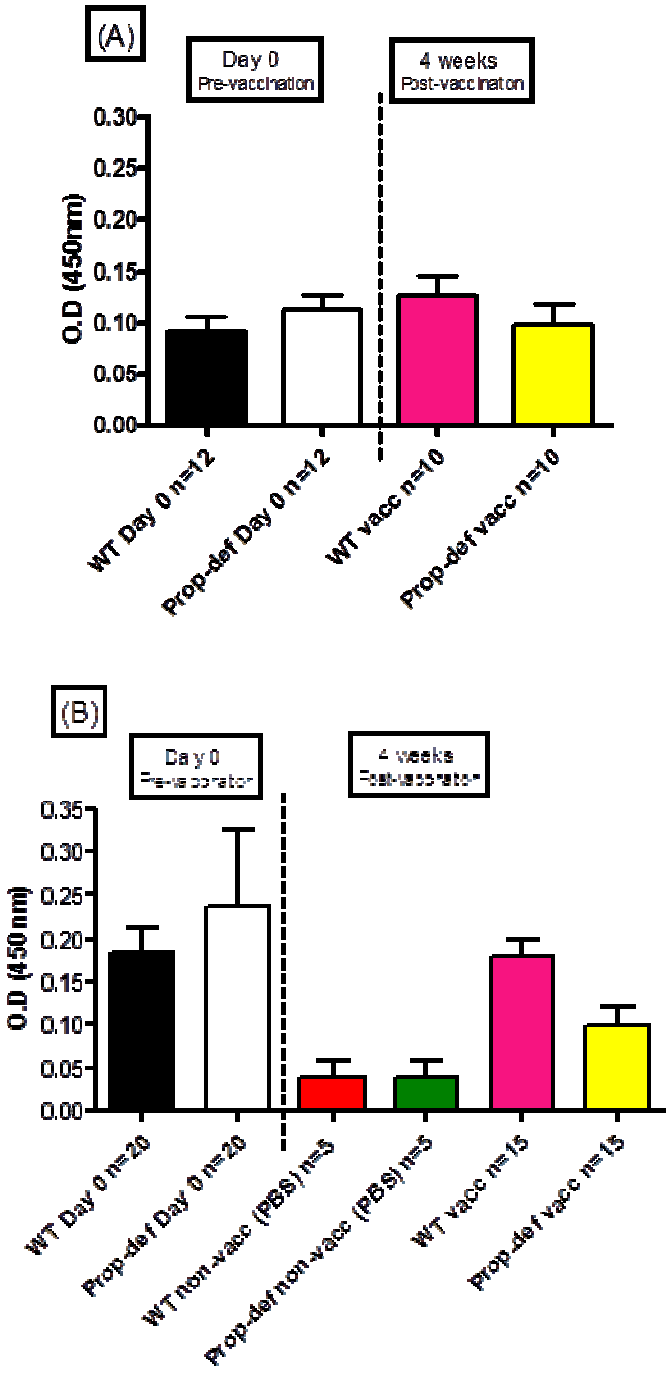


Figure 4.9: Anti-capsular polysaccharide type 2 (PPS2) IgG3 in experimental mice
Levels of IgG3 specific antibodies responses to capsular polysaccharide type 2 (mean±SD) at pre-vaccination and post-vaccination . **(A)** The first experiment (total n=12 of each WT and prop-def) and the second experiment (total n=20 of each WT and prop-def) in **(B).**

4.3.1.3 Differential cell count of peritoneal cell population

Interested to find out the differences increased of IgM level occurred in PBS given mice (Figure 4.6B), a differential cell count for peritoneal cell population were performed. It was thought due to the method of administration of vaccine and PBS was by intraperitoneal administered.

As described in materials and methods section (see Chapter 4.7), mice were culled at the end of experiment. Peritoneal cavity were opened and washed with ice-cold PBS. Cells collected from the peritoneal lavages then were cytospun and stained with Giemsa stain. Microscpic differential cells count was done for monocytes, macrophages, lymphocytes and mast cells. Cells count was expressed in percentage as in Figure 4.10. In overall, total peritoneal cell population for both control (PBS given) of properdin-deficient and wild type vaccinated consisted of lymphocytes as the biggest population (more than 50%), next was macrophages population with approximately 30%, followed by monocytes and mast cells with less than 10%. However in vaccinated properdin-deficient, lymphocytes and macrophages appeared the highest with similar count. Monocytes and mast cells population were similarly with low percentages of cell count in peritoneal of vaccinated properdin-deficient. In comparison, peritoneal lymphocytes population was significantly higher in wild type vaccinated compared to the properdin-deficient ($p<0.05$) whereas peritoneal macrophages population was significantly higher in properdin-deficient vaccinated compared to the wild type ($p<0.05$).

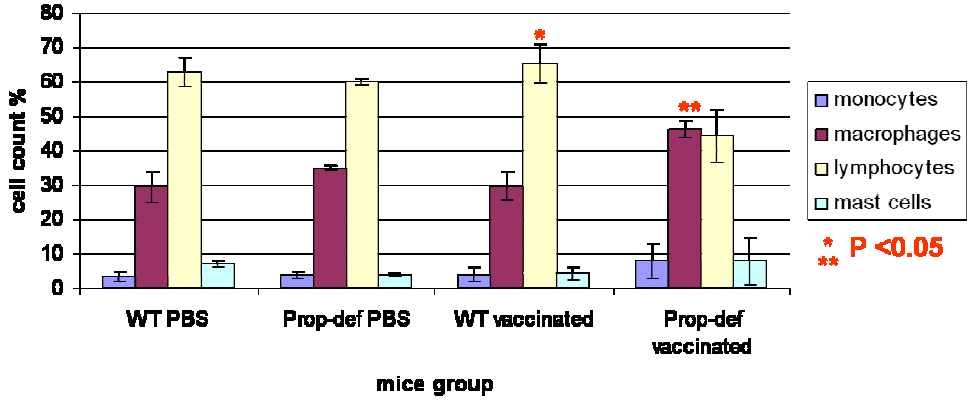


Figure 4.10: Peritoneal cells count in experimental mice
A differential count of cytospun-Giemsa stained cells from peritoneal lavages of the experimental mice. Cell count for monocytes, macrophages, lymphocytes and mast cells are expressed in percentage (mean±SD). Peritoneal lymphocytes population is significantly higher in WT vaccinated compare to the properdin-deficient whereas peritoneal macrophages population is significantly higher in properdin-deficient vaccinated compare to the WT. (*p<0.05, Mann-Whitney non-parametric analysis compared WT and prop-def)(n=2 of each group)

4.3.2 Analysis of immune responses following intranasal pneumococcal infection (in continuation of vaccination study) for properdin-deficient and wild type mice

Following the vaccination stage, the experimental mice were infected intranasally with $1.0x10^6$ *S. pneumoniae* D39. There were 7 days of duration for infection stage and infected mice were monitored twice a day during the infection stage. At the end of the experiment, mice were culled and their blood, lungs and spleen were taken for analysis (see chapter 4.2).

The first experiment was performed with total 12 of wild type while 10 of properdin-deficient as lost 2 mice due to anaesthetize failure. Whereas the second experiment involved 20 mice of each groups, wild type and properdin-deficient.

4.3.2.1 Viable counts of blood at 24 hours post-infection of streptococcal infection

On the infection day, soon after intranasal infection was performed, the number of bacteria infected the mice (dose administered) was confirmed at $1x10^6$ CFU/ml by plating serial dilutions of the inoculum on blood agar.

At 24 hours post-infection, blood from properdin-deficient and wild type were obtained, serially diluted and plated onto blood agar. Colony forming unit (CFU) of bacteria on the plates were counted the next day.

In 1st experiment, two control mice (non-vaccinated (PBS) and infected) of each WT and properdin-deficient showed viable count with 1.67 x 10^5 CFU/ml and 1.23 x 10^5 CFU/ml respectively. However there was no colony of bacteria obtained from the rest of other mice blood.

Whereas in 2nd experiment, control (non-vaccinated (PBS) and infected) of WT (n=2) and properdin-deficient (n=2) demonstrated with mean viable counts, 2.45 x 10^5 CFU/ml and 1.13 x 10^5 CFU/ml respectively. In addition, one of experimental properdin-deficient vaccinated and infected showed 1.3 x 10^4 CFU/ml of bacteria in the blood. Again, there was no colony of bacteria obtained from the rest of other mice blood.

Nevertheless there were no significant difference between the groups, WT and properdin-deficient.

The fact that viable counts of bacteria were recovered from 24 hours post-infection showed an evidence of pneumococcal sepsis and the one with no count of viable bacteria indicated that those mice were able to clear the bacteria from their blood.

(A)

Mice Label	CFU count (CFU/ml)
WT non-vacc (PBS)+inf, n=1	1.67×10^5
Prop-def non-vacc (PBS)+inf, n=1	1.23×10^5
Others experimental mice	Nil

(B)

Mice Label	CFU count (CFU/ml)
WT non-vacc (PBS)+inf, n=2	2.45×10^5
Prop-def non-vacc (PBS)+inf, n=2	1.13×10^5
Prop-def vaccinated +infected, n=1	1.3×10^4
Others experimental mice	Nil

Table 4.3: Viable count of *S. pneumoniae* (CFU/ml) recovered in blood after 24 hours of infection. Data shows of first experiment (A) and second experiment (B).

4.3.2.2 Survival of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae*

During the 7 days of duration for infection stage, infected mice were monitored and their survival rates were presented as survival curve (Figure 4.11).

In the first experiment (Figure 4.11 (A)), a death rate was found in properdin group. At 48 hours post-infection, one of properdin-deficient non-vaccinated and infected was found dead. Whereas one of properdin-deficient vaccinated and infected was found dead at 72 hours. In contrast, all mice of wild type group both vaccinated and non-vaccinated were survived during the infection stage. However there was no difference in the survival between properdin-deficient and wild type mice.

While in second experiment, twenty in total of properdin-deficient and wild type were intranasal challenged with *S.pneumoniae* and monitored for 7 days (Figure 4.11 (B)),. After 24 hours following intranasal challenge with D39, a death rate was observed in control wild type non-vaccinated and infected mice (2 out of 5 mice died), and one of properdin-deficient mice vaccinated and infected was found dead.

At 72 hours post-infection, a death was found in both controls, wild type properdin-deficient non-vaccinated and infected and a death was found in wild type vaccinated and infected at 96 hours post-infection.

At the end of the seven days of infection stage, 9 of 10 for both immunised wild type and properdin-deficient mice survived, whereas there were 2 out of the 5 wild type non-vaccinated and 4 of properdin-deficient mice non-vaccinated survived.

The immunised wild type mice were significantly protected ($P<0.05$) compared to non-vaccinated wild type mice. Non-vaccinated properdin-deficient mice showed greater survival than the wild type mice ($P<0.05$). However there was no difference ($P>0.05$) in the survival of immunised properdin-deficient and wild type mice.

This experiment showed that, the effectiveness of the immunisation and there was a significant advantage for properdin-deficient mice to survive pneumococcal pneumonia.

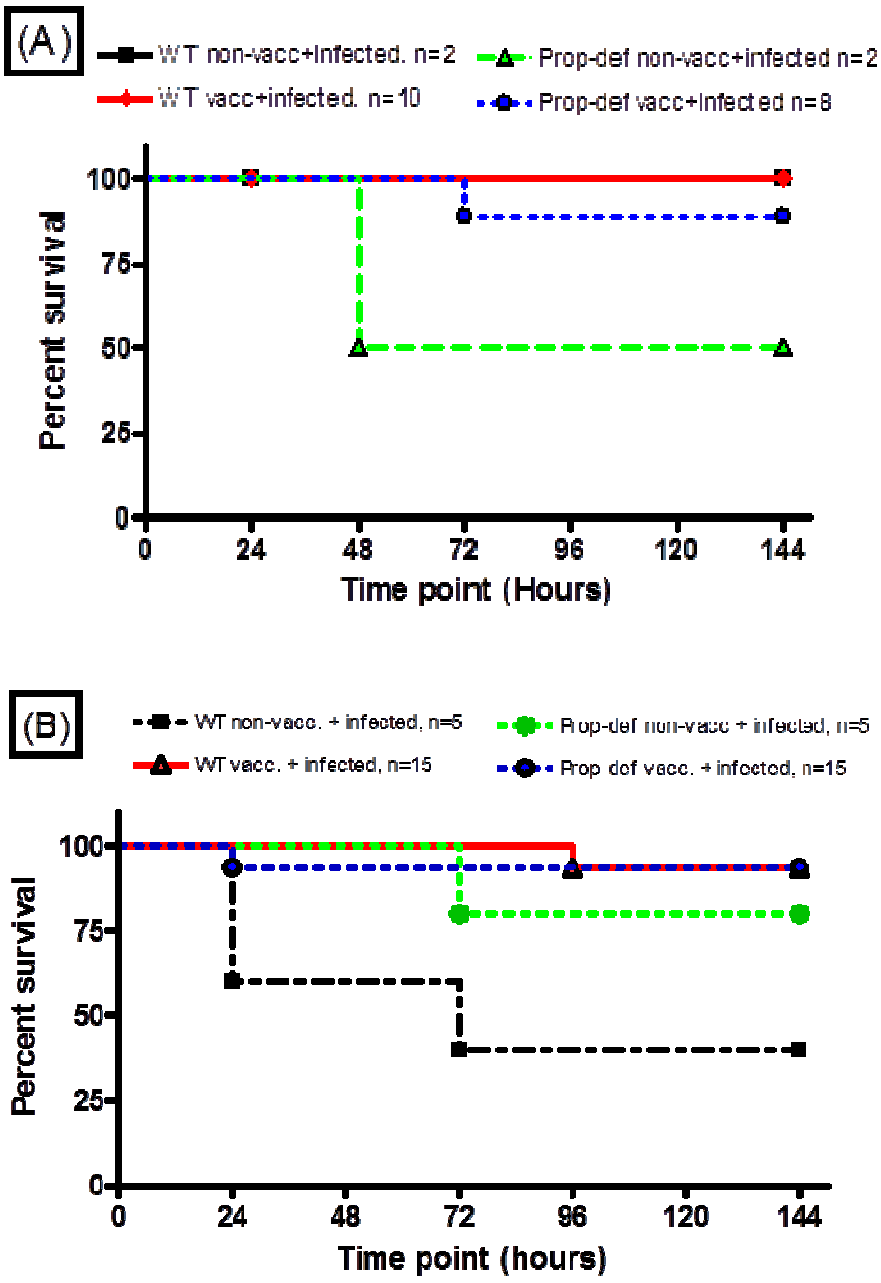


Figure 4.11: Survival curves for experimental mice
Continuing from the vaccination stage, wild type mice and properdin-deficient mice were intranasal challenge with *S. pneumoniae* D39 infected mice were monitored along the duration of infection stage. Data are presented as survival curve and shows of first experiment **(A)** (total n=12 of WT and n=10 of prop-def) and second experiment **(B)** (total n=20 of each WT and prop-def). ($p<0.05$, Kaplan-Meier Log-rank Survival curves Chi-square test).

4.3.2.3 Disease severity of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae*

The infected mice were monitored twice daily and their disease severity been recorded and score (see 4.2.3.2). In relation to the survival curve, disease severity score of experimental mice was observed to aid extra information following the infection. Scoring was start from zero for normal mice and followed increasing in score number parallel by the severity of the condition; e.g 1 for +hunched, 8 for dead and etc. Outcome of survival score correlate with severity, the greater death percentage the higher score observed. Figure 4.12 showed the mean disease severity score over duration of infection stage.

Along the duration of infection in the first experiment, only wild type vaccinated was scored as normal condition significantly ($p<0.05$) compared to others, wild type non-vaccinated and both properdin type, vaccinated and non-vaccinated were found with overall score of 3 (+starey/piloerect).

Whereas over the post-infection stage in the second experiment, the mean disease score was significantly ($p<0.05$) improved in vaccinated wild type compared to non-vaccinated wild type. Properdin-deficient non-vaccinated mice showed significantly ($p<0.05$) lower score than wild type non-vaccinated. In overall non-vaccinated wild type was found to have the worst condition and scored more than 4 (++starey/piloerect). However there was no significance difference on vaccination for properdin-deficient and wild type.

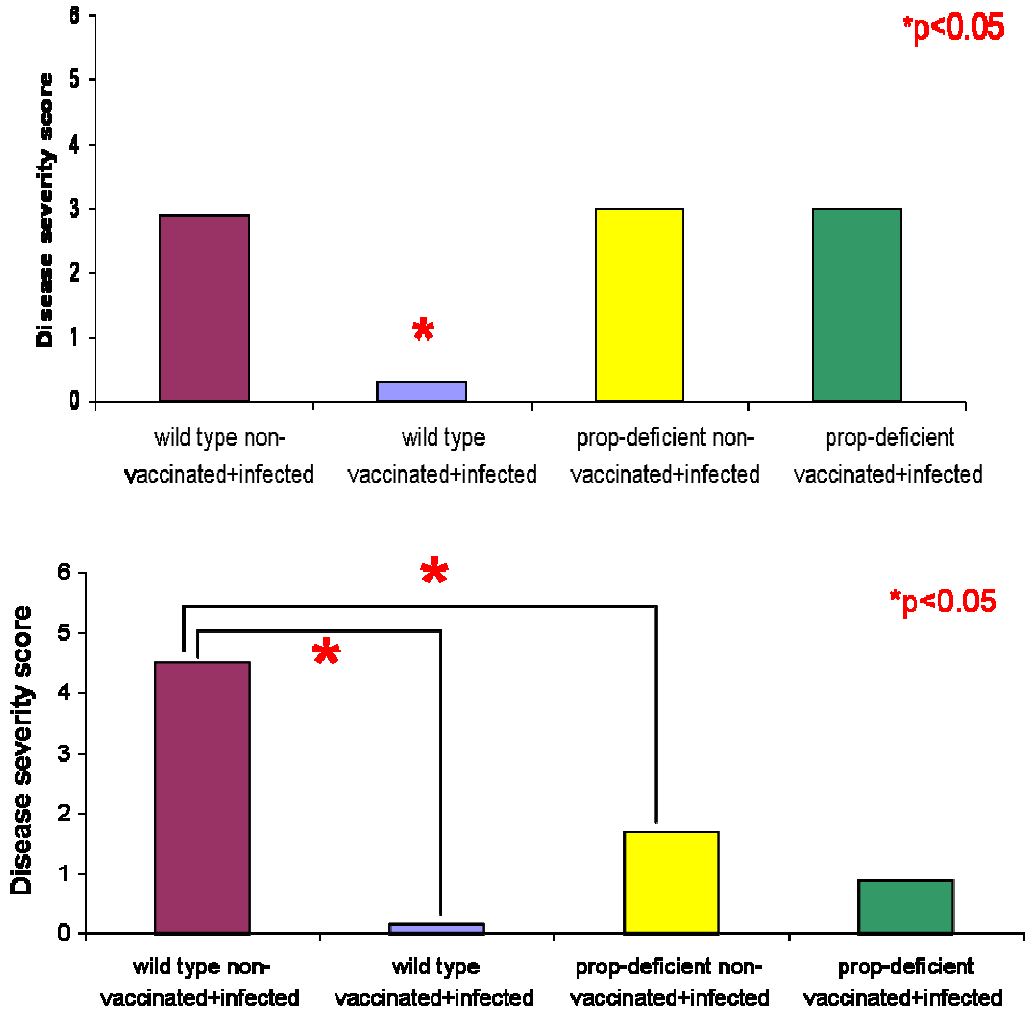


Figure 4.12: Disease severities score of experimental mice
The mean disease severity score over duration of infection stage shows **(A)** first experiment (total n=12 of WT and n=10 of prop-def) with overall experimental mice group is in worst condition except wild type vaccinated and **(B)** the second experiment (total n=20 of each WT and prop-def) shows the mean disease score is low in vaccinated group and in overall wild type non-vaccinated in worst condition. (*p<0.05, Non-parametric Mann-Whitney compared wild type and properdin-deficient).

4.3.2.4 Determination of antibody response following intranasal infection with *S. pneumoniae* D39

4.3.2.4.1 Serum concentration of IgM of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae*

Immunoglobulin IgM plays an important role in the innate immune defense against bacterial infection. As mentioned earlier, the total serum IgM concentrations was measured by using commercial ELISA in Properdin-deficient and wild type mice and was used to determine the primary immune response towards infection measured at time point 24 hours post-infection and 7 days post-infection (end of experiment) which summarised in Table 4.4 (A). Serum level of IgM is an important marker that can vary during infection, so it was important that now known that baseline level, 50-180ng/µl of serum IgM as within the normal range. In continuing from the previous study, the vaccination stage, mice were infected with D39 *Streptococcus pneumoniae* after 4 weeks of vaccination. Total IgM level has acute increase at least with 7 fold increase after 24 hours of infection. IgM level after 24 hours of infection for most of the mice were higher than standard value thus could not quantify reliably. However, at the end of this experiment i.e after a week of infection total level of IgM was decreased (range of 35-90ng/µl for wild type, both non-vaccinated and vaccinated and properdin-deficient mice (non-vaccinated and vaccinated) with range of 40-70 ng/µl). In noted, the value of total IgM level at the end were lesser than the baseline level.

Whereas total serum IgG concentrations were also measured by using established standards in the commercial ELISA but unfortunately only managed to measure IgG level for the time points Day 0 and end of experiment (Post-vacc & post-infection). Table 4.4 summarised data for total serum IgG at the Day 0 and end of experiment.

Basically, IgG concentrations at Day 0 demonstrated the baseline level for both mice groups which is at range of 1200-1700ng/µl for wild type and 1000-1600ng/µl in properdin-deficient. Total serum IgG at the end of experiment (Post-vacc & post-infection) for wild type control non-vaccinated infected demonstrated with mean IgG level of 535ng/µl meanwhile the properdin-deficient with level 380 ng/µl of IgG. In contrast, both properdin-deficient and wild type, vaccinated and infected mice were higher than the control with range 800-3850 for wild type and 1000-2500 for properdin-deficient. Nevertheless, there was no significance difference between properdin-deficient and wild type.

(A) Total serum IgM	**Wild Type**	**Properdin-deficient**
Day 0 (Pre-vacc) **ng/µl**	55-180 (n=12)	60-140 (n=12)
24 hrs (Post-infection) **ng/µl**	500->1000 (n=10)	80->1000 (n=8)
End of exp. **(Post-vacc & post-infection)** **ng/µl**	Non- vacc, (n=2): 42-50 Vacc, (n=10): 35-90	Non- vacc, (n=1): 46 Vacc, (n=8): 40-70

(B) Total serum IgG	**Wild Type**	**Properdin-deficient**
Day 0 (Pre-vacc) **ng/µl**	1200-1700 (n=12)	1000-1600 (n=12)
End of exp. **(Post-vacc & post-infection)** **ng/µl**	Non- vacc+inf (n=2): 520-550 Vacc+inf, (n=10): 800-3850	Non- vacc+inf, (n=1): 380 Vacc+inf, (n=8): 1000-2500

Table 4.4: Range of total serum IgM level **(A)** and IgG level **(B)** in properdin-deficient and wild type mice for the indicated time point following intranasal infection *S. pneumoniae.*

4.3.2.4.2 Determination of serotype specific anti-PPS 2 IgM level following intranasal infection with *S. pneumoniae* D39

Following the intranasal challenged with *S. pneumoniae* serotype 2 strain D39(continuing from vaccination experiment), it was ideally to measure IgM class specific antibodies to PPS2 because of D39 which is polysaccharide antigen type 2 and type 2 polysaccharide is contained in the vaccine. The polysaccharide specific ELISA was setup as before (see chapter 4.2).

In figure 4.13 (A) showed level of specific IgM antibodies responses to capsular polysaccharide type 2 (mean±SD) in first set of experiment with total of 12 of each mice group, properdin-deficient and wild type.

As mentioned before, the mice showed a similar level of IgM at day 0 as a baseline level with range of 0.10 to 0.30.

Following 24 hours of post-infection, non-vaccinated control of WT and properdin-deficient were still in range of baseline level, whereas vaccinated with mice both properdin-deficient and wild type demonstrated a significantly increased anti-capsular polysaccharide type 2 IgM antibodies ($P<0.05$) compared to their control that been given with PBS. There was significantly difference between properdin-deficient and wild type vaccinated mice as anti-capsular polysaccharide type 2 IgM antibodies level in properdin-deficient was higher at least with a fold increases compared to wild type. At the end of the experiment demonstrated an increased of anti-capsular polysaccharide type 2 IgM antibodies in controls mice of wild type and properdin-deficient compared to 24 hours time point. In contrast, both vaccinated wild type and properdin-deficient were decreased in their specific PPS2 IgM level compared to post-vaccination time point. There was no significance difference between properdin-deficient and wild type vaccinated mice as well between the controls.

Meanwhile figure 4.13 (B) showed the mean±SD of specific IgM antibodies responses to capsular polysaccharide type 2 following the intranasal challenged with *S. pneumoniae* D39 in the second set of experiment with total of 20 mice of each properdin-deficient and wild type respectively.

At Day 0, Pre-vaccination sera at baseline level for both group wild type and properdin-deficient mice. Following 24 hours infection, non-vaccinated control of WT was still in range of baseline level, whereas properdin-deficient non-vaccinated control demonstrated a significantly increased anti-capsular polysaccharide type 2 IgM antibodies ($P<0.05$) compared to the WT control. The experimental mice, vaccinated and infected properdin-deficient and wild type mice developed comparable IgM antibodies responses and higher the levels compared to their controls.

At the end of the experiment demonstrated an increased of anti-capsular polysaccharide type 2 IgM antibodies in controls mice of wild type and properdin-deficient compared to 24 hours time point. Both vaccinated wild type and properdin-deficient were increased in their specific PPS2 IgM level compared to post-infection time point. However there was no significance difference between properdin-deficient and wild type vaccinated mice as well between the controls.

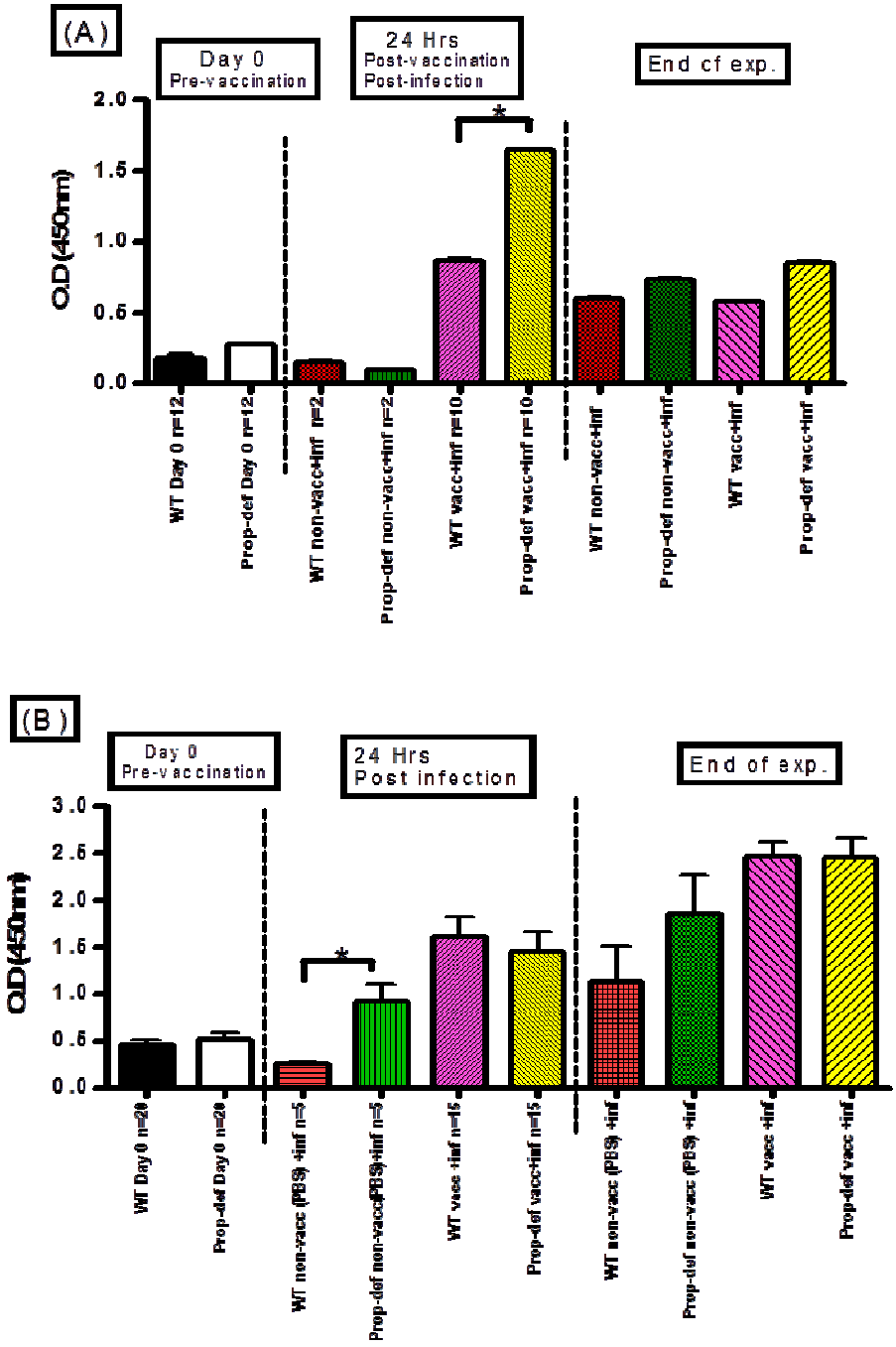


Figure 4.13 Anti-capsular polysaccharide type 2 (PPS2) IgM in experimental mice following intranasal infection with *S. pneumoniae* **D39.** The mean±SD level of specific IgM antibodies responses to capsular polysaccharide type 2 after intranasal infection with *S. pneumoniae* D39 (continuing from vaccination experiment) for experimental mice mice at indicated time points **(A)** first experiment and **(B)** the second experiment. (*p<0.05, Non-parametric Mann-Whitney compared wild type and properdin-deficient).

4.3.2.4.3Determination of IgM level of serotype specific anti-PPS 3 and anti-PPS6B following intranasal infection with *S. pneumoniae* D39

Similarly following the intranasal challenged with *S. pneumoniae* serotype 2 strain D39 (continuing from vaccination experiment), IgM class specific antibodies to PPS3 and PPS6B also been measured. The polysaccharide specific ELISA was setup as before (see chapter 4.2). Data were presented in the table represents the level of the IgM antibodies (mean±SD) responses to the specific polysaccharide.

In table 4.5, non-vaccinated wild type and properdin-deficient appeared to increase their anti-capsular type 3 IgM level at the end of the experiment compared to post-vaccinated time point while both vaccinated wild type and properdin-deficient were consistent in their specific PPS3 IgM level.

Meanwhile in table 4.6, a markedly increased of anti-capsular type 6B IgM level at the end of experiment were observed for both vaccinated group compared to non-vaccinated.

	Wild Type	**Properdin-deficient**
Day 0 (Pre-vacc) **O.D (450nm)**	0.32±0.11 (n=12)	0.34±0.1 (n=12)
End of exp. **(Post-vacc & post-infection)** **O.D (450nm)**	Non- vacc+inf (n=2): 0.58±0.06 Vacc+inf, (n=10): 0.56±0.1	Non- vacc+inf, (n=1): 0.75±0.03 Vacc+inf, (n=8): 0.82±0.1

Table 4.5 Anti-capsular polysaccharide type 3 (PPS3) IgM in experimental mice
Levels of IgM specific antibodies responses to capsular polysaccharide type 3 (mean±SD) at time point of pre-vaccination and post-vaccinated+ post-infected.

(A)	**Wild Type**	**Properdin-deficient**
Day 0 (Pre-vacc) **O.D (450nm)**	0.1±0.03 (n=20)	0.11±0.02 (n=20)
End of exp. **(Post-vacc & post-infection)** **O.D (450nm)**	Non- vacc+inf (n=5): 0.08±0.02 Vacc+inf, (n=15) 1.15±0.2	Non- vacc+inf, (n=5): 0.17±0.02 Vacc+inf, (n=15) 1.09±0.2

Table 4.6 Anti-capsular polysaccharide type 6B (PPS6B) IgM level in experimental mice
Levels of IgM (mean±SD) specific antibodies responses to capsular polysaccharide type 6B at pre-vaccination and post-vaccinated+ post-infected.

4.3.2.4.4 Determination of IgG3 level of serotype specific anti-PPS2 following intranasal infection with *S. pneumoniae* D39

Continuing from vaccination experiment and following the intranasal challenged with *S. pneumoniae* serotype 2 strain D39, IgG3 class specific antibodies to PPS2 been measured. The polysaccharide specific ELISA was setup as before (see chapter 4.2) and the data were presented in the table.

In table 4.7, immunoglobulin G3 levels specific antibodies responses to capsular polysaccharide type 2 in sera at the end of the experiment (post-vaccinated and post-infected) of control groups and vaccinated properdin-deficient were increased compared to post-vaccination time point. However there were no significance difference between properdin-deficient and wild type

4.3.2.5 Serum concentration of C3 of properdin-deficient and wild type mice in intranasal infection with *S. pneumoniae*

Serum C3 is the most abundant component of complement pathway in the blood. Serum C3 was measured to measure innate immune response by complement activity. Low values of serum C3 were interpreted as an indication of utilisation of C3 in the complement activity. The concentration of C3 was measured by commercial ELISA in properdin-deficient and wild type serum at time point end of the experiment (post-vaccinated and post-infected). C3 normal range from the kit was within range of 0.3- 1.15mg/ml (data sheet of mouse C3 ELISA kit, ICL). As seen on figure 4.14, a comparable overall amount of serum C3 was observed in the serum obtained from properdin-deficient and wild type. The concentration of serum C3 in control non-vaccinated and infected properdin-deficient was within the normal range with mean concentration of 1.12mg/ml. Average concentrations from other samples were slightly higher than within the C3 normal range. A slight increase of the C3 level was observed in non-vaccinated and infected WT with mean concentration of 1.95mg/ml. whereas mean concentration of properdin-deficient infected and vaccinated was1.44mg/ml and WT infected, vaccinated was1.26mg/ml. Nevertheless, there was no significant difference between the groups.

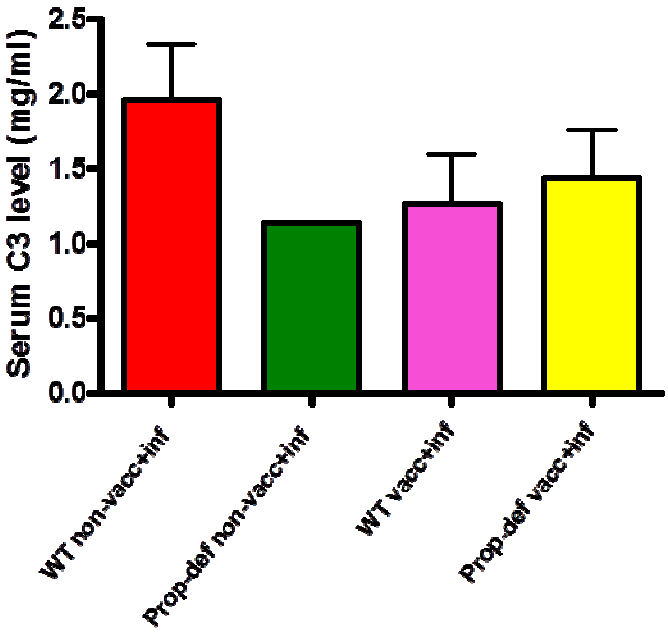


Figure 4.14: Level of serum C3 expressed in mg/ml represents mean±SD at the end of first experiment (total n=2 of WT non-vacc+inf, n=1 of prop-def non-vacc+inf, n=10 of each WT and prop-def vacc+inf)**.** There is no significant difference between the groups.

4.3.2.6 PMN recruitment in the lungs

PMN is one of major components of inflammatory and immunologic reactions in the lung. The normal lung airways generally contain a small number of PMN and sequential infiltration of PMNs to the inflamed areas of lung was observed in H&E stained slides of lungs. The PMN number for the experimental mice demonstrated a comparable overall PMN number in lungs and showed remarkable higher number than normal lungs. Wild type vaccinated group have less number of PMN than the control wild type after infection. While both group of, vaccinated and control PBS has a similar number of PMN. The properdin-deficient both control and vaccinated have less number of PMN than the wild

type group for control and vaccinated. However there was no significant difference between the groups.

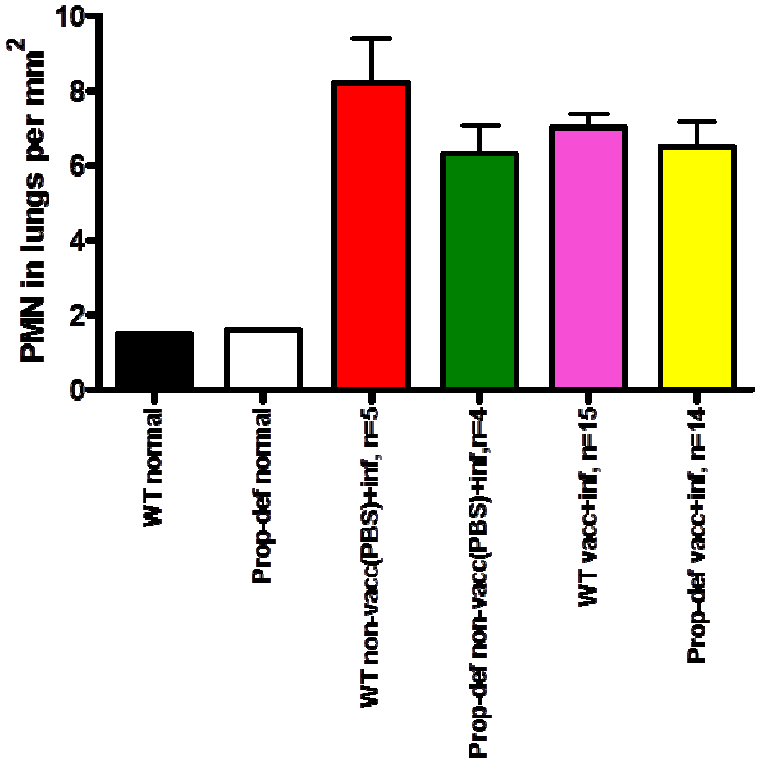


Figure 4.15: Polymorphonuclear leukocytes (PMN) count in H&E slides stained of experimental mice lungs

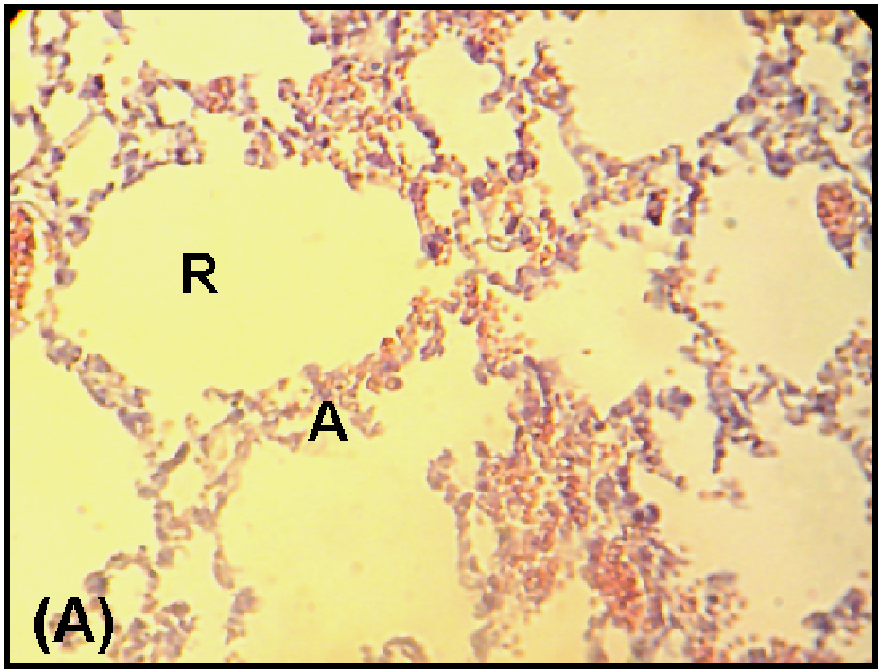
4.3.2.7 Haematoxylin and eosin staining

4.3.2.7.1 Haematoxylin and eosin staining of the lungs

Normal mice lung of WT (fig. 4.15 (A)) and properdin-deficient (not shown) showed H&E stained with artery in the center and portion of bronchiole to left surrounded by thin walled alveoli where gas exchange takes place.

Meanwhile H&E stained of lung of WT both infected, vaccinated and non-vaccinated demonstrated local accumulation wild type vaccinated and infected showed local accumulation with medium cellular infiltration

H&E stain of properdin-deficient lung showed hyperemic tip (blood filled) indicated pneumonia regional. Both properdin deficient infected, non-vaccinated and vaccinated (fig. 4.15 (C) and (E) respectively) demonstrated worst condition with no proper lung structure visible, thickening of inter alveolar spaces with a lot of inflammatory cells and complete infiltration by heavy cellular infiltration of inflammation cells indicated pneumonia.


R
A
(A)

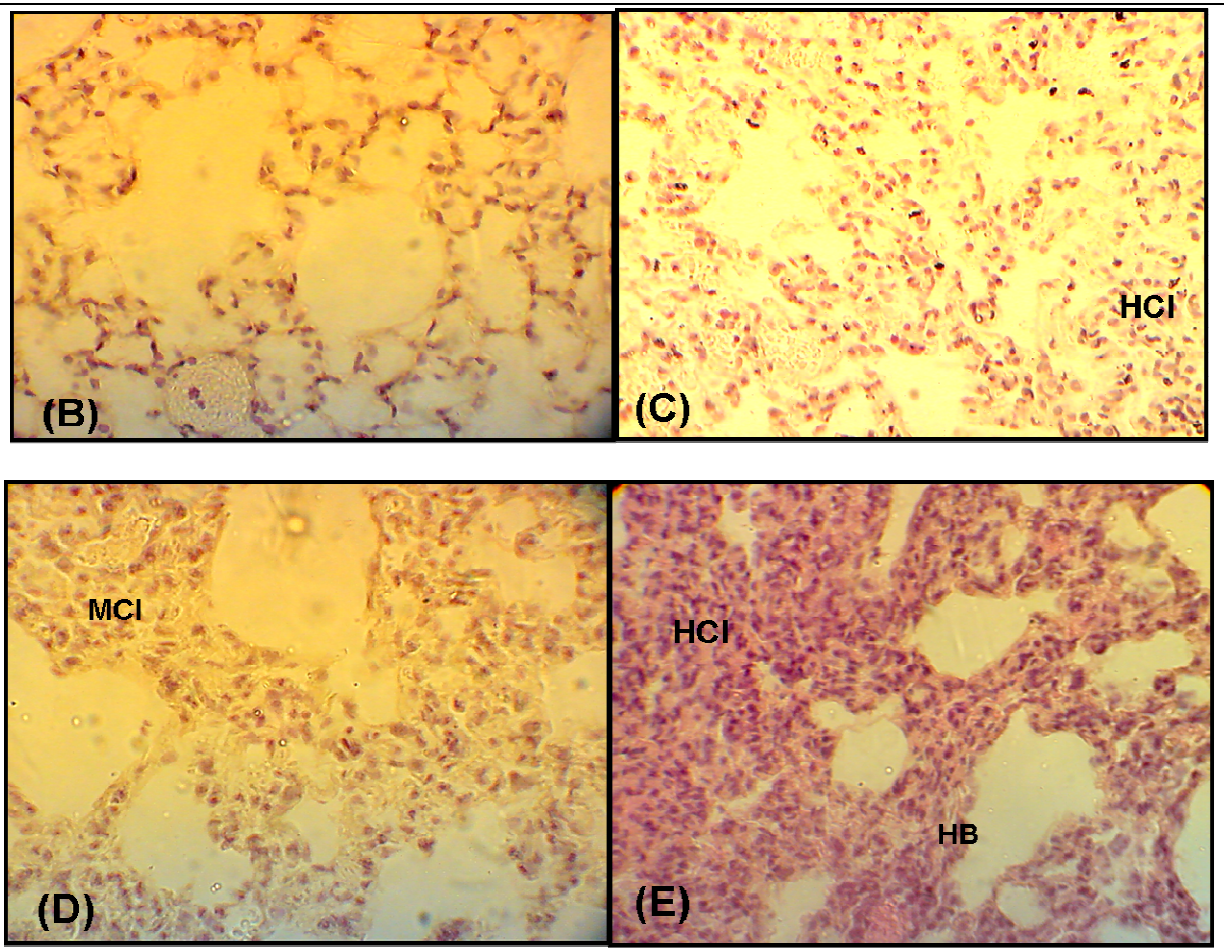


Figure 4.15: Photomicrographs of H&E slides stained of experimental mice lungs at 400x magnification of light microscopy **(A)** normal lung of wild type, **(B)** lung of wild type non-vaccinated (PBS given) and infected, **(C)** lung of properdin-deficient non-vaccinated (PBS given) and infected, **(D)** lung of wild type vaccinated and infected, **(E)** lung of properdin-deficient vaccinated and infected. **R=**respiratory bronchioles, **A=** alveoli, **HCI=** heavy cellular infiltration, **MCI=** medium cellular infiltration, **HB=** hypertrophy of bronchiole wall.

4.4 Discussion

Complement-deficient individuals are susceptible with bacterial infections and it is highly recommended for them to have vaccination. There are a few studies investigate the impact of complement deficiencies on vaccine success and in relation with current study, the role played by properdin, the positive regulator of alternative pathway; during immunisation and bacterial infection was investigated using the properdin-deficient mouse line and wild type littermate.

As mentioned earlier, the study was involved of two set of experiments; first experiment with total of 12 of each mice group, properdin-deficient and wild type while the second experiment was repeated with total of 20 mice of each group.

4.4.1 Role of properdin in immune responses following intraperitoneal polysaccharide vaccination

The IgM natural antibodies play an important role in the first innate immune response towards infection and immunisation.

It has shown for both experiments that baseline level of IgM concentrations at Day 0 pre-vaccinated with at range of 50-180ng/µl which demonstrated as detectable level of natural IgM level that present naturally at time point zero (Figure 4.5 along with Table 4.2). The differences between the experiments were wild type appeared to have higher baseline level

of IgM concentration compared to properdin-deficient samples for the first experiment (figure 4.5 (A)) meanwhile for the second experiment, the properdin-deficient have significantly higher natural IgM compared to wild type (figure 4.5 (B)). Fijen group (Fijen et al., 1998) have shown similar levels of the pre-vaccination serum antibodies against meningococcal capsular polysaccharide in vaccinated-complement-deficient patients and healthy group. These detectable levels of natural repertoire antibody for vaccine pneumococcal polysaccharide most probably acquired during life due to colonisation or previous infection with *S. pneumoniae* or cross-reacting organisms (Fijen et al., 1998) and it also likely found to be derived from among intestinal and pharyngeal bacteria (Heidelberger, 1984, Lee et al., 1991). In addition, a population of B cells in mouse known as B-1a, produced natural antibodies, IgM mostly even without exposure to antigen and in co-operation with the innate immune response can recognize TI-2 antigens (Ochsenbein and Zinkernagel, 2000, Wardemann et al., 2002).

Generally when measured at 4 weeks of post-vaccination (Figure 4.5 along with Table 4.2), pneumococcal vaccination raised IgM antibody levels for the 23 serotypes of polysaccharide vaccine in the vaccinee experimental mice reflected successful of vaccination when compared to both groups of control mice given PBS were still at the same range of concentration as pre-vaccinated.

Structural characteristics of antigens can affect complement activation and determine localisation of antibody response (Jarva et al., 2003). Antigen such as capsular polysaccharide is an important virulence factor of the pneumococcus and they are mostly negatively charged by having acidic components, including D-glucuronic acid and phosphate in phosphodiester bonds (Lee et al., 1991).

The series of serotype specific ELISA have shown successful of vaccination by significance increase of IgM level after 4 weeks of vaccination. Serum of unchallenged wild type and properdin-deficient mice IgM level of anti-capsular polysaccharide 2 has shown as a part of the natural antibody repertoire. In the absence of Properdin, specific anti-capsular polysaccharide antibodies of the IgM type are made. However, vaccinated properdin-deficient mice do not differ from wild type mice in their immunoglobulin response to the polysaccharide vaccine (fig 4.6).

Type 3 strains of pneumococcal are sialic acid rich capsule is among invasive and virulent types (Lee cj 1991). It has shown the level produced for specific IgM against PPS3 (Figure 4.7) were similarly to the levels of IgM specific antibodies responses to PPS 2 (Figure 4.6) by which demonstration of an increased anti-capsular polysaccharide type 3 IgM antibodies compared to their control (PBS given) after 4 weeks post-vaccination and present or absence of properdin does not affect the antibody response to capsular polysaccharide 3. The finding was comparable to finding of BALB/c depleted complement mice which does not affect the antibody response to capsular polysaccharide 3 (Markham et al., 1982).

The sialic acids rich capsule of PPS3 have shown prevents C3b deposition on bacteria surface thus prevent activation of alternative pathway. However by having enough amounts of type-specific anti-capsular antibody able to overcome PPS3 capacity in which allow complement activation by C3b deposition on bacteria surface (Jarva et al., 2003, Markham et al., 1982).

Additional to PPS2 and PPS3, specific ELISA to pneumococcal polysaccharide serotype 6B was performed as well as serotype 6B is also the most immunogenic among the polysaccharide that contained in Pneumovax and amongst common for invasive

pneumococcal disease in children (Vella et al., 1992) as it is most frequently cause drug-resistant pneumococcal infections. It has verified that this serotype 6B is difficult to encounter and cause drug-resistant pneumococcal infections as the findings were no increase of anti-capsular type 6B IgM antibodies for both properdin-deficient and WT which is similarly to non-vaccinated (PBS injected) mice (Figure 4.8).

Studies have shown predominant subclasses protective antibodies to polysaccharide is IgG3 in mice (Harris et al., 2003, Hu and Test, 2004, McLay et al., 2002, Snapper et al., 1992). IgG3 levels were measured but were found to be quite low (figure 4.9) compared to IgM levels. This is consistent with the fact that the intraperitoneal administration of the polysaccharide vaccine would activate primarily peritoneal B-cells which have reduced IgG3 production. Wild type vaccinated demonstrated a significantly higher of anti-capsular polysaccharide type 2 IgG3 level compared to the properdin-deficient.

Germinal centers are formed during an immune response elicited with a so-called T-independent antigen (Lentz and Manser, 2001). This reaction is, however, of short duration. The IgG subclass switching and affinity maturation occurs within germinal centres of spleen. In observations, wild type spleen section of H&E stained showed many follicles and some seen active germinal centre with secondary centroblast which is indicating active proliferating activity of the spleen. It has shown in presence of properdin importance in influence of class switching and affinity maturation in response to type 2 T-independent antigens as it is includes binding to surface polysaccharide lead to induces multivalent membrane immunoglobulin cross-link on b cell surface, complement fixation and interaction with host cellular Fc receptors (McLay et al., 2002) in contrast of lack of function in absence of properdin.

The intraperitoneal route of administration for vaccination been widely used in experimental animals probably as most effective site of antigen deposition as the method led to the local response to resident B cells by appearance of antibody secreting cells and drainage of the antigen via lymphatic into the circulation; results of a systemic antibodies response.

Additional findings of microscopic differential cells count of peritoneal lavages demonstrated an intraperitoneal vaccination is characterised by recruitment of inflammatory cells and it seems in presence of properdin has increased recruitment of lymphocytes. In contrast, in absence of properdin lymphopenia is induced and increased recruitment of macrophages (Figure 4.10).

Since natural antibody presents in the serum at low titers, the findings of increasing antibody levels with at least a fold to 4 fold rises following vaccination are clearly preparing for later adaptive response (Wernette et al., 2003).

The 23-valent Pneumococcal capsular polysaccharide vaccine is classified as T-cell-independent type-2 (TI-2) antigen and is moderately effective due to poor immunogeneicity. Following immunisation with this TI-2 antigen, a protective immunity is generated and mediated by antibodies against capsular polysaccharide (usually simultaneously of the IgM and IgG type, within 3 days) (Hu and Test, 2004). This T1-2 antigen can interact with B cells directly to initiate clonal expansion and antibody production, then a persistent stimulation of B-cells can provide long lasting antibody levels but demonstrate lesser avidity, opsonophagocytic and bacteriolytic activities, compared to antibodies elicited by immunisation with T-dependent antigens (Harris et al., 2003, Hu and Test, 2004, Lee et al., 2001). Immune responses to capsular polysaccharide antigens elicits

antibodies in mice that are isotype restricted which in orders of IgM, followed by IgG3, IgG2b and IgA-specific antibodies (McLay et al., 2002, Tian et al., 2007).

4.4.2 Role of properdin in immune responses following intranasal challenge of vaccinated mice

Generation of antibodies to capsule polysaccharide is essential for the survival of animal challenged with *S. pneumoniae*. The complement system plays a key role during innate immune response towards pathogens, including *S.pneumoniae*.

In current study, the experimental animal model of properdin-deficient and WT is on a C57Bl/6 background, which is known susceptible to the *S.pneumoniae* D39 infection as there were variation of susceptibity to pneumococcal disease in according to the mouse strains (Kadioglu and Andrew, 2005).

The vaccination procedure proved efficacious following to intranasal infection. The clearance of pneumococcoci by phagocytosis is dependent on anti-capsular antibody and facilitated by complement activation (Catterall, 1999). In orchestra, resident alveolar macrophages during the early stage of infection followed by efficient polymorphonuclear leukocytes (PMN) to play a phagocytic role (Catterall, 1999).

The 24 hours following intranasal infection with *S.penumoniae*, vaccination helps to clear bacterial loads from blood as the mice that been vaccinated has no CFU count (Table 4.3). The vaccinated mice were protected from *S.penumoniae* as shown by better survival and low severity score (Fig. 4.11 and 4.12).

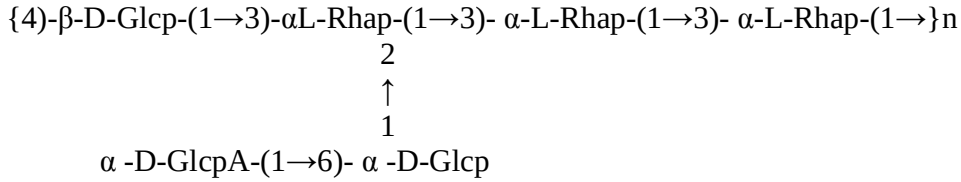
Taken of antibody response for vaccinated mice following 24 hours post-infection, vaccinated mice of both properdin-deficient and wild type demonstrated a significantly high concentration of total IgM as primary antibody against infection but then reduced after a week of post-infection compared to Day 0 pre-vaccinated level (Table 4.4A). At 24 hours after intranasal infection, serum levels for the specific anti-capsular polysaccharide type 2 IgM demonstrated a high level of specific anti-capsular type 2 IgM seem comparable to those of day 28 of the vaccination schedule as primary antibody against *S.penumoniae* D39 and was consistent at the end of experiment. These antibodies signifies the contribution elicited by the vaccine

As seen during the first experiment, the properdin-deficient appeared to have a significantly high level of specific anti-capsular type 2 IgM compared to wild type (Fig.4.13). It seems that in absence of properdin helps in maintenance and boosts the humoral memory response to encounter the infection.

A markedly increased of anti-capsular type 6B IgM level was seen at the end of experiment against *S.penumoniae* D39 for both vaccinated group compared to non-vaccinated.

It is surprised that antibody type specific of PPS6B can recognised *S.pneumoniae* D39 type 2 although there were no similarities of the chemical structures between the pneumococcal polysaccharide as shown below (Lee et al., 1991, Park et al., 2009):

Pneumococcal polysaccharide serotype 2:



Pneumococcal polysaccharide serotype 6B:
{2)- α- D -Galp-(1→3)- α- D -Glcp-(1→3)- α- L -Rhap-(1→4)-D-ribitol-5-P-(O→}n

Probably the explanation can be, unless the antibody type specific of PPS6B were made as recognising α-L-Rhap-(1→3) in serotype 2 which is can be found in serotype 6A (Lee et al., 1991, Park et al., 2009). Perhaps further investigation can be done in order to investigate the specificity of the antibody response to towards the specific polysaccharide.

At the end of experiment, immunoglobulin class switching happen when the secondary antibody, IgG demonstrated a significantly high concentration of total level for both vaccinated properdin-deficient and wild type mice (Table 4.4B). Immunoglobulin G3 levels in sera at the end of the experiment (post-vaccinated and post-infected) were found moderate increased and there were no significance difference in absence or in presence of properdin (Table 4.7).

Protection from *S. pneumoniae* and sepsis is provided by natural IgM and anti-polysaccharide IgM binding to *S. pneumoniae* also activation of the complement pathway. At a high infectious dose, however, the immune response turns against the host by causing tissue damage. Through out all, properdin-absence was noticeable in second experiment, as one of properdin-deficient vaccinated were contained bacterial loads in blood which is consistent with the survival rate showing a death at 24 hours time point. In absence of properdin prevent positive regulation of alternative pathway and less inhibit surface binding of bacterial thus unable to clear the bacteria from blood. In correlation although with high population of peritoneal macrophages which demonstrated previously during

vaccination and perhaps due to over workload of inflammatory reaction which can be illustrated by lung H&E microphotograph (Fig.4.15) with heavy cellular infiltration of cells, still unable to clear the bacteria, gave a harmful reaction to the host and results a death.

Importantly, the survival data show that the vaccination scheme has worked for the wild type that been protected from the infectious challenge. In relation to the better outcome of wild type vaccinated, this group have less PMNs in lungs than control wild type after infection. While both group of properdin-deficient, vaccinated and control PBS has a similar number of PMN which reflects their survival outcome, too, no difference.

Nevertheless taken altogether the current findings have shown there were no vaccination failure although there was variation in the specific antibody response among the experimental mice similar to findings of previous study of properdin individuals as mentioned earlier in introduction. Also recent finding (Guttormsen et al., 2009)showed MBL deficient mice model not impaired in their antibodies response to *S. pneumoniae* polysaccharide intaperitoneal immunisation. In some study involving two C1q-deficient patients demonstrated the naturally acquired anti-polysaccharide antibody response showing low class switching ability in these patients by consisted of elevated IgM levels compared to normal but lesser IgG and IgA levels (Griffioen et al., 1991).

Previous published study demonstrated the role of alternative pathway in killing of *N.meningitidis in vitro* as shown effective bactericidal activity against serogroup W135 *N.meningitidis* in presence of properdin which describes the association of properdin-deficient with *N.meningitidis* (Soderstrom et al., 1991, Sjoholm et al., 1991). However upon vaccination markedly reduced the need for the properdin in alternative pathway-

mediated killing of the meningococcal shown in published study of properdin-deficient sera that the *N.meningitidis*-killing was moderate but vaccination clearly enhanced opsonophagocytosis in the sera (Soderstrom et al., 1989).

The comparable results of vaccinated controls and vaccinated properdin-deficient demonstrated in the presence of a high concentration of anti-capsular antibody, the lack of properdin in properdin-deficient is masked by the antibody-dependent role by the classical pathway as the vaccination promoted classical pathway-mediated killing in serum and opsonisation of serogroup W135 *N.meningitidis* in which described the classical pathway take over the alternative pathway (Soderstrom et al., 1989). In relation, it is clarify the properdin-deficient patients do not develop recurrent infections of *N.meningitidis* (Fijen et al., 1999) and in return, it is advised for properdin-deficient individuals to have vaccination for every 3 years with tetravalent ACWY meningococcal capsular polysaccharide to protect properdin-deficient individuals towards *N.meningitidis* infection (Fijen et al., 1998).

As explained, the absence of properdin only partially impaired the alternative pathway and the role of the pathway can be replaced by classical pathway in defence against the bacterial infection. By vaccination, specific antipolysaccharide is produced, mediated opsonophagocytosis and facilitated bacterial clearance The complement system bridges both arms of immune system, the innate and adaptive in which provide additional enhancement of TI-2 response and facilitate B-cell bind of antigen (Vos et al., 2000).

By contrast other complement component, as showed three out of four C3-deficient patients demonstrated negligible levels of anti-pneumococcal capsular polysaccharide antibodies and suffered from recurrent pneumococcal sepsis (Hazlewood et al., 1992). Hereditary C3-deficiency predisposes to infections with not only encapsulated, but a

variety of microorganisms, reflecting the central role of C3 and its activation/degradation products in the complement activation cascades.

4.4.3 Unexpected antibodies raised in absence of properdin following intraperitoneal PBS

The mice used as controls for the vaccination received PBS intraperitoneally and gave unexpected result: the properdin-deficient group raised a significantly higher concentration of total serum IgM (Fig. 4.5B) and anti-capsular type 2 IgM (Fig. 4.6B) compared wild type control mice which were at the same range of concentration as pre-vaccinated.

These interesting differences lead to an experiment of differential cell count for peritoneal cell population as it was thought due to the method of administration by intraperitoneal. Nevertheless both wild type and properdin-deficient has comparable count of microscpic differential cells count of peritoneal lavages that was done for monocytes, macrophages, lymphocytes and mast cells (Figure 4.10). Peritoneal administered of tetanus toxoid, a safe purified antigen, demonstrated most peritoneal cells were lymphocytes followed by monocytes/macrophages, polymorphonuclear leukocytes and mesothelial cells (Lue et al., 1994).

It is thought that in the absence of properdin an inhibitory signal in counteracted and antibody production increased. This is masked when polysaccharides crosslink the surface of B-cells. It should be noted that these properdin-deficient have higher natural antibody. Because day 0 levels are comparable it seems the only explanations is that through the

application route of the carrier liquid (PBS) in a non-sterile way (no alcohol swabbing etc), resident skin bacteria were introduced which in their limited numbers were able to provoke this phenotype.

4.4.4 Role of properdin in immune responses following intranasal challenge of non-vaccinated mice

A comparable bacteria load were found in the blood of both properdin-deficient and wild type and in effort of pneumococcal clearance, data of PMN count in experimental mice lungs compared to normal lungs demonstrated inflammatory and immunologic reactions in the lung in response to the infections. Nevertheless there was no significant difference both properdin-deficient and wild type indicated the presence or absence of properdin did not seem to impact on pneumococcal clearance.

In one set of experiment showed low survival rate with worst severity disease in the wild type compared to the infected properdin-deficient mice (fig. 4.11B and 4.12B). Therefore it seemed that the presence of properdin appeared to be detrimental as properdin was playing its role in alternative pathway activation loop to clear the bacterial count in the blood but then the system had failed to clear the infection probably as more pneumococcal could be re-seeded into the lungs via blood circulation resulted pneumonia and sepsis among the wild type mice.

Lack of the complement activation in the blood of the properdin-deficient mice was due to the absence of properdin in the alternative pathway activation loop, resulting in a poorer

clearance of the pneumococcal bacteria in the blood. However the current finding demonstrated properdin-deficient mice had a benefit to survive pneumococcal pneumonia and sepsis with better condition of the infected mice independent of vaccination compared to their wild type littermates (fig. 4.11B and 4.12B). In comparable of previous study of intranasal *S.pneumoniae* D39 infection in other infected complement-deficient; C1qa, factor B, C4-deficient (C57Bl/6 background) showed a higher survival rate than their wild type (Kerr et al., 2005, Brown et al., 2002).

In absence of vaccination, humoral antibody can be developed as consequences of natural exposure to bacteria (Casal and Tarrago, 2003) and that antibody repertoire to pneumococcal polysaccharide is seemed quite sufficient to defence against challenge.

At 24 hours after intranasal infection, serum levels for anti-polysaccharide PPS2 IgM of controls mice seem comparable to those of day 28 of the vaccination schedule. At 7 days post-intranasal infection, however, all IgM levels have further increased.

These antibodies are specific because: 1) the levels increase up to day 28 for wild type and properdin-deficient mice vaccinated and 2) the difference in levels between vaccinated with control of properdin-deficient mice and wild type signifies the contribution elicited by the vaccine. However these antibodies also can be not specific because they can be provoked by intraperitoneal PBS.

Therefore, the ELISA might detect two sorts of anti-polysaccharide IgM –the anti PPS2 that are intended by the study experiment, but also an increase in natural, low affinity IgM production on stimulation of peritoneal B-cells. This effect is obviously big enough to cause a change in level in the serum.

At the end of the experiment of post-infection the wild type control demonstrated a significantly higher of anti-capsular polysaccharide type 2 IgG3 level compared to the properdin-deficient which comparable when they were vaccinated. The finding demonstrated natural class switching without vaccination during infection showed a similar response to TI-2 antigen.

Importantly, it is both classical and alternative pathway of complement play the important role in defence against pneumococcal infection. However it is thought the classical pathway plays the dominant role rather than alternative pathway (Brown et al., 2002, Jarva et al., 2003).

4.4.5 Role of properdin in this study

This study therefore showed that in addition to the beneficial role as a positive regulator of complement pathway, properdin could possess detrimental role and results a host-damage in response to infection and by the vaccination efficacious it protects the experimental mice from pneumococcal disease when intranasal infected of *S.pneumonia*.

Chapter 5: The role of properdin in maturation of dendritic cells in vitro

5.1 Introduction

Complement and DCs cooperate to modulate T cell (Zhou et al., 2007) and B cells (Leslie and Nielsen, 2004) responses. However research still need to be done to see relationship of complement properdin on DC differentiation and maturation.

5.1.1 Dendritic cell

Dendritic cells (DCs) were firstly identified by Steinman in 1972 (Steinman, 2007). DCs play a key role in host defenses that able to induce primary immune responses by acting as antigen presenting cells (APCs). Main function of dendritic cells is to process ag material and present it on the surface to other immune cells. DCs involved in cell-mediated immunity as the DC responsible activate naïve T cells and generate for cell responses (Banchereau and Steinman, 1998, Reis et al., 2007). DC also function as tolerance inducer by eliminating self-reactive T cells (Banchereau and Steinman, 1998, Tan and O'Neill, 2005).

However DCs exist in different maturation stages (Reis et al., 2007) can only become fully functional APC when they are activated and mature.

5.1.1.1 Development and functional properties of dendritic cells

Dendritic cells maybe immature or mature. This distinctions relates to their phagocytic activity, cytokine production and surface expression of co-stimulatory molecules (Banchereau et al., 2000).

5.1.1.2 Immature dendritic cells

Immature DCs reside within non-lymphoid tissues. In the immature state, iDC actively capture and process antigen. Immature DCs capture antigens by several pathways including macropinocytosis, phagocytosis (involving CD36 and integrins) and receptor-mediated endocytosis (via Fc- and C-type lectin receptors)(Banchereau et al., 2000, Banchereau and Steinman, 1998). Immature DC is characterised by low expression of MHC-II, co-stimulatory molecules and low CD40, CD54, CD58, CD80 and CD86 (Winzler et al., 1997, Steinman and Young, 1991, Banchereau and Steinman, 1998). Immature DCs lack or have low levels of several important accessory molecules that mediate binding and stimulation of T cells. Other characteristics of immature DC are highly organised cytoskeleton, slow motility, efficient antigen uptake and processing of soluble proteins (Winzler et al., 1997, Steinman and Young, 1991, Banchereau and Steinman, 1998). Immature DCs rich with MHC II products within intracellular compartments also react rapidly to inflammatory cytokines and microbial products (Satthaporn and Eremin, 2001).

5.1.1.3 Mature dendritic cells

As the function of DC depends on the state of activation, DC activation or maturation can be induced by either exogenous or endogenous factors such as foreign antigen or pathogens, contact with inflammatory stimuli (e.g. cytokines), infectious agents and signals such as lipopolysaccharide (LPS) (Banchereau et al., 2000, Reis et al., 2007).

Upon activation, DCs switch their pattern of chemokine receptor and adhesion molecule expressions permiting their migration to secondary lymphoid tissues, such as the lymph nodes, spleen or thymus. Once migrated, iDC is transform to become mature DC. DC maturation is final stage of DC differentiation and goes through several changes that reflect the changing functional role of the cells.

In contrast to iDCs, mature DCs have lost ability to efficiently take up and process antigen but are extremely efficient in antigen presentation and stimulating T cells (Winzler et al., 1997, Steinman and Young, 1991, Banchereau and Steinman, 1998**).** Mature DCs express high levels of surface MHC II proteins, CD80, CD86, CD40 and others related with antigen presentation. Mature DC change MHCII distribution and alter expression of adhesion molecules (Rescigno et al., 1997, Tan and O'Neill, 2005, Winzler et al., 1997). They also change morphology by having dendrites or branch projections.

5.1.1.4 Migration, maturation and function dendritic cells

As an APC, DCs interact with T cells and B cells by combining interaction of co-stimulatory molecules, cytokines and presentation of peptides to T and B lymphocytes (Reis et al., 2007). Mature DCs present processed antigens in association with MHC and CD1 molecules (Winzler et al., 1997).

The DC responsible for T cell responses and this happens when the T cell receptors (TCRs) on T lymphocytes recognise fragments of antigens (Ags) bound to molecules of the major histocompatibility complex (MHC) on the surfaces of DCs. There are two types of peptide binding proteins: MHC class I and II, which interact with and stimulate cytotoxic T lymphocytes (CTL, CD8) and T helper cells (Th, CD4), respectively (Banchereau and Steinman, 1998, Moser and Murphy, 2000). They activate helper T cells and Cytotoxic T cells and also activate B cells (Reis et al., 2007) by presenting them with Ag derived from pathogens. When encountered, the pathogens are phagocytosed and their proteins are processed and expressed on DC surface to be presented to T cells (Reis et al., 2007).

Appropriate Th cell activation requires the recognition of MHC-peptide complexes on DCs by antigen-specific TCR together with the interaction of co-stimulatory molecules such as CD80 and CD86 present on the DC surface and CD28 on T cells. T cells can also activate DCs by the interaction of CD40 and CD40 ligand, which up-regulates CD80 and CD86 expression and cytokine release (Banchereau et al., 2000).

The maturation pathway is subsequently modulated during DC–T cell interaction, involving a combination of signals resulting from the interaction of co-stimulatory molecules as well as the production of cytokines. The different types and levels of

cytokines produced by DCs such as IL-12, IL-10, IL-6, IFN-γ and TNF-α (Moser and Murphy, 2000, Pasare and Medzhitov, 2003a, Reis et al., 2008, Pasare and Medzhitov, 2003b, Nolan et al., 2004) upon pathogen recognition. Subsequently the immune cells destroy the pathogens as immune responses. These illustrated in figure 5.1.

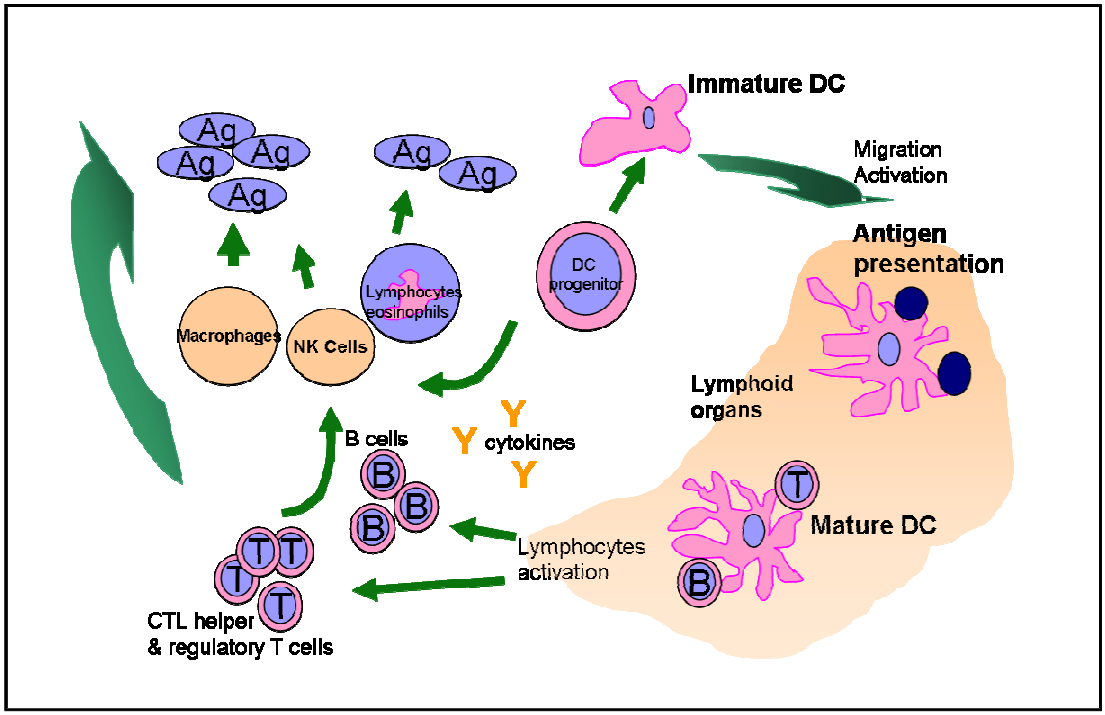


Figure 5.1: Migration and activation of DC from non-lymphoid tissues to secondary lymphoid organ. DCs reside in peripheral tissue in immature state. In presence of antigen, immature DCs become activated and migrate to secondary lymphoid organ. Upon migration, DC become mature DC and help activate T helper cells, NK cells and B cells by presenting them with Ag derived from pathogens. Figure was adapted from Banchereau 2000 (Banchereau et al., 2000).

5.1.2 What's known about dendritic cells and complement

Dendritic cells and complement system have been shown to be important and cooperate in the interface between the innate and the adaptive immune response (Banchereau et al., 2000, Carroll, 2004, Wills-Karp, 2007). During activation, dendritic cells become important antigen presenting cells. Complement plays a role herein.

The interface between innate and adaptive immunity is a topical research area and has documented a role of complement in lowering the threshold of signaling for B-cells. The way this is achieved by activation of the classical and alternative pathway of complement, yielding preferentially C3dg or C3b/iC3b, respectively. These fragments bind to CR2, CD21, which is a B-cell receptor co-receptor, and thereby enhance immunoglobulin secretion (Leslie and Nielsen, 2004).

The dendritic cells belong to both, innate and adaptive immune response arms, depending on their state of activation, and furthermore they have been shown to express a variety of complement components. Reis's group (Reis et al., 2006) have shown human monocytes-derived DC express several complement proteins such as C3, C5, C9, Factor I, Factor H, B, D, properdin mRNA, in a stimuli-specific manner independent of DC- maturation.

In addition, they found human immature and mature DCs to express C1q (Castellano et al., 2004, Reis et al., 2007), C7, C8, C4b binding protein and complement receptors (CR)-3 and CR4 (Reis et al., 2007). Work by Nolan and group (Nolan and Reid, 1993) has shown that properdin expression decreases significantly on LPS maturation of bone-marrow derived dendritic cells from wild type mice.

Interestingly, bone marrow-derived dendritic cells has shown synthesise C3. The significant functional impact of this was shown in a study investigating dendritic cell-dependent allospecific T-cell response in a transplant model using C3-deficient mice (Peng et al., 2006). Then, C3-deficient dendritic cells expressed less MHCII and co-stimulatory B7.2, were impaired in alloreactive T-cell stimulation, and skewed the cytokine response of T-cells to favor Th2 development. Further, bone marrow-derived dendritic cells purified from mice deficient in Factor B (an essential factor of the alternative pathway), C3aR (receptor for C3 split product C3a, an anaphylatoxin) or C1q (an essential component of the classical pathway) showed impaired T-cell allostimulation (Castellano et al., 2007, Peng et al., 2006). C3 was shown to be a necessary factor for cytokine-induced differentiation and lipopolysaccharide-induced maturation of monocyte-derived dendritic cells as previous work showed that the lack of C3 inhibits maturation of monocyte-derived dendritic cells and reduced production of cytokines; IL-6 and IL-12 (Reis et al., 2008). In addition, the C3 deficient DC have reduced T cell stimulation and impaired allograft rejection (Zhou et al., 2007).

However few studies have investigated properdin expression in murine or human tissues. The fact that properdin was found to be expressed by limited tissues suggested properdin exerts a tissue-specific function.

Therefore the aim of the study was to assess whether properdin is essential in the differentiation and maturation of dendritic cells derived from mouse bone marrow and spleen.

5.2 Materials and Methods

Dendritic cells can be generated *in vitro* by culture in growth medium, either derived from pluripotent precursor cells of bone marrow or splenocytes or from blood-derived monocytes in influence of serum, GM-CSF (Inaba et al., 1992, Sallusto and Lanzavecchia, 1994, Shortman and Liu, 2002) and IL-4 ((Inaba et al., 1992, Sallusto and Lanzavecchia, 1994)

5.2.1 Materials

Mice (wild-type and properdin deficient) were used at 6-10wk of age.

All the chemicals used were purchased from: (Unless otherwise stated in the text).

Culture media: RPMI 1640 +25mm HEPES+L-glutamine (2mM), (GIBCO, Fisher Scientific, Loughborough, UK).

Culture media was supplemented: Streptomycin (100μg/ml) (Sigma-Aldrich, Gillingham, UK), Penicillin (100U/ml) (Sigma-Aldrich, Gillingham, UK), 10% heat-inactivated FCS (Sigma-Aldrich, Gillingham, UK) and termed as 'cell culture media'.

Cytokines: rmGM-CSF (recombinant murine granulocyte-macrophage colony-stimulating factor) and IL-4 (Interleukin-4), (R&D Systems, Abingdon, UK).

Density gradient separation: OptiPrep (Axis-Shield, Oslo, Norway)

LPS (Lipopolysaccharide *Escherichia coli* serotype 0111:B4; 1mg/ml; Alexis Biochemicals, Lausen, Switzerland)

Monoclonal antibody (anti-mouse) for surface marker: Phycoerythrin (PE)-conjugated, Fluorescein isothiocyanate (FITC), (BD Biosciences Pharmingen, Oxford, UK)

Tube label	Concentration
IgG PE	0.2mg/ml
IgG FITC	0.5mg/ml
CD11c PE	0.2mg/ml
CD40 FITC	0.5mg/ml
CD80 FITC	0.5mg/ml
CD86 FITC	0.5mg/ml
MHCII FITC	0.5mg/ml

5.2.2 Dendritic cell culture

Methods for generation of dendritic cells were kindly advised by Dr. M. Browning (University of Leicester). All preparations were performed in a laminar flow hood for sterility.

5.2.2.1 Bone marrow preparation

Bone marrow derived dendritic cells were generated as described previously with minor modifications (Inaba et al., 1992, Lutz et al., 1999).

Femurs and tibiae of C57BL6 (wild type and properdin deficient mice) were removed and purified from the surrounding muscle tissue (using sterile forceps and scissors). Thereafter intact bones were left in 70% IMS (industrial methylated spirit) for 2–5 min for disinfection and washed with PBS (phosphate buffered saline). Then both ends were cut with scissors and the marrow flushed with PBS using a Syringe with a 0.45 mm diameter needle (Terumo Europe NV, Leuven, Belgium). Clusters within the marrow suspension were disintegrated by vigorous pipetting and were left in PBS to settle down to the bottom of collection tube. Leukocytes were obtained after a wash in PBS.

Bone marrow dendritic culture

Cells were incubated in tissue culture flask (25cm^2 tissue culture flask, Nunc Scientific Lab Supplies, Wilford, UK) overnight at 37°C in humidified incubator with 5% CO_2. Cell culture media were prepared consisting of RPMI 1640 + Penicillin (100U/ml) + Streptomycin (100µg/ml) + L-glutamine (2mM) + 10% heat-inactivated FCS.

At day 0, bone marrow leukocytes were seeded 2 x 10^6/ml in a final volume of 7ml of cell culture media with addition of cytokines: rmGM-CSF (10ng/ml) and IL-4(10ng/ml) per flask.

However, before culturing the cells with the cyokines, at least 1x 10^5/ml cells were kept aside for flow cytometry analysis to give starting population as whole cell population marker for Day 0 without any stimulation.

Cells were fed with same media (RPMI 1640+ penicillin+ Streptomycin+ L-glutamine + 10% heat-inactivated FCS+rmGM-CSF+IL-4) throughout the culture. At day 3 and 6, half of cell were collected and centrifuged. Cell pellet then were resuspended into 10ml fresh media + rmGM-CSF + IL-4 and back into original flask.

At day 7, non-adherent cells were gently collected and were split into two equal aliquots. Half of the cells were termed "immature dendritic cell" (iDC) were harvested and stained for flow cytometry analysis. The other half was maintained in cell media+ rmGM-CSF+IL-4 and 1µg/ml LPS for further 48 hours which activates the cells to become so-called "mature dendritic cells" (mDC). At day 9, these cells were harvested. Cells that were adherent were collected by using Trypsin-EDTA (see section 2.1.4). Harvested cells were stained for flow cytometry analysis to check for maturity.

5.2.2.2 Spleen

Methods were adapted from Leenen (Leenen et al., 1998).

Spleen preparation

Spleen (wild type and Properdin deficient) were obtained and cut or minced into small pieces. Suspensions were passed through 100µm filter (Nylon cell strainer, BD Falcon, BD Biosciences Europe, Erembodegem, Belgium) and centrifuged. Cell pellets were

resuspended in lysis buffer (17mM Tris HCl- 14mM NH_4Cl, pH 7.2) and were incubated at 37ºC for 5-10 minutes to lyse the red blood cells. Cells were then washed with cell culture media. Cells were kept incubated in tissue culture flask for overnight at 37°C in 5% CO_2 humidified incubator.

Spleen Dendritic cell culture

Same as bone marrow culture, some of the splenocytes were kept aside for flow cytometry analysis to give starting population of whole cell population marker for Day 0 without any stimulation. After overnight incubation, non-adherent cells were collected for spin and resuspended in cell culture media at 1-2 x 10^7 cells/ml. For DC enrichment, density gradient separation was applied by layered 2ml of DC suspension on top of 2ml 'OPTIPREP' and centrifuge after for 20 min at 530 x g. Cells on the interface were collected and routinely maintained.

Same as bone marrow culture, splenocytes were fed in cell culture media (RPMI 1640+ penicillin+ Streptomycin+ L-glutamine + 10% heat-inactivated FCS+rmGM-CSF+IL-4) throughout the culture. At day 3 and 6, half of cell were collected and centrifuged. Cell pellet then were resuspended into 10ml fresh media + rmGM-CSF + IL-4 and back into original flask.

At day 7, non-adherent cells were gently collected and were split into two equal aliquots. Half of the cells as "iDC" were harvested and stained for flow cytometry analysis. The other half as "mDC" was maintained in cell media+ rmGM-CSF+IL-4+1µg/ml LPS for further 48 hours. At day 9, these cells were harvested. Cells that were adherent were

collected by using Trypsin-EDTA (see section 2.1.4). Harvested cells were stained for flow cytometry analysis.

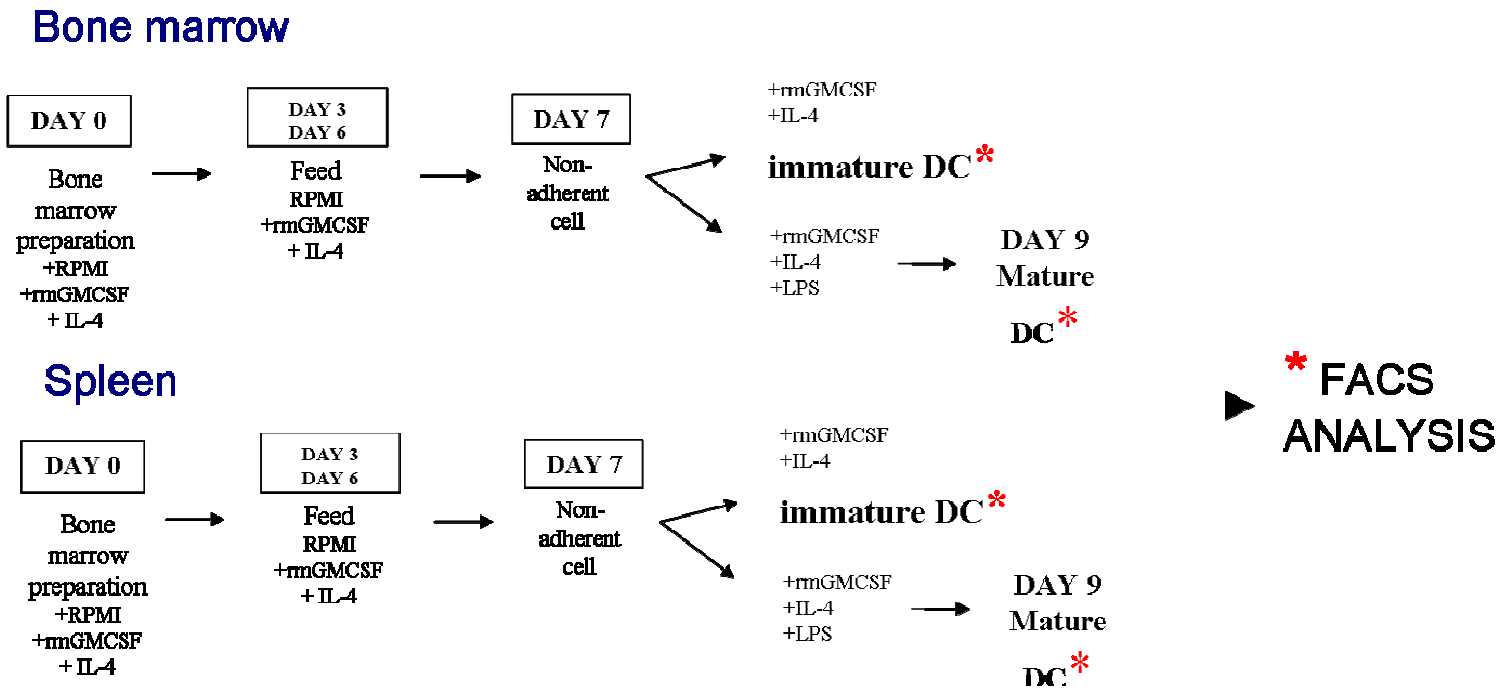


Figure 5.2: DC preparation derived from bone marrow and spleen can be illustrated as schedule above. * shows cells were harvested and stained for flow cytometry analysis.

5.2.3 Flow cytometry analysis of cell surface markers.

Cells were collected and checked for positivity and maturity as dendritic cells by using flow cytometry analysis of cell surface markers.

Flow cytometry is based on principles of light absorbance and emission and is a technique to measure properties of individual particles in suspension by counting and examining the microscopic particles. Flow cytometry analysis tells about a cell's relative size, granularity or internal complexity, by measuring fluorescence intensity and epitope expression of a cell or particle by laser light or fluorescence emitted by a fluorochrome (dye of conjugated antibody that directly targets epitope of interest to allow its biological and biochemicsl properties to be measured).

Briefly in principle, when a laser beam of flow cytometry hits the cells as the sample liquid containing the cells pass through the flow chamber, the fluorochromes attached to the cells absorb light and then emit a specific colour of light depending on the type of fluorochrome. The way the light bounces off each cell gives information about the cell's physical characteristics. Light bounced off called forward scatter (FSC) indicates the size of the cell while light off in other directions called side scatter (SSC) tells about cell's granularity or internal complexity. Then detectors collect the light emitted and the data from the detectors is sent to a computer and plotted on a graph.

In this study, direct immunofluorescence using FITC- or PE conjugated mouse monoclonal antibodies (mAb) to CD40, CD80, CD86, MHCII, and CD11c was performed to stain for cell surface marker expression in comparison with isotype control antibodies by flow cytometry.

5.2.3.1 Monoclonal antibody staining and flow cytometry

Briefly, cells populations were harvested and analysed. Cells were counted to be at least $1x10^5$/ml – $1x10^6$/ml per sample tube.

Cells sample were washed by centrifuging at 1500rpm for 7 minutes in 2ml of ice cold PBS. Supernatant were removed and cell pellet were resuspended in remaining drop of buffer.

Staining using FITC- or PE conjugated mouse monoclonal antibody (mAb) to CD40, CD80, CD86, MHCII, CD11c or with the appropriate isotype control antibodies (IgG PE and IgG Fitc) by 1-20µl MAb (to cell sample with concentration use up to 1µg/µl) were added to cell samples and similar volume of PBS to cell controls. Cells samples were mixed by vortex. Then cells were incubated for 30 mins at 4°C in dark. Next cells were washed twice by centrifugation. Lastly cells were fixed by 500µl ice cold fix buffer (PBS+ paraformaldehyde 2% + 2% sodium azide). Cells sample without any antibody acted as a control as an overview of whole population through FITC (CC FITC) or PE (CC PE) channel of flow cytometry.

The same buffer was used throughout for the incubation with antibodies as well as for all washes and using a standard technique, followed by flow cytometry (FACSCalibur, BD Biosciences). Data were analysed using Flow-JO software (USA).

During the analysis, the forward and the side scattered showing the possible all cell population and gating is performed by placing a gate on the wanted cell population (excluded cell debris). By gating the wanted cell population, data of positive cells can be obtained and given in percentage.

Ideally FACS histogram produced a single distinct peak that can be interpreted as positive data. FACS histogram produced several peaks if having mix population of cells. So by having negative Isotype control alongside act as a control that isolate non-specific expression thus help to separate positive population from the mix population.

As for positive dataset, by running an appropriate isotype control and overlaying image on the histogram, the positive dataset is identified on the right of the isotype control.

5.2.4 Determination of TNF-alpha production

Quantification of the cytokine TNF-alpha (TNF-α) produced by cultured cells was analysed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (BD OptEIA™ Pharmigen, UK).

For the determination of TNF-α in culture fluid, supernatants from DCs were collected and stored at -80°C.

The ELISA was carried out according to the manufacturer's protocol.

Basically, the BD OptEIA™ test is a solid phase sandwich ELISA. Firstly, ELISA diluent was added to a 96-well plate coated with TNF-α. Then, standards and samples were added to the wells and left for incubated for 2 hours at room temperature. The wells were washed for 5 times with wash buffer. The detection antibody, namely biotinylated polyclonal anti-mouse TNF antibody was added and the plate incubated for 1 hour at room temperature. After a second wash, streptavidin-horseradish peroxidase was added and left to react for 30 minutes at room temperature. The wells were washed again and a proxidase substrate solution TMB One-Step Substrate Reagent was added, which produces a blue colour in direct proportion to the amount of TNF-α present in the initial sample. The Stop Solution acidifies the reaction, and the wells were read at 450 nm within 30 minutes.

5.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc.,USA) and data are expressed as mean ± SEM. The differences in mean values were analysed by Mann-Witney nonparametric test. Values of $p < 0.05$ were considered statistically significant.

5.3 Results

In the study to characterise the immune response of properdin-deficient mice, the work was interested in the relevance of properdin in dendritic cell maturation. To achieve this (as described in Chapter 5-Materials and Methods section), dendritic cells were derived from bone marrow flushes and spleens of wild type and properdin-deficient mice by incubation in growth medium RPMI with recombinant murine GM-CSF and IL-4 at 37ºC in 5% CO_2 for seven days to generate immature dendritic cells (iDC). At this time point, the so-called iDC were stimulated with lipopolysaccharide (LPS, 1µg/ml) for the next 48 hours, to become mature dendritic cells (mDC).

5.3.1 Generation of dendritic cells

In generation of DC, the preparations and analysis for both wild type and properdin deficient were done in parallel as been presented together in a graph to make a parallel comparison.

5.3.1.1 Phenotypic analysis of dendritic cells by FACS histogram

FACS was performed in order to assess expression of the surface markers on the characterised cell. FACS analyses were done by staining using the following mAB: CD40, CD80, CD86, MHCII, and CD11c, with PE or FITC conjugated.

FACS analyses for starting population were done on the very first day without any stimulation of cytokines influence. For iDC, FACS was done after seven days of culture in RPMI growth media together with rmGm-CSF+IL-4 while for mDC, FACS were done on the last day of culture after the next 48 hours of LPS stimulation.

Dendritic cells were phenotypically characterised by FACS analyses.

Representative FACS histograms of experiments are shown in figure 5.3 depicting the expression of DC overlayed on the isotype control. Red line for open histogram represents isotype control-IgG same class of the marker to show background non-specific binding of cell epitopes. Blue line for open histogram represents profiles of the specific cell surface marker. Any shift to the right of isotype control means positive expression of the specific cell surface marker (CD40, CD80, CD86, MHCII, and CD11c).

These panels shows that the cytokines mediated differentiation is working about to the same extent of wild type and properdin-deficient DC and LPS stimulation appears to give different pattern of wild type and properdin-deficient DC.

In Figure 5.3(A) demonstrated a representative FACS histogram for BMDC of wild type (i) and properdin deficient mice (ii).

Briefly, for start population of wild type bone marrow-derived cells in normal growth media without any stimulation, FACS histogram showed only a weak expression of CD80+ and MHCII+. The rest of selected markers such as CD40, CD86 and CD11c were same as isotype control. Compared to start population, flow cytometry analysis of BMDCs culture of wild type at 37ºC in 5% CO_2 incubator for 7 days in RPMI media together with rmGM-CS and IL-4 demonstrated surface and functional phenotypes characteristic of iDCs by having positive expression of CD80, CD86, MHCII and CD11c,

indicate by the percentage value of positive cell from cell population, 31.4%, 16.5%, 54.2% and 40.8% respectively.

Meanwhile addition of LPS for the next 48 hours (9 days in total of incubation) generates cells with characteristics typical of mature DCs defined by a well-characterised surface phenotype marker with an increase of DC-specific marker CD11c with 76% of the cell population (clearly distinguished from iDC) and increased of co-stimulatory molecules such as CD40 (28.9%), CD80 (74.1%), CD86 (54%) and MHC class II (62%).

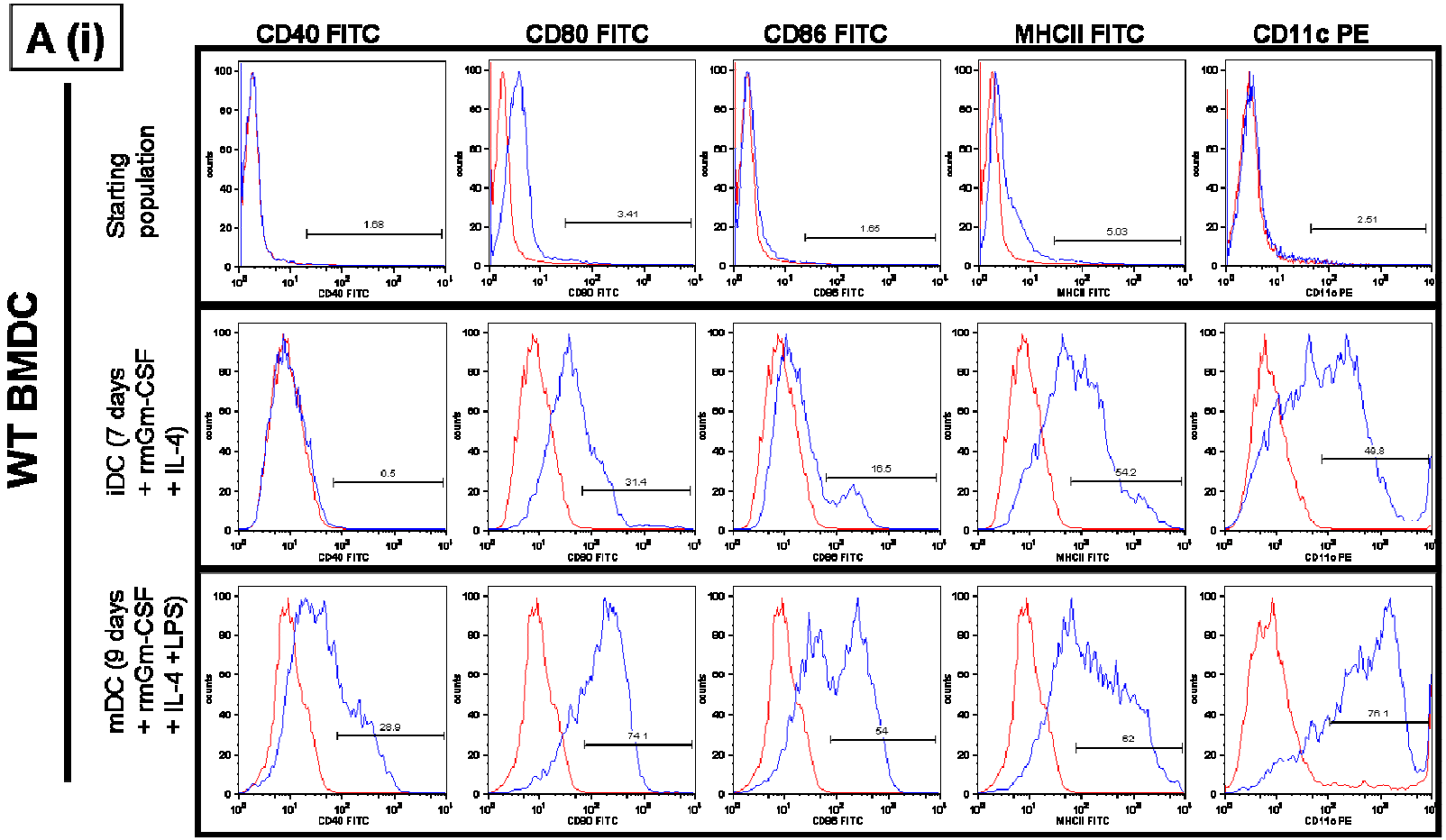
Properdin-deficient BMDC yield the following results (fig. 5.3 A (ii)). As for starting population cells in normal growth media without any stimulation, FACS histogram profile demonstrated a weak expression of CD80+ and MHCII+. Then, properdin-deficient BMDC culture for IDC (7 days in rmGm-CSF+IL-4) demonstrated a weak CD80+, CD86+, MHCII+ and CD11c+ Meanwhile mDC properdin-deficient BMDC demonstrated a weak positive for all the marker; CD40+, CD80+, CD86+, MHCII+ and CD11c+ with 0.47%, 1.92%, 0.84%, 2.75% and 9.01% respectively gating from the cell population.

Figure 5.3 (B) shows a representative FACS histogram for SpDC of wild type (i) and properdin deficient mice (ii). In contrast to bone marrow-derived DC, splenocytes DC show a different staining pattern.

The wild type splenocytes (Fig.5.3 B(i)) on starting populations showed expression of CD80+, MHCII+, CD11c+ and a weak CD86+. Here, wild type SpDCs differentiated for 7 days in RPMI medium supplemented with rmGM-CSF and IL-4 showed the

characteristic CD11c+ by 56% of cells, co-stimulatory CD80+ (40.9%), CD86+ (43.9%) and MHC II+ (78.9%). Then, on induction of rmGM-CSF, IL-4 and LPS after 9 days incubation showed a moderate to strong expression of all markers; CD40+ (31.9%), CD80+ (89.8%), CD86+ (76.9%), MHC II+ (82.6%) and CD11c+ (66.1%).

While FACS histogram profiles of Properdin-deficient SpDC ((Fig.5.3 B(ii)) expressed only MHCII + on start population and iDC. In addition profiles of mDC showed a weak expression of CD80+, CD86+ and MHCII+ (only with 4.95%, 2.91% and 24.8% respectively).


A (i)
WT BMDC
CD40 FITC
CD80 FITC
CD86 FITC
MHCII FITC
CD11c PE
Starting population
iDC (7 days + rmGm-CSF + IL-4)
mDC (9 days + rmGm-CSF + IL-4 +LPS)
1.68
3.41
1.65
5.03
2.51
6.5
31.4
16.5
54.2
49.8
28.9
74.1
54
62
76.1
counts

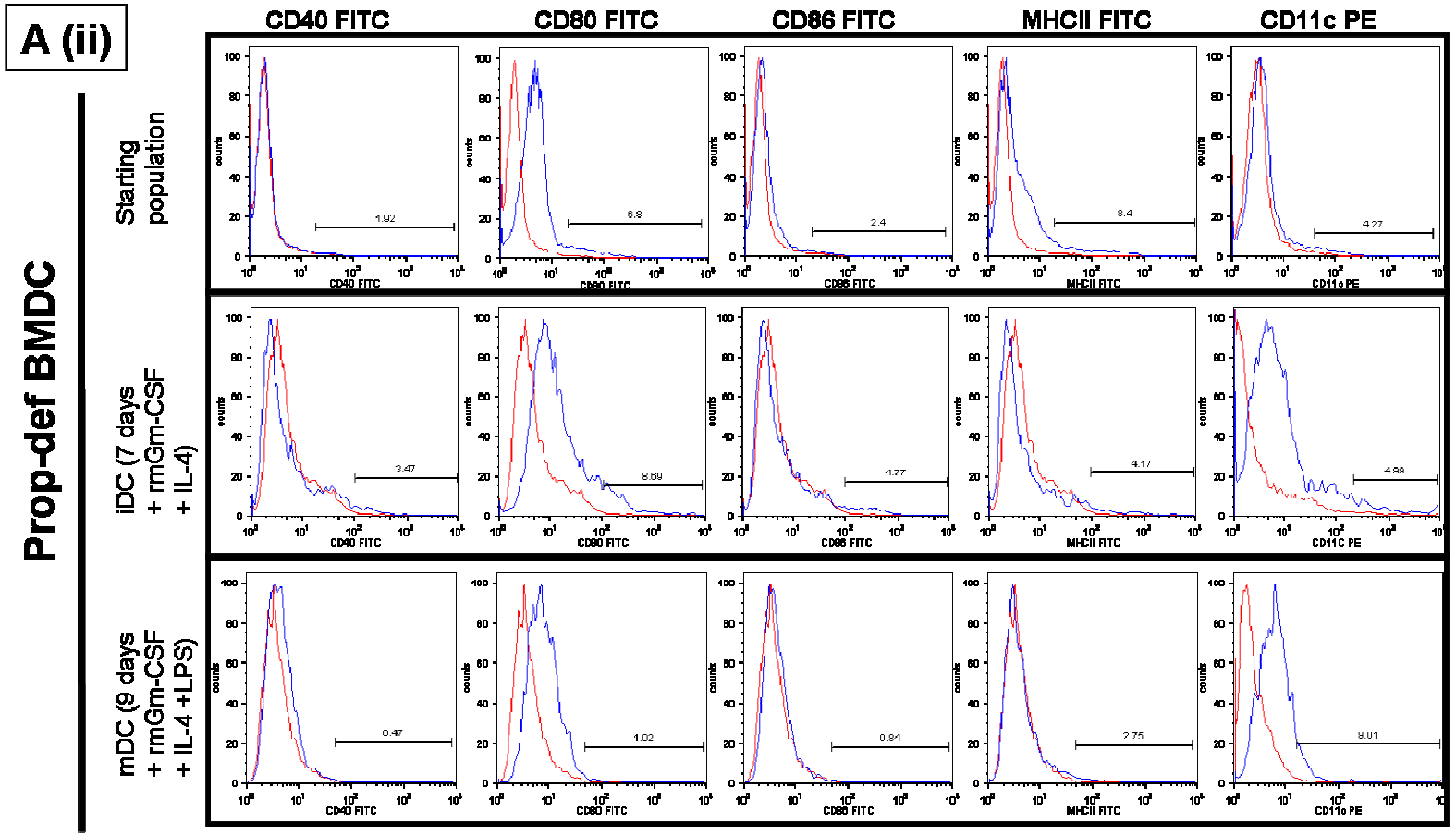
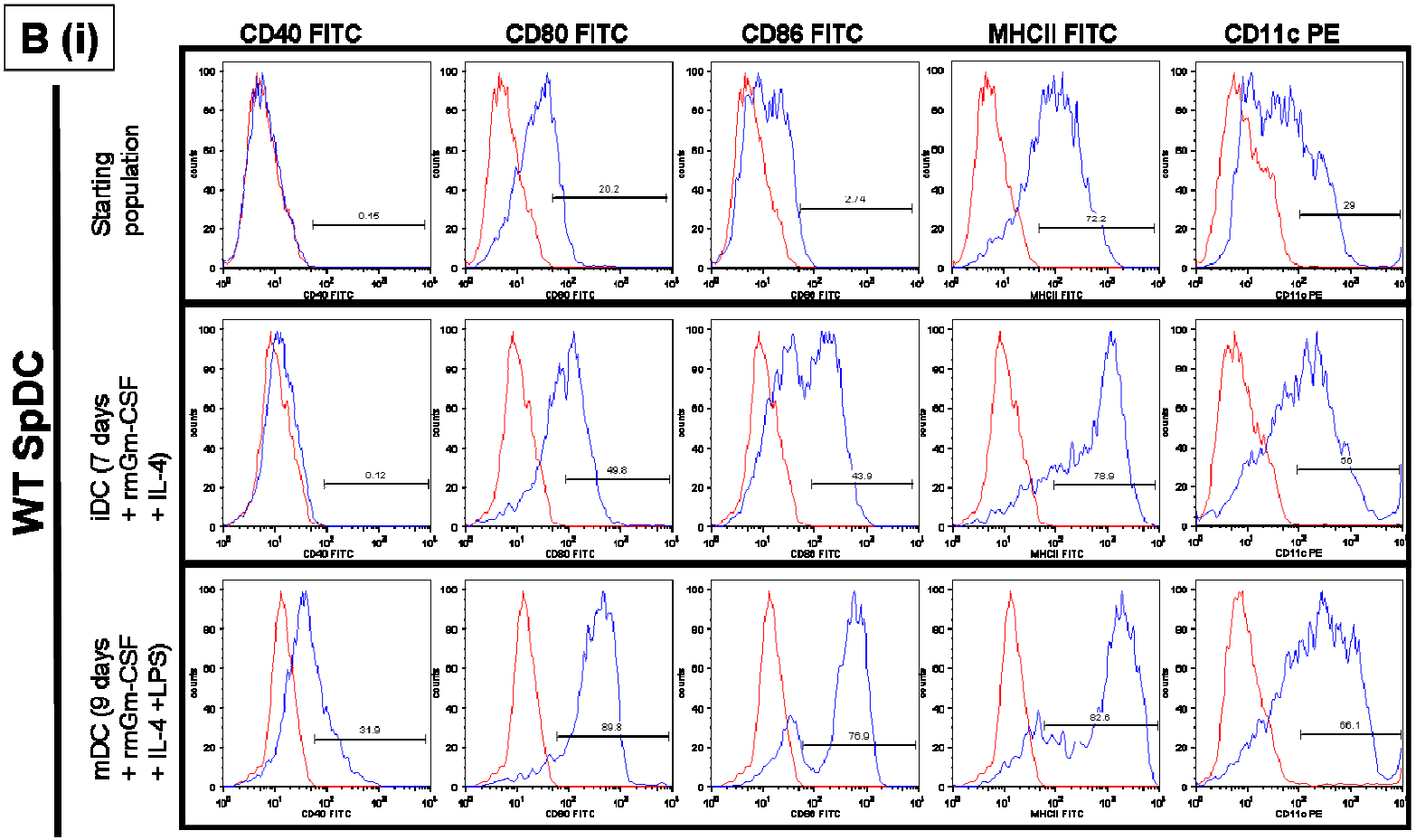


Figure 5.3 (A) : Representative FACS histogram of BMDC A (i) wild type derived, A (ii) properdin-deficient derived.
Phenotypic analysis of surface markers of dendritic cells (CD40, CD80, CD86, MHCII, CD11c) in state of start populations, inactivated immature (iDC) (7 days+rmGm-CSF+IL-4); and activated matured (mDC) (9 days+rmGm-CSF+IL-4+LPS). The open red histogram shows the profiles obtained with isotype control mAb. The open blue histogram shows cells stained with mAbs for the specific surface markers for (CD40, CD80, CD86, MHCII, CD11c). Value on the histogram indicates the percentage of cells expressing positively the surface markers. (Representative of at least three set of experiments).


B (i)
WT SpDC
CD40 FITC
CD80 FITC
CD86 FITC
MHCII FITC
CD11c PE
Starting population
iDC (7 days + rmGm-CSF + IL-4)
mDC (9 days + rmGm-CSF + IL-4 +LPS)
0.15
20.2
2.74
72.2
29
0.12
49.8
43.9
78.9
30
31.9
89.8
76.9
82.6
86.1

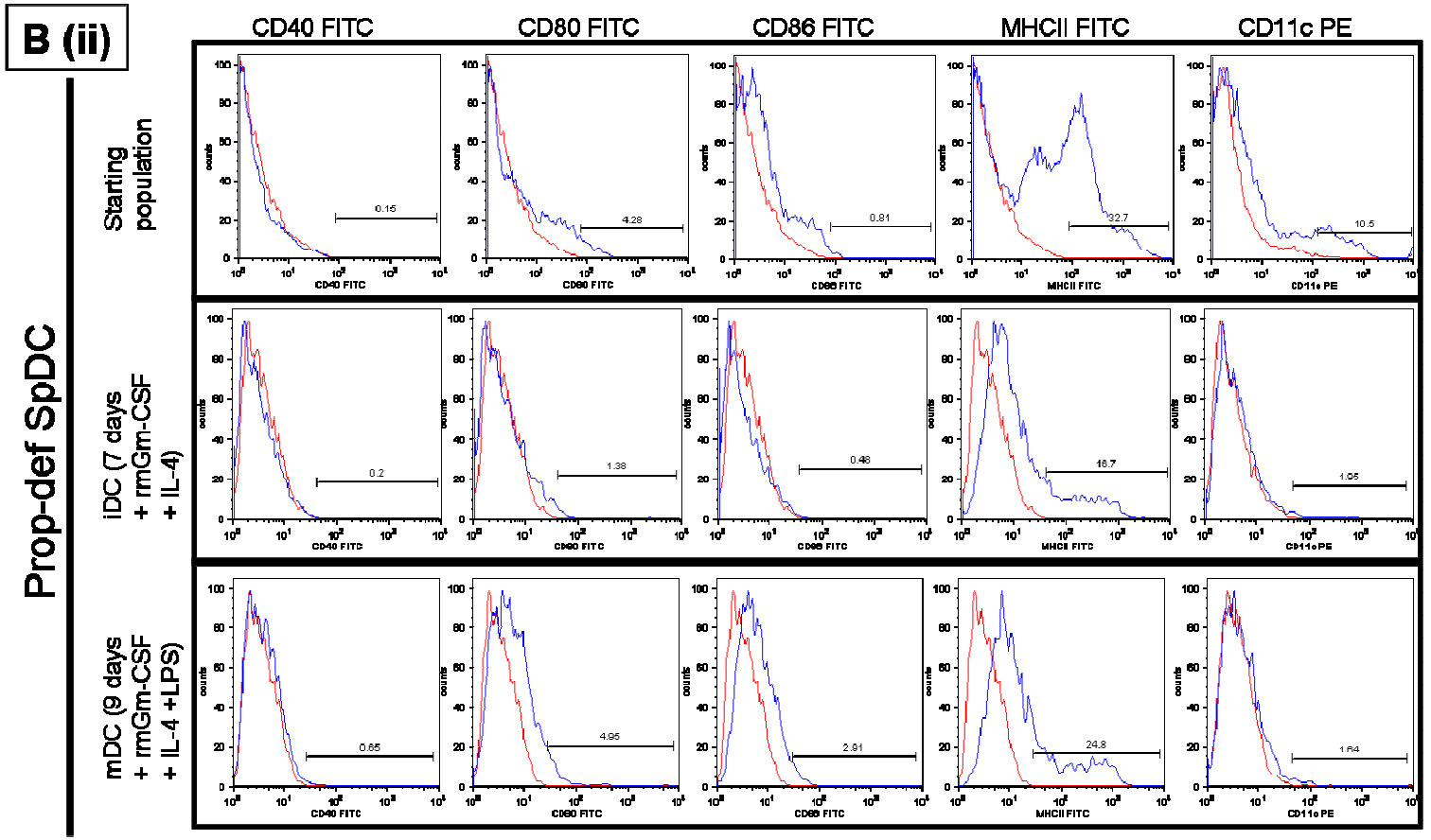


Figure 5.3 (B) : Representative FACS histogram of SPDC B(i) wild type derived, B (ii) properdin-deficient derived.
Phenotypic analysis of surface markers of dendritic cells (CD40, CD80, CD86, MHCII, CD11c) in state of start populations, inactivated immature (iDC) (7 days+rmGm-CSF+IL-4); and activated matured (mDC) (9 days+rmGm-CSF+IL-4+LPS). The open red histogram shows the profiles obtained with isotype control mAb. The open blue histogram shows cells stained with mAbs for the specific surface markers for (CD40, CD80, CD86, MHCII, CD11c). Value on the histogram indicates the percentage of cells expressing positively the surface markers. (Representative of at least three set of experiments).

5.3.2 Maturation of dendritic cells by lipopolysaccharides (LPS).

Generated dendritic cells were capable to differentiate into activated mature dendritic can be analyse by having high positive expression of cell surface marker such as CD40, CD80, CD86, MHCII and CD11c. DC exist in different functional and maturation stage upon contact with pro-inflammatory cytokines and bacteria products induces maturation.

As maturation of dendritic cells can be triggered by LPS stimulation, generated iDCs were stimulated with LPS (1µg/ml) for the next 48 hours to activate iDC becoming mDC. The phenotype of mature DC was analysed by FACS on the very last day of culture.

Although as earlier both BMDC wild type and properdin-deficient were seen capable to characterised to be a dendritic cells by FACS histogram but in need to see whether the characterised DC able to mature upon LPS induction.
Calculations of the induction for the cells to become mature cells were simply done by minus the percentage of positive cells upon the LPS stimulation (9 days) with the one without LPS stimulation (7 days) for the each dendritic cell surface marker.

Table 5.1 (A) & (B) represent percentage of cells that positive of the cell surface markers increased for maturation of mDC (9 days, rmGm-CSF+IL-4+LPS) when compared to iDC (7 days, rmGm-CSF+IL-4) after LPS (1µg/ml) induction.

In table 5.1 (A) demonstrated wild type BMDC were able to mature upon LPS stimulation as cells have shown increased of markers by nearly 50% of cells increased for marker CD80, CD86 and DC marker CD11c. Meanwhile properdin deficient BMDC only had 4% of cells increased their positivity for CD11c surface marker. LPS challenge had only a modest impact in the CD40, CD80, CD86, MHCII and CD11c expression levels of properdin deficient BMDC as shown was barely increased and comparable to the wild type derived BMDC.

In table 5.1 (B) demonstrated wild type SpDC were also able to mature following LPS stimulation. Cells of wild type SpDC have shown an increased of cells population with positive co-stimulatory surface markers of CD40, CD80, CD86 and MHCII by more than 30% but a small increased of CD11c.

In the meantime, properdin deficient SpDC culture following DC differentiation demonstrated a small increased of CD40, CD80, CD86 and MHCII and none of CD11c.

It is concluded that BMDC and SpDC from properdin-deficient mice show less maturation from wild type mice.

(A)

Phenotypic Marker	Wild Type BMDC	Prop-def BMDC
CD40 FITC	28.4%	-
CD80 FITC	42.7%	-
CD86 FITC	37.5%	-
MHCII FITC	7.8%	-
CD11c PE	49.8%	4%

(B)

Phenotypic Marker	Wild Type SpDC	Prop-def SpDC
CD40 FITC	31.78%	0.45%
CD80 FITC	40%	3.57%
CD86 FITC	33%	2.43
MHCII FITC	37%	8.1%
CD11c PE	10.1%	-

Table 5.1 Effects of LPS on DC maturation. Percentage of positive cells of dendritic cell surface markers increases for maturation when compared mDC (9 days, rmGm-CSF+IL-4+LPS) to iDC (7 days, rmGm-CSF+IL-4) derived from bone-marrows **(A)** and spleens **(B)** of wild type and properdin deficient mice after LPS (1µg/ml) induction. (Data shown are from the representative histogram).

5.3.3 Morphology assessment of the characterised dendritic cells.

Tissue culture of dendritic cells derived from bone marrow and spleen of wild type and properdin-deficient mouse were established. The cells were isolated and grown under culture conditions as previously described, which allowed direct comparisons between the cell types. The appearance of dendritic cells morphology was assessed by phase contrast microscopy.

Morphologically, immature DCs for both wild type and properdin-deficient at seven days of incubation were seen as floating cells in round shape cells and some seen with veils and irregular shape. These cells were relatively small in size (data not shown).

As maturation of dendritic cells can be triggered by LPS stimulation, generated iDCs were incubated with LPS (1µg/ml) for the next 48 hours to stimulate iDC becoming mDC. Representative cell culture micrographs (Fig. 5.4) illustrating dendritic cells after 9 days of incubation in RPMI medium supplemented with rmGM-CSF, IL-4 and LPS.

In figure 5.4 (A) & (C) shown wild type bone marrow-derived and spleen-derived culture with cells with large veils and long dendrites seen 48 hours after stimulation with LPS. Upon maturation, some of the cells were seen formed large cells aggregate. Moreover observations, cells with dendrites have become attached to the flasks. However, equivalent cultures derived from properdin-deficient mice (Fig. 5.4 (B) & (D)) show an impairment of phenotypic differentiation with less dendrite were formed. Notably, dendritic cells from

wild type mice seem to produce more dendrites portrays positive as mature dendritic cell than those from properdin-deficient mice.

It has found that both bone marrow and spleen-derived dendritic cells of wild type as expected on addition of LPS.

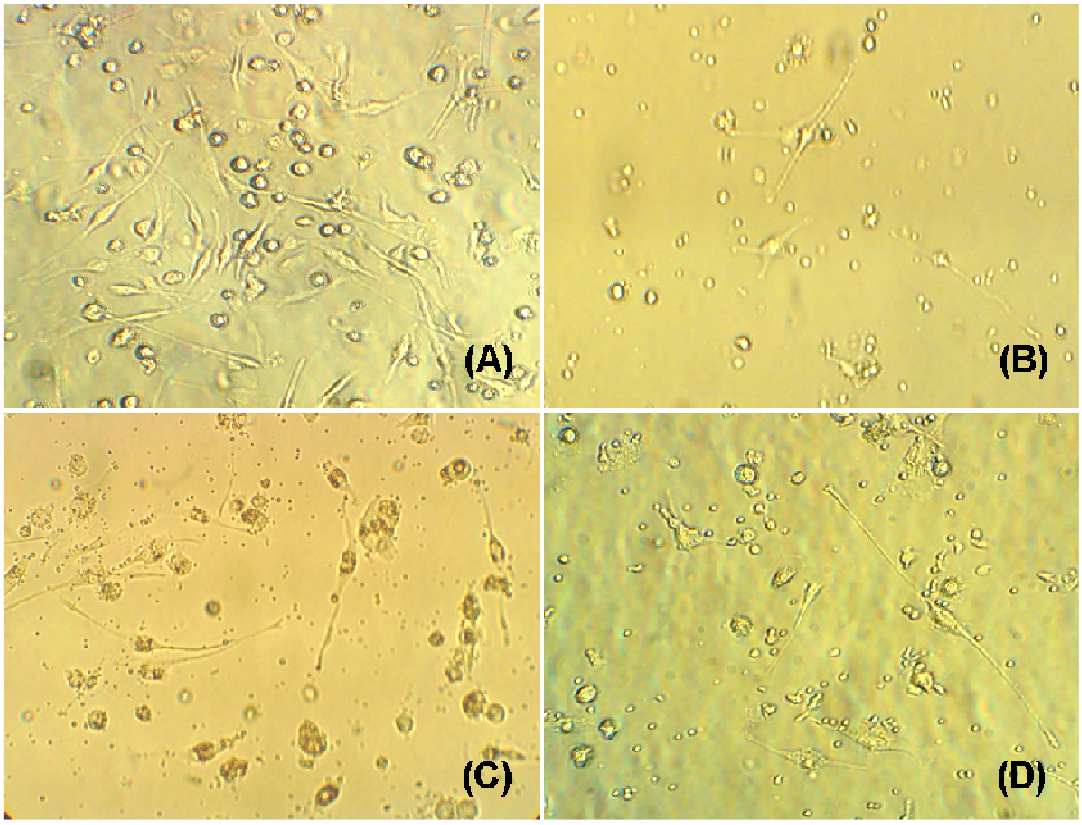


Figure 5.4: Assessment morphology of dendritic cells after stimulation with LPS.
Representative cell culture photomicrographs of (A) Wild type BMDC (B) Properdin-deficient BMDC (C) Wild type SpDC (D) Properdin-deficient SpDC.
Each frame pair represents taken with phase contrast microscopy at x200 magnification. After 9 days of incubation in RPMI medium supplemented with rmGM-CSF, IL-4 and LPS (1µg/ml), dendritic cells were morphologically polydendritic and formed long branching dendrites (A, C). In contrast, properdin-deficient cell culture (B, D) with less dendrites were formed.

5.3.4 Measurement of cytokine TNF-α secretion by characterised dendritic cells.

Dendritic cells produced different types and levels of cytokines as part of immune response to microenvironment (Steinman, 2007). Important cytokines produced by DCs are those of the IL-12 family, IL-10, IL-6 and TNF-α (Moser and Murphy, 2000, Pasare and Medzhitov, 2003a, Reis et al., 2008, Pasare and Medzhitov, 2003b, Nolan et al., 2004). Quantification of TNF-α by commercial ELISA were established from supernatant of BMDC and SpDC culture of dendritic cells derived from bone marrow and spleen of wild type and properdin-deficient mouse as previously described (section 5.2.4). Cell media RPMI only acted as a negative control.

Figure 5.5 shown TNF-α cytokines measurement produced by bone marrow-derived and spleen-derived culture supernatant of wild type and properdin deficient mice. As a negative control, cell media RPMI showed no TNF-α production.

In figure 5.5 (A) TNF-α was hardly expressed for both immature bone marrow-derived dendritic cells culture of wild type and properdin-deficient. Then, after stimulation with LPS, level of TNF-α increased significantly for both mDC of wild type and properdin-deficient. Wild type bone marrow-derived culture expressed significantly higher level of TNF-α compared to the properdin-deficient (*$P<0.05$ for iDC vs. mDC)

Similar observation to wild type SpDC culture was seen in TNF-α measurement in spleen-derived dendritic cells culture (fig. 5.5 (B)). TNF-α level from immature SpDC was expressed low for both wild type and properdin-deficient. Upon LPS induction, level of TNF-α increased significantly for both mDC wild type and properdin-deficient (*P<0.05 for iDC vs. mDC).

As seen previously maturation wild type was increased , wild type cells produced more TNF-α than properdin-deficient cells after stimulation with LPS (*P<0.05 for WT vs. Prop-def).

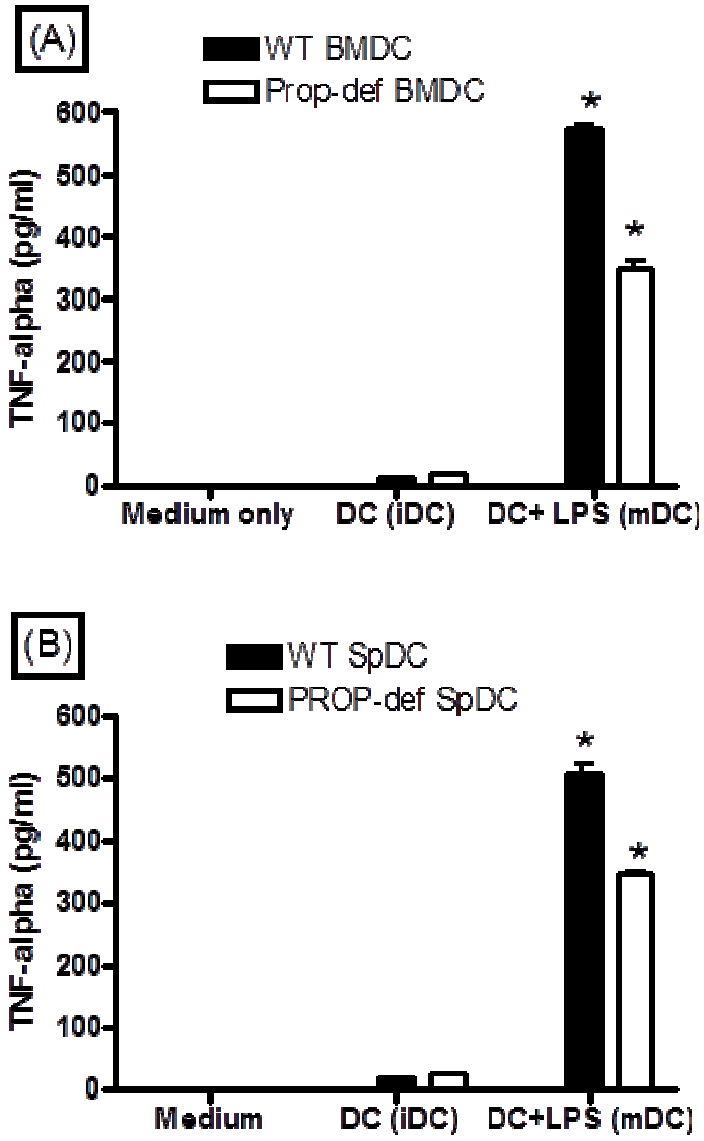


Figure 5.5: TNF-α cytokines measurement by commercial ELISA produced by bone marrow-derived (A) and spleen-derived (B) culture supernatant of wild type and properdin deficient (*P<0.05 for iDC vs. mDC, *P<0.05 for WT vs. Prop-def; Mann-Witney analysis).

5.4 Discussion

The study presented here to see relationship of complement component properdin in dendritic cells characterisation and maturation.

There are two types of DC derived; Lymphoid-DC and myeloid-DC (Banchereau et al., 2000, Cella et al., 1997, Steinman et al., 1997) which is differs in primary precursors, cytokine requirements, phenotype, surface marker expression, locations and functions (Leenen et al., 1998).

Dendritic cells of hematopoietic origin can be derived from primary lineages as pluripotent precursor cells of the bone marrow, blood-derived monocytes, or from secondary lymphoid organs thymus or spleen (Inaba et al., 1992, Shortman and Liu, 2002) and can be generated *in-vitro* by culture in medium supplemented with serum, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Inaba et al., 1992)and IL-4 (Sallusto and Lanzavecchia, 1994).

For the first time in the laboratory, a protocol for the generation of dendritic cells has developed. DCs were successfully purified and differentiated *in vitro* from bone marrow and spleen of wild type and properdin-deficient mice in induction of serum, growth factors and cytokines. Immature DCs are initially generated in the presence of rmGM-CSF and IL-4 with 7 days culture. Additional culture with LPS, rmGM-CSF and IL-4 for next 48 hours results in a maturation of the DCs.

5.4.1 Phenotypic analysis of characterised dendritic cells

Dendritic cells phenotype can be characterised by expression of DC marker-CD11c, MHCII, DC-SIGN molecules and co-stimulatory markers; CD40, CD80, and CD86 (Sallusto and Lanzavecchia, 1994, Banchereau et al., 2000). LPS was shown to upregulate dendricity and DC activation (Banchereau et al., 2000, Banchereau and Steinman, 1998, Reis et al., 2008).

DCs were capable of differentiation into mature DCs as reflected by the upregulation of DC markers. As reviewed in Banchereau et al., 2000 (Banchereau et al., 2000), immature and mature DC differ in phagocytic activity, cytokine production and expression of surface marker.

In cell differentiation into DC, DC phenotype is associated with the increase of the CD11c marker. CD11c is known as major DC marker as a key to distinguish DC from macrophages (Metlay et al., 1990). CD11c marker expression on the cell surface in mouse study showed extensively expressed and induced during inflammation functioning as an endocytic receptor (Bullard et al., 2007). Meanwhile expression of co-stimulatory markers and MHCII are widely known as characteristics to differentiate immature and mature DC. Again as mentioned earlier, immature DCs express low MHCII and co-stimulatory markers and portrays in an activity of antigen-capture. High levels of expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules including CD80, CD86 and CD40 considered contributing to the efficiency of DCs as APCs.

5.4.2 The findings on the bone marrow-derived dendritic cells

DC originating from BM cells yield homogenous DC colonies enters the blood as progenitors and colonised from blood stream, lymphoid and non-lymphoid (Winzler et al., 1997). In mouse bone marrow cultures, immature DCs were found to phagocytose particles including bacteria and lost this capacity upon migration and maturation of DC in secondary lymphoid tissues (Inaba et al., 1993, Lutz et al., 1999). Mature DCs are recognised as the most potent APC and the considered a key determinant of the outcome of T cell activation leading to T cell tolerance or T cell immunity.

Looking at expression as expected, iDC of wild type bone marrow-derived presented lower expression levels of markers CD40, CD80, CD86, MHC class II and CD11c molecules when compared to mature DC (Fig. 5.3A (i) and Table 5.1A). The immunophenotypic profile consistent with a mature DC phenotype was expressed by WT BMDC following maturation protocols with characteristics typical of mature DCs defined by an increased of surface phenotype marker CD11c and increased of co-stimulatory molecules such as CD40, CD80, CD86 and MHC class II (Fig. 5.3A (i) and Table 5.1A). along with dendritic morphology (Fig. 5.4A). The study presented here showed that wild type BMDC expressed positive CD11c and made them clearly distinguishable from iDC (Fig. 5.3A (i) and Table 5.1A).

The findings of wild type BMDC are consistent with previous studies. Similarly findings seen in normal mouse BMDC by (Mascanfroni et al., 2008) and mouse BMDC by (Lutz et

al., 1999) with high expression of MHCII and CD11c together with dendritic morphology seen upon LPS stimulation.

In comparison to wild type BMDC, properdin deficient BMDC presented fewer expression of markers CD40, CD80, CD86, MHC class II and CD11c (Fig. 5.3A (ii) and Table 5.1A). The CD maturation markers were hardly expressed following DC differentiation and the maturation of properdin-deficient is less compared to the wild type (Table 5.1A) with less dendrite morphology of properdin-deficient SpDC in culture (Fig.5.4). Overall, properdin deficient BMDC did not show positive characteristic as mature dendritic cells.

5.4.3 The findings on the splenic dendritic cells

The spleen is a secondary lymphoid organ, which filters antigens and apoptotic cells from blood by splenic marginal zone dendritic cells. This efficient function is thought to be dependent on complement receptors and effect on cytokine production (Tan and O'Neill, 2005). Mature DCs in secondary lymphoid tissues, such as lymph nodes and spleen, have a lower capacity to capture antigens, but are efficient in antigen presentation and stimulating naïve T cells.

There are two subpopulations of DC in mouse spleen characterised by a difference in location, phenotype, phagocytic ability. DCs are predominantly located at the border of marginal zone and splenic red pulp, as marginal DC. Meanwhile a minor population is located in the white pulp zone known as interdigitating DC (Leenen et al., 1998).

It is thought that functional DC maturation ends by apoptotic cell death and no reversion to iDC (Winzler et al., 1997). In contradiction, studies have found splenic DC was under steady state condition. It is shown DCs in the spleen would also be capable of responding to infection by maturation via activation from danger signals similar during steady-state conditions and during infection. Upon LPS stimulation *in vivo*, marginal DC was found by expressing macrophages marker and able to phagocytose as typical function of macrophages (De Smedt et al., 1996). Marginal DC was then found migrated to T cell areas of spleen when LPS *in vivo* administration induces maturation (Leenen et al., 1998) and also found migrated from spleen to the liver into hepatic lymph when studied rat phagocytosed *in vivo* upon i.v latex particles (Matsuno et al., 1996). It is thought that pathogen signals would stimulate splenic DCs to migrate to the T cell area of the spleen and T cells is then stimulated by activated DCs. This would prime T cells to form effector T cells. In addition DCs may also become activated by inflammatory cytokines released by activated DCs and take an additional part in T cell priming (Tan and O'Neill, 2005).

This study reveals that spleen-derived cells in the thesis have positive marker for dc as spleen is a main location for mature DC.

As shown (Fig.5.3 B (i) and Table 5.1B, expression of CD11c of wild type SpDC increased upon LPS stimulation Wild type SpDC seems undergo steady state condition as mentioned earlier. As expected as typical spleen DC stages, the expression showing as mature DC on starting population with high MHCII and CD11c. However expression marker decreased on iDC stage and then upon LPS stimulation increase again CD40, CD80, CD86, and CD11c but lesser expression of MHCII to that starts population.

The data indicated that current study generates comparable populations of SpDC to those obtained by others in previous finding. Leenen group studies (Leenen et al., 1998) found freshly isolated SpDC to express high MHCII and N418/CD11c and showed decrease to increase expression of markers upon culture.

In contrast spleen-derived DCs from properdin-deficient mice were markedly impaired in their up-regulation of DC markers compared to spleen-derived DC from WT mice. The properdin-deficient SpDC fewer in mature expression on starting population but then increase expression during iDC and did not show changes upon LPS. This indicated properdin-deficient did not show positive characteristic as mature dendritic cells in keeping with less dendrite morphology (Fig. 5.4(D)). Interestingly as a novel finding, especially by looking at CD11c properdin-deficient SpDC showed significantly less of CD11c at start population and mDC compared to wild type (Fig. 5.3(B)). Possibly properdin-deficient SpDC has impaired in function and going through apoptotic instead undergo steady state condition resembling wild type SpDC.

5.4.4 Cytokines TNF-α production

Dendritic cells produced several essential cytokines produced are such as the IL-12, IL-10, IL-6 and TNF-α (Moser and Murphy, 2000, Pasare and Medzhitov, 2003a, Reis et al., 2008, Pasare and Medzhitov, 2003b, Nolan et al., 2004) in response to microenvironment which are important in the elicitation of an immune response.

In current study, TNF-α was hardly expressed for both immature bone marrow-derived and spleen-derived dendritic cells culture of wild type and properdin-deficient ((fig. 5.5(A) &

(B)). Level of TNF-α was increased after stimulation with LPS for both mDC bone marrow-derived and spleen-derived of wild type (fig. 5.5 (A) & (B)). Surprisingly both of properdin-deficient mDC and SpDC were able to secrete TNF-α by induction of LPS. However both wild type BMDC and SpDC produced more TNF-α than properdin-deficient. It is thought that wild type-derived BMDC should have more TNF-α because of the stabilising effect of properdin effecting greater signalling of Tlr-mediated TNF-α release. TNF-α level was measured in the Ivanovska et al (Ivanovska et al., 2008) showed the levels seemed to correlate with survival outcome. Comparable to the finding (Ivanovska et al., 2008) of TNF-α of *in vivo* LPS model of properdin-deficient and wild type mice, while current study was *in vitro* study and differences (TNF-α) are likely due to preactivation of cells analysed *ex vivo* in this paper.

LPS stimulates a variety of cells to produce cytokines and chemokines, for example GM-CSF, TNF-a, IL-1, MIP-1a and b. (Banchereau and Steinman, 1998). These products are known to modulate DC movement and maturation. In communicating with T cells, TNF-α can be produced by T cells (Lutz et al., 1999)instead the DC itself. These may influence the presented findings of properdin deficient culture as measurement of TNF-α were obtained from supernatant of the culture.

5.4.5 Limitations of the study

Other than time constraint, the current study has to encounter several limitations. As a primary culture, DCs are difficult to maintain in culture due to contamination and short lifespan. The study was a time consumed as all the culture work and analysis were done in parallel. Trial of ELISA to measure IFN-γ than TNF- α were in performed but unfortunately could not be proceed due to some technical error, limited sample availability. It is a shame could not proceed to measure DC important cytokines production such as IFN-γ or IL-12. In addition, study by Tan and O'Neill (Tan and O'Neill, 2005) stated that limitations and difficulties of the experiment are during generation of immature DCs which impacts on capacity to differentiate to other stage of maturation and limitations to create *in vitro* totally similar environment as *in vivo*.

Method of separation of generating DC by sedimentation and based on adherence-non-adherence separation that used could limit the study as major contaminant were granulocytes that also respond to growth factor GM-CSF stimulation (Lutz et al., 1999) and less obtained purified DC as probably risk of loss expanding DC. Method of separation can be improved to obtain more purified DC and larger volume of cells by magnetic separation technique which is more costly.

5.4.6 Properdin essential to allow dendritic cells to develop their activated phenotype

In findings, wild type bone marrow and spleen derived DC culture shows positive as mature dendritic cells however properdin deficient culture did not show positive characteristic as mature dendritic cells. The data so far suggest that have identified properdin as another relevant player in differentiation as well as maturation of bone marrow-derived and spleen-derived dendritic cells, based on the differences obtained using cells purified from wild type and properdin-deficient littermate mice.

The study finding presented here could lead the answer to the questioned of susceptibility and prone to diseases amongst properdin-deficient patient which properdin-deficient patient may impair in their dendritic cells that probably due to DC amount or impaired functional DC. In suggestion, perhaps in future properdin-deficient patient can go for genetic screening for screening of functioning DC.

The findings shed new light on determinants for the properdin-deficient mice in septic models of disease. The mechanism by which properdin, a secreted protein, achieves this, needs to be investigated further. Therefore the study presently concludes that the presence of properdin is essential to allow dendritic cells to develop their activated phenotype and properdin is a relevant player in dendritic cell mediated immune response.

Chapter 6: The role of properdin in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG

6.1 Introduction

The complement system plays a role in defence against pathogen and also involved in the role is dendritic cells as antigen presenting cells. In relation, it is interesting to study mycobacteria-dendritic cells interaction in relation with the complement pathway.

6.1.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis. Mtb are facultative intracellular pathogen and non-motile organism also known as acid-fast bacteria as retaining the carbolfuchsin dye during the Ziehl-Neelsen staining. Mycobacterial cells exist in rods or slightly bacilli in shape.

The mycobacterial cell surface is made up of lipid bilayer covered by a peptidoglycan. Attached to the peptidoglycan are the cell wall made of arabinogalactans, arabinomannans, glycolipids and mycolic acids (Carroll et al., 2009, Daffe and Draper, 1998). The complex characteristic of cell wall with extraordinarily high lipid content contribute cell hydrophobic to solutes (i.e. acids, alkalis) and gave protection to the cell in escaping immune system killing mechanism and drug treatments (i.e antibiotics resistant).

6.1.2 Modulation of immune response: Mtb and the dendritic cells

Dendritic cells functioning as antigen presenting cells (APC) play a role to activate T cell responses in initiation of specific immune responses to pathogens.

The DC is activated through the release of cytokines i.e IFN-γ by activated T cells or through action of TLR (transmembrane proteins that recognise microbial products in triggering the host defence mechanisms)(Hickman et al., 2002).

Mycobacterium is able to enter the DC through a number of different receptors (such as Fc receptor, TLR and mannose receptor) and also invades the host system by phagocytosis then resides inside the phagosome (a membrane bound cytoplasmic vacuole) of DC of the host (Bodnar et al., 2001, Amer and Swanson, 2002). As intracellular bacteria, mycobacteria are resistant to direct complement attack, escape antibody-mediated process as antibody cannot diffuse into the phagocytic cell and are able to survive in phagocytic cells (Carroll et al., 2009).

Once inside the cell in a phagosome only some are killed however others still viable and multiply. Mtb make use of DC as immunity or escape target (Martino, 2008) by remaining in the phagosome. They are capable of preventing phagosome-lysosome fusion, which protects the bacteria in the phagosome from destruction (Carroll et al., 2009). These are overcome by the DC when it becomes activated by the innate immune response. Activation allows the DC to overcome Mtb replication. DCs are unable to kill the mycobacteria efficiently compared to macrophages, which are able to eliminate the Mtb but DC could inhibit their growth (Bodnar et al., 2001).

Mycobacterium leads to DC maturation and migration to secondary lymphoid organs thus initiating priming of immune responses to *M. tuberculosis* (Mtb). Mtb in turn has adapted strategies and evolved further mechanism to survive within DC. This permit evasion by utilise of antimycobacterial products released by the DC (Tufariello et al., 2003)and arrest the development immune response (Russell et al., 2007) thus prevent DC activation. DCs may serve as a reservoir for *M. tuberculosis* in tissues, and the viable *M. tuberculosis* could use dendritic cells as transportation to migrate to the lymph node (Bodnar et al., 2001) leading to their persistence within the cells.

It has been demonstrated that DCs internalize various microbes including *Listeria monocytogenes* (Guzman et al., 1996) and *M. bovis* BCG (Inaba et al., 1993).

Previous findings showed that mycobacteria may alter the functions and phenotype of DCs. DC showed a mature phenotype after peripheral blood-derived DCs (human) phagocytosed Mtb (Henderson et al., 1997). In contrast other studies showed impairment in immune response by delayed development of CD4+ T cell responses, lack of CD11c (Tian et al., 2005) and less MHCII presentation (Wolf et al., 2007) in demonstration of DC contained Mtb in human (Tailleux et al., 2003) and mouse tissues in *in vivo* (Humphreys et al., 2006).

Following mycobacterial infection also seen to alter cytokines production which is important for the interaction with T cells and modulation of immune responses by DCs in the production of antimycobacterial immune response. Mycobacterial infection of DC results in the upregulation of the IL-2, IL-1, IL-6 and TNF-α (Henderson et al., 1997, Hickman et al., 2002).

6.1.3 Relationship of complement pathway in mycobacterial infection

Previous studies have demonstrated that *M. tuberculosis* and *M. bovis* BCG can activate complement and enter into the host cell may be facilitated by interaction with complement receptors (Carroll et al., 2009, Schlesinger, 1993, Schlesinger et al., 1990).

Briefly reviewed in Schlesinger 1998 (Schlesinger, 1998) mentioned mononuclear phagocytes secreted complement proteins and presence of complement activation via complement receptors CRl, CR3 and CR4 helps enhanced Mtb phagocytosis leads to opsonisation of the bacterium with the C3 activation products C3 b and C3 bi. Then the activation of the classical and alternative pathways resulted in the deposition of C3b on the surface of the bacterial cell.

Comparable findings to Mtb, previously shown *M. bovis* BCG activates complement via both classical and alternative pathways in which also resulting the deposition of C3b on the surface of the bacterial cell. Activation via the classical pathway is shown by C1q binding in *in vitro* BCG infected serum and demonstration by MBL and L-ficolin binding to the bacterium in *in vitro* BCG infected serum has been demonstrated indicating lectin pathway activation (Carroll et al., 2009).

Here needs to go a rationale, why complement deficiency was evaluated to cellular reaction to Mtb e.g: CR3/Mtb (Melo and Stokes, 2000), C5a/BCG (Moulton et al., 2007), CR3/ Mtb (Hu et al., 2000), C3/ *M. avium*(Bohlson et al., 2001).

The aim of this study is to investigate further function of generated BM-derived DC of wild type and properdin-deficient mice towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

6.2 Materials and methods

6.2.1 Infection of Dendritic cells with Mycobacterium tuberculosis H37Rv and Mycobacterium bovis

This work was carried out in collaboration with Dr. Galina Mukamolova (Department of Infection, Immunity and Inflammation, University of Leicester). *Mycobacterium tuberculosis H37Rv* is an ACDP category 3 hazardous pathogen and all work with this bacterium was carried out in a Class 1 or Class 2 microbiological safety cabinet, within the Containment laboratory suite and in accordance with the suite's Code of Practice.

Mycobacteria used for infection

Mycobacterium tuberculosis H37Rv or *Mycobacterium bovis* (BCG, Glaxo strain) were grown in 7H9 medium containing 10% v/v OADC supplement (Becton, Dickinson and Company) and 0.05% w/v Tween 80 at 37ºC with shaking to mid-exponential phase. Bacteria cells were centrifuged at 2000g for 20 min, washed twice in warm medium (RPMI) and resuspended in the same medium. Bacterial aliquots determined at $5x10^6$ cells/ml were frozen and kept at -80 ºC. For infection, bacteria cells were thawed, centrifuged and washed 2 times with fresh RPMI medium. Bacterial suspensions were passed though 23G gauge needle 10 times to disperse clumps.

6.2.2 Bone marrow-derived Dendritic Cell (BMDC) Culture

Dendritic cells of wild type and properdin deficient mice were prepared in parallel as outlined in materials method chapter 5.2.2. For this experiment, medium without antibiotics was used.

The cell concentration of the cultures was adjusted $1.0x10^6$ cells/ml and cells were placed in triplicate into 24 wells plate (1ml/well) prior to the infection step.

6.2.3 Infection of dendritic cells

Infection of the dendritic cells (DC) was performed similarly as previously described (Bodnar et al., 2001) with some adjustments such as days of DC culture preparation and number of multiplicity of infection (MOI) of mycobacterium.

After culture for 7 days, dendritic cells (both wild type and properdin deficient-BMDC in parallel) (at 10^6/ml/well in triplicate in RPMI medium plus rm-GM-CSF+IL-4) were infected in 24 well plates with *Mycobacterium tuberculosis H37Rv* or *Mycobacterium bovis*, BCG at a multiplicity of infection (MOI) of 5 to 10.

The plate was left for incubation at 37°C in 5% CO_2 incubator in CAT3 suite. The infected cultures were left incubated at various time point of post-infection.

At time point after 24hour incubation (post-infection), extracellular bacteria (floating bacteria) were separated from cells by low speed centrifugation (300g for 10 min); DCs

attached to well bottom were washed with the medium 3 times. Non-attached infected DCs were pooled together and then 1ml aliquots of cell suspension were placed in wells with "washed" attached DC.

The infected cells were resuspended in final volume of RPMI and the washings were saved and pelleted at 2000xg for 15minutes. The resulting pellets were resupended in medium (extracellular Mtb).

Along with the all the experimental setup, cultures of dendritic cells (without any infection) in RPMI act as a control (to check any contamination / to monitor health of cells).

6.2.4 Intracellular load of mycobacterium within bone marrow-derived-dendritic cells

Samples for quantification of intracellular bacteria were taken at the end of the infection period after removal of extracellular bacteria. After removal of supernatants, lysis of cells was performed by the addition of 0.06% w/v SDS and shearing through a 23G needle. The intracellular load of Mycobacterium within the dendritic cells was determined by CFU count.

Colony-forming unit count

Colony counts were carried out by the drop plate method, which is a modified version of the Miles and Misra method.

Ten-fold serial dilutions of broth culture or cell suspension were made in aliquots. DC lysates were serially diluted in 7H9 supplemented medium. Four 10 µl drops for each dilution were spotted on duplicate 7H10 supplemented agar plates (Becton, Dickinson and Company). After complete absorption of liquid, the plates were sealed with sealing film (Nescofilm, Osaka, Japan), inverted and incubated in double bags at 37°C for up to 3 weeks. Individual colonies were counted in appropriate dilutions. Colonies were counted using a dissection microscope at 4xmagnification.

Final counts of colonies were used for the final calculation of CFU shown below:

CFU/ml= (average colony count per 10µl drop) x (dilution factor) x50

6.2.5 Determination of TNF-alpha production

Quantification of the cytokines TNF-alpha produced by cultured cells was analysed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (BD OptEIA™ Pharmigen, UK). The ELISA was carried out according to the manufacturer's protocol as performed in Chapter 5.2.4.

For the determination of TNF-alpha in culture fluid, supernatants from mycobacterium-infected DCs were collected at different times post-infection, filtered (0.2µm pore size, Millipore, Hertfordshire, UK) and stored at -80°C.

6.2.6 Ultrastructural analysis of infected murine dendritic cells

Electron microscopy was carried out at the Electron microscopy unit (School of Biological Sciences) at the University of Leicester with the technical expertise from Stefan Hyman and Natalie Allcock

Preparation and use of fixatives and stains during specimen processing was carried out in dedicated fume hoods and in accordance with the COSHH guidelines, due to the hazardous nature of chemicals used.

Procedure for processing Mycobacteria for electron microscopy:

Methods of fixation were adapted from Sharbati-Tehrani et al (Sharbati-Tehrani et al., 2005).

For transmission electron microscopy (TEM) infected or uninfected DCs as described (see 6.2.3) were harvested by spun down at 250g for 5 minutes and washed twice in PBS prior to being pelleted.

There, cells were fixed in 2.5% (v/v) glutaraldehyde/0.05M HEPES ph7.2 for 1 h at room temperature and kept at 4°C. Then the fixed samples were sent to the Electron microscopy Laboratory.

After 3 washes in 0.05M HEPES with 10 minutes each wash, cells were post-fixed in1% osmium tetroxide in 0.05M HEPES for 90minutes, thoroughly washed with distilled de-ionised water for 3 times for 10 minutes each wash.

The samples were dehydrated through a graded ethanol series (70%, 90% and 100% ethanol). The 100% ethanol was replaced with propylene oxide (PO) twice for 15 minutes each and then transferred through changes of PO: agar low viscosity resin mix with 3:1 ratio, 1:1 ratio and lastly with 1:3 ratio for 30 minutes each changes. Then the PO: agar low viscosity resin mix -infiltrated samples were transferred to 100% agar low viscosity for overnight. The resin was changed the following day to remove any traces of PO. Finally the samples were filled with fresh agar low viscosity resin and the resin was polymerised at 60°C for 5 hours to produce a solid block for ultrathin sectioning.

The embedded samples were then cut into ultrathin sections (approximately 80nm) using Reichert Ultracut S ultramicrotome (Leica Microsystems). Sections were collected onto copper mesh electron microscope grid (Athene, Agar Scientific), stained with 2% aqueous uranyl acetate followed by Reynolds' Lead citrate (Reynolds 1963). Then the grids were observed and recorded using JEOL 1220 Transmission Electron Microscope using an accelerating voltage of 80kV. Digital images were recorded at various magnifications using SIS Megaview III Digital Camera (Olympus-SIS).

6.2.7 Statistical analysis

Statistical analyses were performed using InStat3.0 and GraphPad Prism 4 (GraphPad Software Inc., USA). Data are expressed as mean±SD (standard deviation). Mann Whitney non-parametric test was used. P-value was set at 95% confidence and differences with $P \leq 0.05$ were regarded as significant.

6.3 Results

6.3.1 Determination of mycobacterial viability within murine bone marrow-derived dendritic cells during the course of in vitro infection.

6.3.1.1 Infection of dendritic cells with *Mycobacterium tuberculosis*

BMDC prepared from properdin-deficient and wild type was infected with *Mycobacterium tuberculosis* H37Rv in RPMI culture medium free of antibiotics. Viable intracellular bacteria within the dendritic cells were quantified by serial dilution and colony counting.

Figure 6.1 presented data of three independent experiments of viable count of intracellular *Mycobacterium tuberculosis* H37Rv within bone marrow derived-dendritic cells of wild type and properdin-deficient at different time point of post-infection. Each measurement of the CFU count comes from an individual infection performed in triplicate for each time point. Bone marrow-derived dendritic cell cultures were infected at time point zero. Presences of mycobacterium at 24 hours were less than infecting dose in all experiments.

In Figure 6.1A, for wild type BMDCs at 24 hours post infection, initial mycobacterium was taken up at 1.9x 10^5 CFU/ml. The number increased massively to 7.1 x 10^6 CFU/ml at 72 hours post-infection. This was followed by a steady increase next two time points in fact till 240 hours and then the number of bacteria in the BMDCs declined at 288hours post-infection. In parallel Figure 6.1A, there was no difference observed in properdin-deficient BMDC. There appeared to be more counts from properdin-deficient BMDC as start uptake at 3.0 x 10^5 CFU/ml but were not statistically significant.

Figure 6.1B, experiment is repeated as before but with shorter post-infection period until 168 hours. An obvious difference in uptake were seen as a relatively higher uptake at 24 hours post infection, CFU count of *M. tuberculosis* start at x10^6 for the both wild type and properdin-deficient infected BMDC. It is appeared the uptake is present but low in number.

Mycobacterium was taken up at 4.5x 10^5 CFU/ml by wild type BMDCs at 24 hours post infection (Figure 6.1C). The number increased to 2.3 x 10^6 CFU/ml at 72 hours post-infection and followed by a steady increase next two time points.
A relatively higher uptake for properdin-deficient BMDC, viable bacterial cell number start at 8x 10^5 CFU/ml at 24 hours and reached plateau at 96 hours with 4.9 x 10^6 CFU/ml (Figure 1C).

Following three experiments of dendritic cells culture infected with *M. tuberculosis* it is conclude that properdin has no role in intracellular survival of mycobacterium.

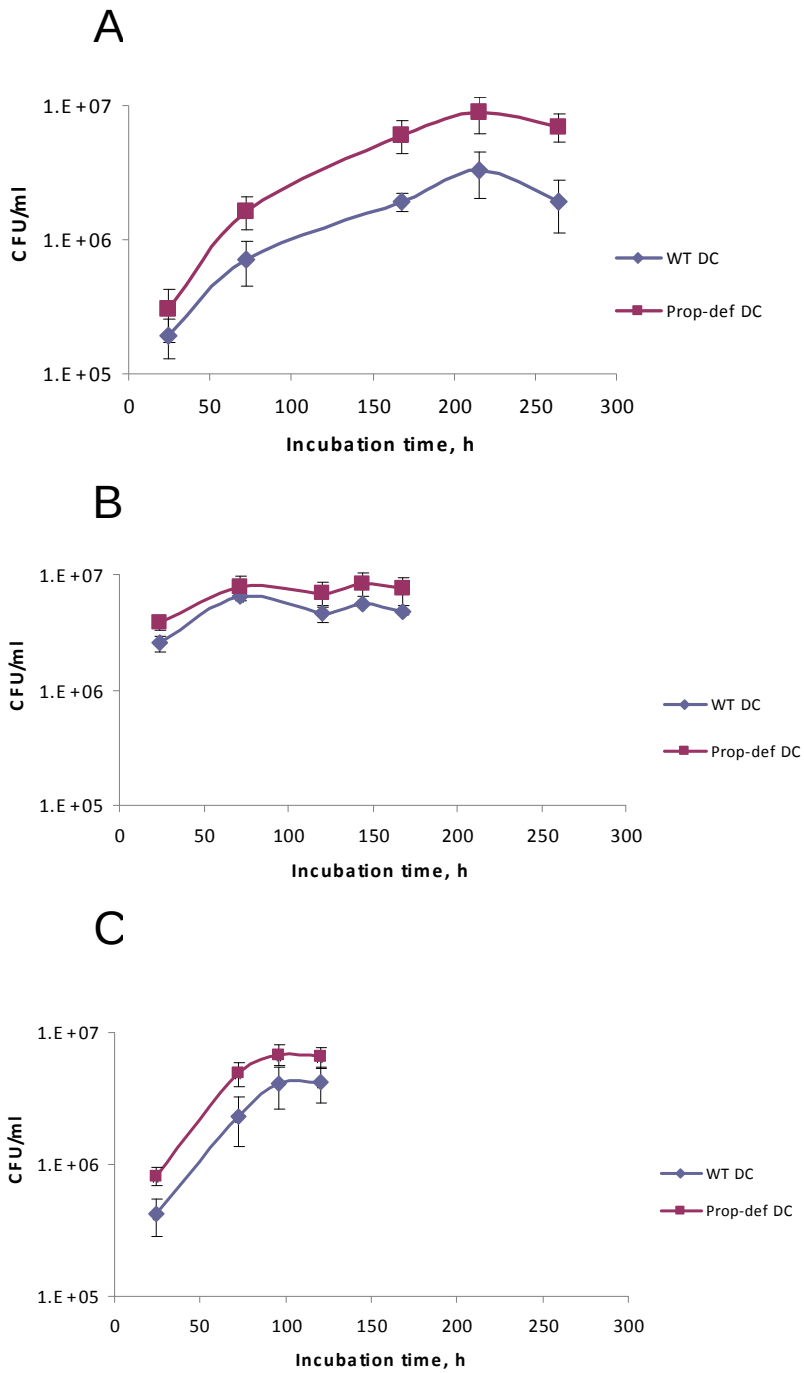


Figure 6.1: Number of intracellular *Mycobacterium tuberculosis* H37Rv within bone marrow derived-dendritic cells of wild type and properdin-deficient mice measured by the colony-forming unit assay of culture lysates at different time point of post-infection. Time point zero is time point of infection. Data represent the mean ± SD counts from three separate well. Figure 6.1A, B and C shows three independent experiments respectively.

6.3.1.2 Infection of dendritic cells with Mycobacterium bovis BCG

In the series of experiment, BMDC derived from properdin-deficient and wild type mice were infected with *Mycobacterium bovis BCG*. Again, same as infection with *M. tuberculosis,* culture from BMDC derived from properdin-deficient and wild type mice were infected with *Mycobacterium bovis* (BCG). Lysates were obtained and CFU counts were carried out.

Bone marrow-derived dendritic cell cultures were infected at time point zero. However for technical reason, the experiment only able to have 24 hours time point.

The graph shows result of infected BMDC culture with *M.bovis BCG*. WT BMDC showed at 24 hours post infection, mycobacterium was taken up at 5x 10^4 CFU/ml and properdin-deficient BMDC bacteria load was at mycobacterium was taken up at 7.5x 10^4 CFU/ml. Nevertheless, there are no significant difference between properdin deficient-BMDC and the wild type.

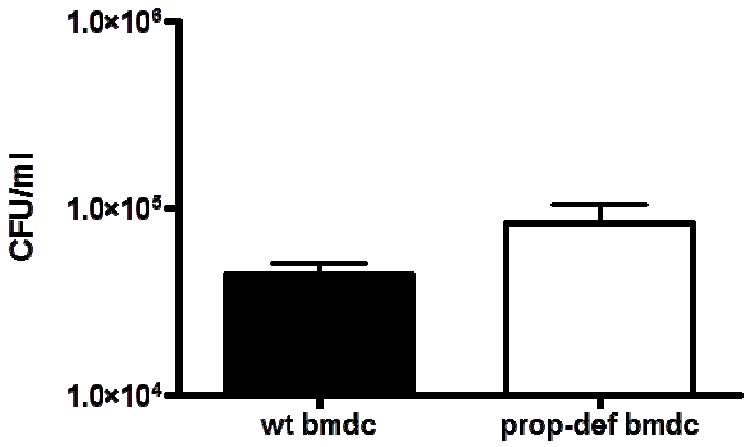


Figure 6.2: Viable counts of intracellular *Mycobacterium bovis BCG* within bone marrow derived-dendritic cells of wild type and properdin-deficient mice measured by the colony-forming unit assay of culture lysates at 24 hours post-infection. Data represent the mean±SD counts from three separate experiments.

6.3.2 Level of cytokine TNF-α by BMDC infected with mycobacteria.

In order to measure cytokine that involved in mechanism in this infection, supernatants from mycobacterium infected DCs were harvested post-infection and commercial sandwich ELISA was used to detect inflammatory cytokine TNF-α in the supernatants.

Figure 6.3, medium control shows no TNF detected and non-infected DCs produced insignificant amounts of TNF-α.

After infection with *M. tuberculosis* at 24 hours, both *M. tuberculosis* infected WT and properdin-deficient BMDC produced significantly higher TNF-α levels compared to controls; medium RPMI and non-infected BMDC (Figure 6.3A).
There were no difference of TNF-α production between wild type and properdin-deficient BMDC.

In addition, TNF-α produced highly by *M. tuberculosis* - infected BMDCs at 24hours post-infection, seen with a decreased production over time yields by other measurements. At 120 hours TNF-α level for both WT and Properdin-deficient drop down approximately with 40 to 70 folds compared to the 24 hours post-infection with production of TNF-α at ±18pg/ml and ±9.8pg/ml of TNF-α respectively (data not shown).

In figure 6.3B, lower levels of TNF-α is made for BCG infected BMDCs (both wild type and Properdin-deficient) compared to Mtb infected BMDCs.

Both WT and Prop-deficient BMDC BCG infected produced significantly high TNF-α levels compared to controls; medium RPMI and non-infected BMDC.

Wild type BMDC infected with BCG produced 375pg/ml of TNF-α meanwhile properdin-deficient BMDC- BCG infected produced 190pg/ml of TNF-α and there were no significant difference of TNF-α production between wild type BMDC and properdin-deficient.

In comparison for both *M. tuberculosis* and BCG challenged BMDCs a relatively higher TNF-α levels measured when infected with *M. tuberculosis* compared to *M. bovis* BCG. In overall levels, MTb infected BMDC are 4-5 folds higher than those measured after infection with BCG. This goes along with BCG being a more attenuated strain.

Levels of TNF-α could merely reflect bacterial load in cells (fig. 6.1 and 6.2).

It is concluded that properdin has no role in TNF-α secretion after infection *M. tuberculosis* and *M. bovis* BCG.

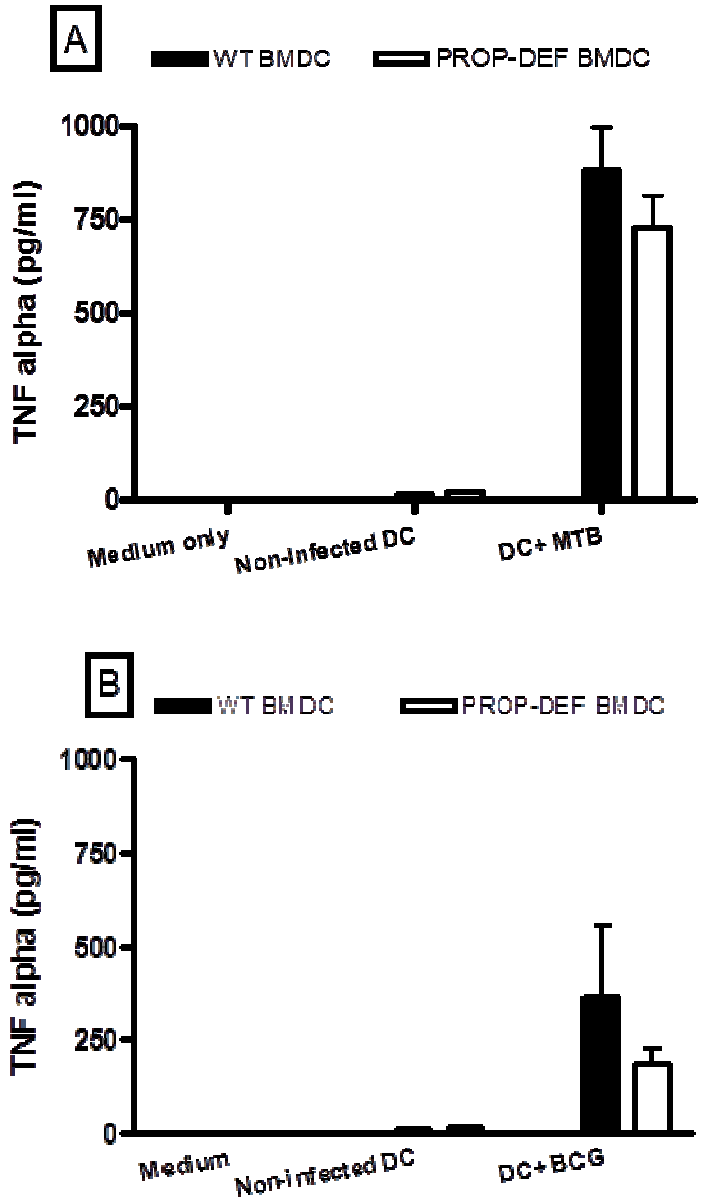


Figure 6.3: Levels of TNF-α in supernatants of infected BMDC, (A) infected with *M. tuberculosis* and (B) with *M. bovis* BCG were measured by ELISA at time point 24 hours post-infection. Medium RPMI was included in the as a control to substract background reading and to confirm no cytokines production comes from the medium. Data represent the mean±SD counts from three separate experiments. ($P \leq 0.05$, non-infected DC *v.s* infected DC, Mann-Witney non-parametric analysis).

6.3.3 Ultrastructural analysis of Mycobacterium bovis (BCG) infected BMDC.

Transmission electron microscopy (TEM) was used to examine infected dendritic cells as the TEM could demonstrate distinct ultrastructural features of the infected DCs.

Samples were prepared for TEM following the protocol set out in the Materials & methods section. For this TEM examination, only *Mycobacterium bovis* (BCG)-infected dendritic cells with 24hours post-infection were able to be carried out. Figure 6.4 shown representatives of transmission electron micrographs showing *Mycobacterium bovis* (BCG) – infected BMDC.

Non-phagocytosed free mycobacterium was seen within the culture with bacilli in shape. There were seen a lots of electron dense bacteria (dark intense bacilli) and electron less dense ones (figure 6.4(A)).

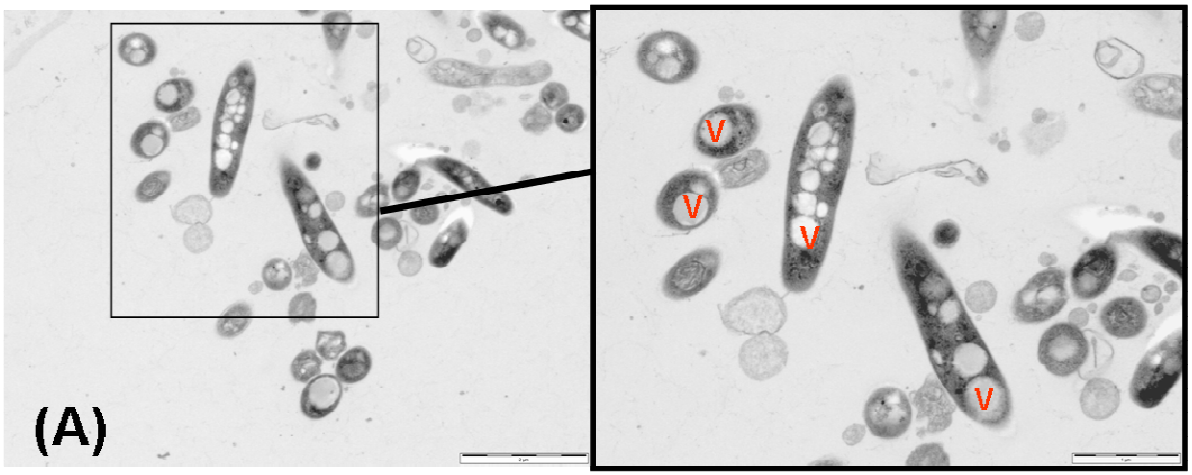
Characterised cultures are immature dendritic cells as the cultures were infected at day 7 of generated DCs. Typical dendritic cells are round in shape with dark nucleus and contain lysosomes within the cytoplasm. Figure 6.4 (B) showed non-infected wild type bone marrow-derived dendritic cell round in shape with small veils in morphology and contained a lot of non-electron dense vesicles.

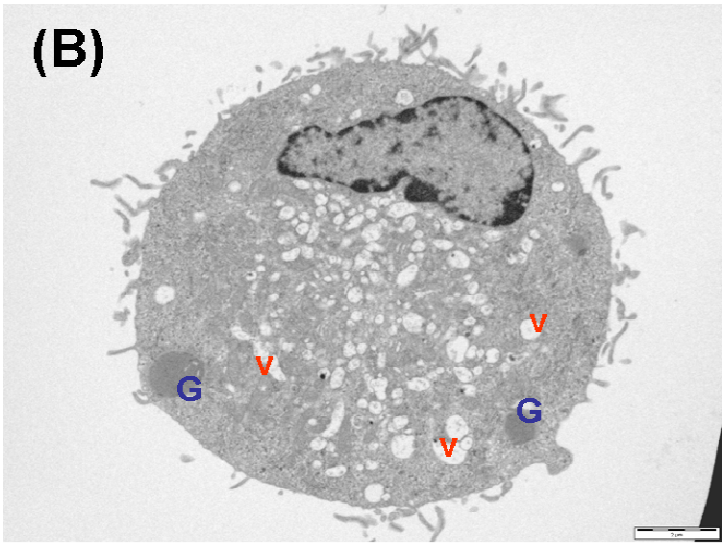
As shown in electron micrograph 6.4 (D), wild type BMDC seen in round shape with protruding dendrites and lobulated nuclei at 24 hr after BCG–infection. Mycobacterium was observed within a membrane-bound phagocytic vacuole in the cytoplasm of the cells and no free bacteria were observed in the cytoplasm. Golgi area also can be seen in this micrograph. In panel 6.4(E), TEM verified in BCG- infected BMDC that the mycobacteria were internalised by showing WT BMDC engulfed mycobacterium

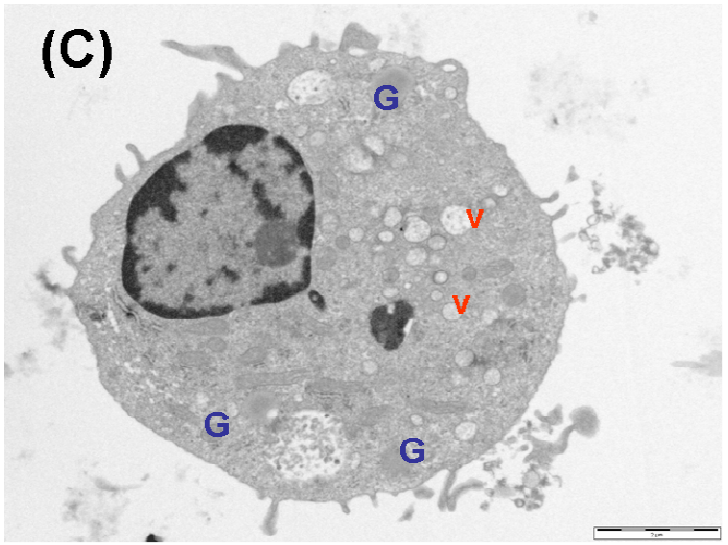
Non-infected properdin-deficient bone marrow-derived dendritic cell appeared round in shape and contained a few of non-electron dense vesicles (figure 6.4 (C)). A large numbers of *Mycobacterium bovis* (BCG) were present within the Properdin-deficient BMDC and some were observed within vacuoles of the cell (panel figure 6.4(F)). Multivesicular phagosomes were observed containing a few numbers of mycobacterium. Some seen that a vesicle can take up up to three bacteria. There were about as many electrons dense mycobacterium as electron less dense mycobacterium in the Properdin-deficient BMDC. Observed too there were lots of dividing BCG (binary fission) with presence of dividing septae of bacteria and also presence of free viable intracellular bacteria.

Some did not have a vacuole membrane around and some with the double membrane of the phagocytic vacuole. Having been internalised by the DC, the mycobacterium were seen still intact in morphology. Figure 6.4 (G) showed cells engulfing mycobacterium and vesicle wrapped out the bacteria when taken up.

Following the infection with *M. tuberculosis* and *M.bovis*, microscopy observation (phase contrast microscopy, not shown) demonstrated morphology of both BMDC-infected culture were having dendrites together with large cells aggregation compare to non infected cells. In overall demonstrated the dendritic cells able to respond to the infection and that there is no role of properdin in mycobacterium containment, phagosome location and appearance of bacteria.


(A)
V
V
V
V


(B)
V
V
G
G
V


(C)
G
V
V
G
G

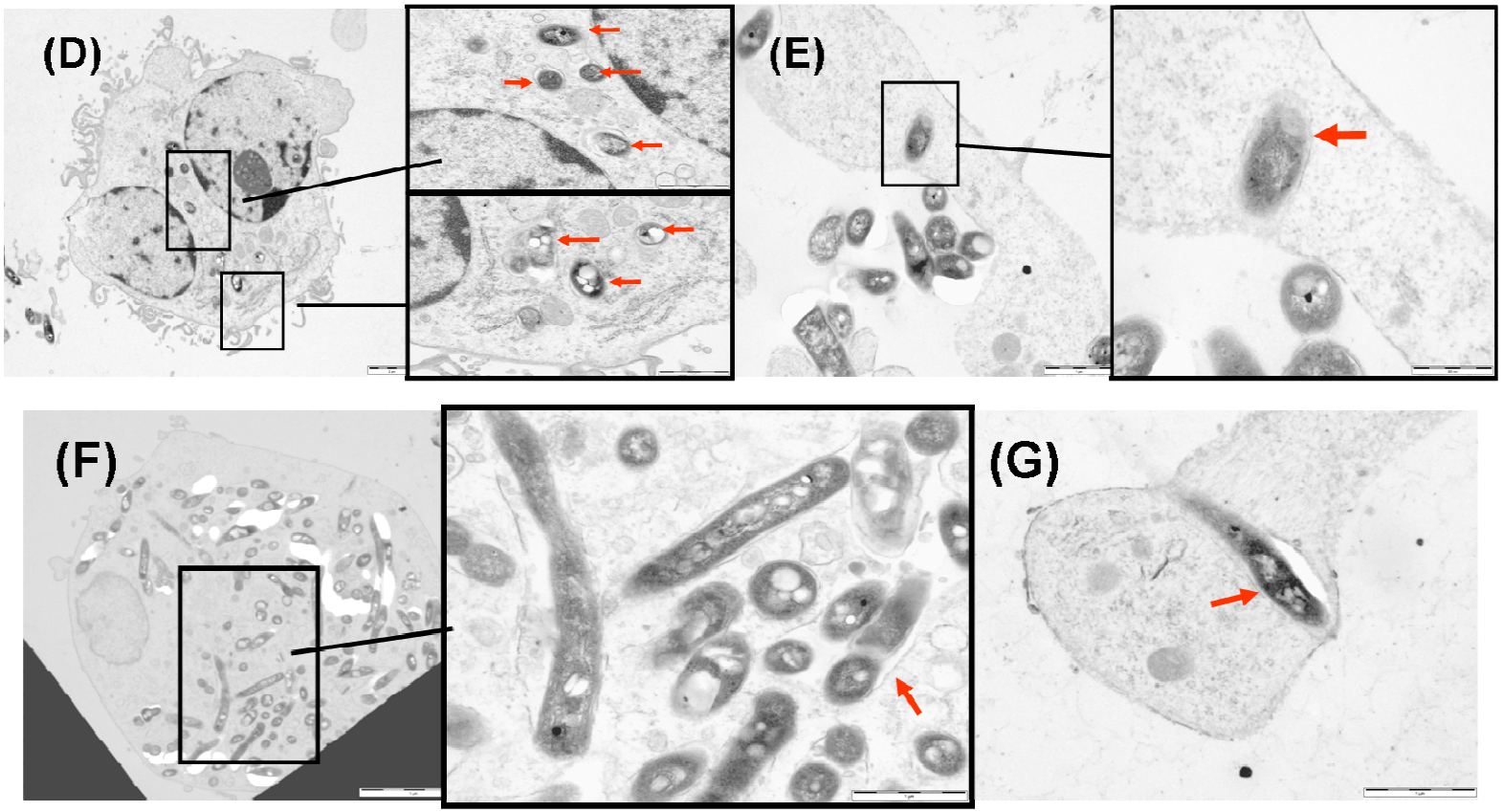


Figure 6.4 Representative of transmission electron micrographs of *Mycobacterium bovis* (BCG) –infected BMDC of WT and properdin-deficient.
(A): free mycobacterium within the culture, (**B):** non-infected WT BMDC WT (**C):** non-infected Properdin-deficient BMDC, (**D):** WT BMDC infected with BCG, showing mycobacterium within the BMDC, (**E):** WT BMDC engulfed mycobacterium (**F):** Properdin-deficient BMDC contained intracellular mycobacterium, (**G):** Properdin-deficient BMDC engulfed mycobacterium. Arrows show individual mycobacterium within a phagocytic vacuole in the cytoplasm of the BMDC. **G: electron-dense granules V: non-electron dense vesicles.**

6.4 Discussion

In the current thesis have successfully generated dendritic cells derived from bone marrow of wild type and properdin-deficient mice (see chapter 5). Following the success, it is interested to know further functional and antimicrobial effects of the characterised DCs.

It is known that *M. bovis* BCG and *M. tuberculosis* can activate the classical and alternative pathways but little is known about the mechanism of the interaction (Carroll et al., 2009, Schlesinger, 1993, Schlesinger, 1998, Schlesinger et al., 1990).

6.4.1 The role of properdin in mycobacterial viability within murine bone marrow-derived dendritic cells during the course of in vitro infection.

Mycobacterium is quite fussy to grow *in vitro*. Usually they only grow on Lowenstein-Jensen medium or Middlebrook medium (broth and agar) and doubling their growth within 24 hours.

Non-infected cell in RPMI medium at all the time points are setup to ascertain the infected BMDC having the extracellular *M. tuberculosis* growth

Following three independent experiments of dendritic cells culture infected with *M. tuberculosis*, results showed comparable viable counts for both wild type and properdin-deficient BMDC (Figure 6.1).

There appeared to be more counts from properdin-deficient BMDC (Figure 6.1A and 6.1B), however there is no significant difference between properdin deficient-BMDC and

the wild type in intracellular growth of mycobacteria. The study did not look at uptake mechanisms (limitation).

Comparable intracellular viable count levels the DCs represent that *M. tuberculosis* grew equally well within the DCs thus confirming the ability of DCs to support the growth of intracellular *M. tuberculosis*.

Viable *M. tuberculosis* were still present in the DC at day 5 after the infection (Figure 6.1A); however, the number of viable bacteria decreased suggesting that these DCs were able, to some extent, to control the growth of the tubercle bacilli.

Interestingly in overall of all the experiments, the bacteria cell counts were never greater than $1x10^7$ CFU/ ml (Figure 6.1).

Although repeat that same numbers of bacteria was added, but an initial difference in uptake develops into distinct growth curves which run parallel. These can be seen in figure 6.1B. The number of viable bacteria in activated DCs did not increase over time, confirming that these cells can inhibit mycobacterial replication. It is thought that differences in initial uptake may due to bacteria preparation, BMDC preparation and differences derivation of generated DCs from different mice pairs.

BMDC derived from properdin-deficient and wild type mice were also infected with *Mycobacterium bovis*. Unfortunately, the experiments are only able to have 24 hours post-infection time point. This is due to technical reason such as contamination of the culture, contamination of the bacteria preparation and etc. Result of infected BMDC culture with

M.bovis was comparable with initial Mycobacterium *tuberculosis* (Figure 6.2). BCG-infected properdin-deficient BMDC appeared to have high viable counts. . Nevertheless, there are no significant difference between properdin deficient-BMDC and the wild type.

Jiao X (Jiao et al., 2002) have shown that mycobacterium can survive within the dendritic cells up to 2 weeks when DC are infected *in vivo* by *M. bovis* and thought it could due to dendritic cells antimicrobial and bacteriostatic activity. In addition, *in vitro* studies shown that DC has poor bactericidal activity leading to *M. tuberculosis* growth in human and murine DC (Bodnar et al., 2001, Henderson et al., 1997).

6.4.2 The role of properdin in secretion of TNF-α by BMDC infected with mycobacteria.

Dendritic cells as antigen presenting cells (APC) communicate with T helper cells and become activated in response to Mycobacterium infection. Next the activated dendritic cells able to initiate the T cells to secrete inflammatory cytokines as initiation of an immune reaction to bacterial products (Bodnar et al., 2001, Lutz et al., 1999). Cytokines (eg: IFN-γ, TGF-β, TNF-α, etc) secreted by cells of the immune system to enhance specific immune response. In addition these molecules play a major role in protective immunity against Mtb infection, as next to the adaptive specific response.

TNF-α one of cytokines with major function as an acute phase response as an alert involved in systemic inflammation and also can cause cell death by apoptosis. TNF-α production lead to inflammatory process as leukocytes recruitment suppresses bacteria spreading. In addition, TNF-α important induce nitric oxide as nitric oxide is importantly for intracellular killing mechanism.

TNF-α production was upregulated following *in vitro M. tuberculosis* infection murine DCs (Bodnar et al., 2001, Demangel et al., 1999). In this experiment, TNF-α was determined as thought as cytokines that control of mycobacterial infection and require for inhibition growth of mycobacterium (Bodnar et al., 2001).

The current study demonstrated WT and properdin-deficient BMDC BCG and Mtb infected produced significantly high TNF-α levels compared to controls; medium RPMI and non-infected BMDC.

Relatively to control of non-infected DC that are immature dendritic cells, those infected dendritic cells which are mature, produce approximately ten fold more TNF-α (fig.6.3).

TNF-α levels Mtb-infected DC supernatant derived from both WT and properdin-deficient seen with a decreased production over times i.e levels drop down at 120 hours, verified the production of TNF-α is a very acute response. Bodnar 2001 also showed TNF-α production of Mtb-infected DC supernatant was decreasing over time.

But those bacteria that escape its effect, are able to continue to grow along a proliferation line that has a comparable slope (the proliferation rate depends on the starting numbers) (fig. 6.1A). This phase then seems TNF-α independent.

In comparison for both *M. tuberculosis* and BCG challenged BMDCs a relatively higher TNF-α levels measured when infected with *M. tuberculosis* compared to *M. bovis* BCG. In overall observation, MTb infected BMDCs are 4-5 folds higher than those measured after infection with BCG (fig. 6.3). Significantly high levels of TNF-α produced in *M. tuberculosis* infected BMDC compared infection with BCG indicating TNF-α production dependent on the strain or pathogenic of the mycobacterium.

The finding of this present study is comparable with Bohlson SS et al 2001 study that showed similar TNF-α level generated from both wild type control littermate and complement C3-deficient macrophages as cytokine response against *M. avium* infection.

However in overall there are no significant differences between the WT and Properdin-deficient BMDCs indicating properdin is not importantly require and has no role in TNF- α production after in vitro infection with Mtb/BCG.

Besides TNF-α measurement, in trial did nitric oxide measurement but unfortunately cannot proceed due to technical error and sample availability. However managed for

preliminary data and showed there were no significantly different between the groups (data not shown).

6.4.3 The role of properdin in intracellular location and containment of mycobacterium in BMDC.

Bodnar KA et al (Bodnar et al., 2001) shown *M. tuber*culosis grew equally well within nonactivated DCs, whereas the *M. tuberculosis* within activated DCs were limited in growth. As well by electron micrograph shown *M. tuber*culosis bacilli internalised dendritic cells and displayed phenotypic and functional changes.

Only infections with BCG proceed for electron microscope as due to technical matters (CAT3 protocols and etc).

The electron micrograph of demonstrated both non-infected wild type dendritic cells in round shape (Fig. 6.4 (B) and (C)) represent as an immature dendritic cells.

BCG-infected of wild type BMDC seen in round shape with lots of protruding dendrites (fig.6.4 (D)) contained a few mycobacteriums within a membrane-bound phagocytic vacuole in the cytoplasm and able to engulf mycobacterium (fig.6.4(E)).

Whereas infected properdin-deficient BMDC were in round shape with no dendrites also seen engulfing mycobacterium (Fig. 6.4 (G)) and contained a large numbers of viable *Mycobacterium bovis* (BCG) within the cells (figure 6.4(F)).

DCs encountering Mycobacterium might not be activated initially, and thus the intracellular bacteria would be able to multiply within the cells offered a thought that maybe properdin-deficient-derived BMDC lack in killing activity and less functioning as activated DC and being as reservoir to mycobacteria. However these have to investigate further with larger sample size to give significance difference.

Factor of controlling containment of mycobacterium within the dendritic cells is depends of intracellular killing activity, antigen presentation to T-cell or escape mechanism by clever mycobacterium (Wolf et al., 2007). In addition, dendritic cells antimicrobial activity depends on maturity and microenvironment upon stimulation for activation.

It is confirmed by electron micrograph that dendritic cells from both wild type (Fig. 6.4(D) and (E)) and properdin-deficient (Fig. 6.4(F) and (G)) functionally phagocytose mycobacterium as an immature dendritic cells. Through all criteria eg: electron density, bacterial division, presence of cytoplasmic bacteria, phagocytic vesicles, in summarised that the immature dendritic cells are highly phagocytic and readily take up various microbes. Altogether, the finding further confirms that DC is able to initiate the innate immune response as well as the primary T cell responses in the early period of BCG infection.

Nevertheless there is no significantly difference between WT and properdin-deficient BMDC as in overall of current study demonstrated the both dendritic cells able to respond to the infection.

6.4.4 The role of properdin in the immune response of BM-derived DC towards Mycobacterium tuberculosis and Mycobacterium bovis BCG

Taken all into consideration by findings of mycobacterial viability, secretion of TNF-α and intracellular containment of mycobacterium in BMDC, it is concluded that properdin has no role of in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

Chapter 7: Conclusion

Properdin (Factor P) as a component of the alternative complement pathway which acts as an important positive regulator of complement activation that stabilizes the alternative pathway convertases (C3bBb) and C3bBb5b in the feedback loop of the alternative pathway, protecting them from rapid inactivation.

Study of properdin promoter in human gene

The findings appeared to have activity in the 670bp properdin plasmid construct in non-LPS transfection but the transfection upon LPS induction was not successful.

In the future, if the study is repeated and able to obtain positive findings, the study will benefit in understanding of regulation and properdin expression in term of cell lineage. If the promoter construct is expressed well, the regulation of infectious situation can be understand by targeted motifs

With modification or gene deletion of the protein in tissue specificity can act as a tool to look the effects of hypoxia, also stimulation of cytokines, LPS or other PAMPS.

The promoter mapping can be done upon the complete promoter characterisation will lead to determination of transcription factor of a gene that bind to promoter regulatory.

Perhaps study of response elements eg. in inflammation cases can be perform by doing gel shift with labelled oligos.

In up-regulation promoter activity in patients will lead a way to increase production of the protein in combating deficiency of the protein in patients.

Structural and function analysis of the gene will added a new beneficial knowledge to the field.

The role of properdin in the immune response in an intraperitoneal model of vaccination

In vaccination studies demonstrated that vaccination proved efficacious as both properdin-deficient and WT had increased in total IgM level and specific IgM level after the vaccination as measured by commercial ELISA for total IgM and specific ELISA for PPS2 IgM. In the absence of Properdin, specific anti-polysaccharide antibodies of the IgM type are made. Vaccinated properdin-deficient mice do not differ from wild type in their immunoglobulin response to the pneumococcal polysaccharide vaccine. Properdin-deficiency had a benefit in survival, independent of vaccination as able to survive via natural antibody. This study lead to knowledge in some disease such as auto immune disease, it could be better for not having properdin. In the future perhaps, the experiment can be repeated for another type of vaccine such as Prevnar, a conjugate pneumococcal vaccine (T cell dependent type) in comparison of current used vaccine, the Pneumovax (T cell independent type).

The role of properdin involved in the *in vitro* characterisation and maturation of dendritic cells

Both bone marrow and spleen dendritic cells derived from properdin deficient mice are impaired to be activated and mature as dendritic cells compared to wild type littermate mice. Therefore the study presently concludes that the presence of properdin is essential to

allow dendritic cells to develop their activated phenotype and properdin is a relevant player in dendritic cell mediated immune response.

The impairment in properdin deficient derived dendritic cells needs to be analysed further to understand the mechanism behind the impairment.

In a link, LPS and CD11c that involved during the study can be study further perhaps to study the molecular link or pathway that could be involved

If it is due to sensitivity problem or due to signal transduction as LPS is involved the TLR4, maybe a signal transduction experiment can be performed further.

The role of properdin in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG

It is concluded that properdin has no role of in the immune response of BM-derived DC towards *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

Ivanovska *et al* 2008 study involved LPS shock on macrophages derived of properdin deficient model and showed a different cell response. In these shock model, less inflammation were observed in the properdin-deficient model with less TNF production.

In the future, perhaps can perform experiments involving the cell signalling or pathway studies to understand the molecular mechanism such as cell signalling in response to LPS and also to look on with other intracellular organism that easier to handle eg: *Listeria*.

Summary

In conclusion, having properdin is essential to help the immune system as part of defence mechanism against infection. Additionally, properdin could play a ‘dual-edged’ role.

AMER, A. O. & SWANSON, M. S. (2002) A phagosome of one's own: a microbial guide to life in the macrophage. *Curr Opin Microbiol,* 5**,** 56-61.

BANCHEREAU, J., BRIERE, F., CAUX, C., DAVOUST, J., LEBECQUE, S., LIU, Y. J., PULENDRAN, B. & PALUCKA, K. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol,* 18**,** 767-811.

BANCHEREAU, J. & STEINMAN, R. M. (1998) Dendritic cells and the control of immunity. *Nature,* 392**,** 245-52.

BENTLEY, C., FRIES, W. & BRADE, V. (1978) Synthesis of factors D, B and P of the alternative pathway of complement activation, as well as of C3, by guinea-pig peritoneal macrophages in vitro. *Immunology,* 35**,** 971-80.

BODNAR, K. A., SERBINA, N. V. & FLYNN, J. L. (2001) Fate of Mycobacterium tuberculosis within murine dendritic cells. *Infect Immun,* 69**,** 800-9.

BOHLSON, S. S., STRASSER, J. A., BOWER, J. J. & SCHOREY, J. S. (2001) Role of complement in Mycobacterium avium pathogenesis: in vivo and in vitro analyses of the host response to infection in the absence of complement component C3. *Infect Immun,* 69**,** 7729-35.

BONGRAZIO, M., PRIES, A. R. & ZAKRZEWICZ, A. (2003) The endothelium as physiological source of properdin: role of wall shear stress. *Mol Immunol,* 39**,** 669-75.

BREUKELS, M. A., ZANDVOORT, A., RIJKERS, G. T., LODEWIJK, M. E., KLOK, P. A., HARMS, G. & TIMENS, W. (2005) Complement dependency of splenic localization of pneumococcal polysaccharide and conjugate vaccines. *Scand J Immunol,* 61**,** 322-8.

BROWN, J. S., HUSSELL, T., GILLILAND, S. M., HOLDEN, D. W., PATON, J. C., EHRENSTEIN, M. R., WALPORT, M. J. & BOTTO, M. (2002) The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proc Natl Acad Sci U S A,* 99**,** 16969-74.

BROWN, S. L., BARRAULT, D. V., PHYTHIAN-ADAMS, A. & KNIGHT, A. M. (2006) Lack of induced co-stimulation as a result of complement receptor 2 (CR2) ligation on mouse splenic B cells. *Int Immunol,* 18**,** 69-78.

BULLARD, D. C., HU, X., ADAMS, J. E., SCHOEB, T. R. & BARNUM, S. R. (2007) p150/95 (CD11c/CD18) expression is required for the development of experimental autoimmune encephalomyelitis. *Am J Pathol,* 170**,** 2001-8.

CARROLL, M. C. (2004) The complement system in regulation of adaptive immunity. *Nat Immunol,* 5**,** 981-6.

CARROLL, M. V., LACK, N., SIM, E., KRARUP, A. & SIM, R. B. (2009) Multiple routes of complement activation by Mycobacterium bovis BCG. *Mol Immunol,* 46**,** 3367-78.

CASAL, J. & TARRAGO, D. (2003) Immunity to Streptococcus pneumoniae: Factors affecting production and efficacy. *Curr Opin Infect Dis,* 16**,** 219-24.

CASTELLANO, G., WOLTMAN, A. M., NAUTA, A. J., ROOS, A., TROUW, L. A., SEELEN, M. A., SCHENA, F. P., DAHA, M. R. & VAN KOOTEN, C. (2004) Maturation of dendritic cells abrogates C1q production in vivo and in vitro. *Blood,* 103**,** 3813-20.

CASTELLANO, G., WOLTMAN, A. M., SCHLAGWEIN, N., XU, W., SCHENA, F. P., DAHA, M. R. & VAN KOOTEN, C. (2007) Immune modulation of human dendritic cells by complement. *Eur J Immunol,* 37**,** 2803-11.

CATTERALL, J. R. (1999) Streptococcus pneumoniae. *Thorax,* 54**,** 929-37.

CELLA, M., SALLUSTO, F. & LANZAVECCHIA, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol,* 9**,** 10-6.

CHOY, L. N. & SPIEGELMAN, B. M. (1996) Regulation of alternative pathway activation and C3a production by adipose cells. *Obes Res,* 4**,** 521-32.

COLEMAN, M. P., MURRAY, J. C., WILLARD, H. F., NOLAN, K. F., REID, K. B., BLAKE, D. J., LINDSAY, S., BHATTACHARYA, S. S., WRIGHT, A. & DAVIES, K. E. (1991) Genetic and physical mapping around the properdin P gene. *Genomics,* 11**,** 991-6.

DAFFE, M. & DRAPER, P. (1998) The envelope layers of mycobacteria with reference to their pathogenicity. *Adv Microb Physiol,* 39**,** 131-203.

DE SMEDT, T., PAJAK, B., MURAILLE, E., LESPAGNARD, L., HEINEN, E., DE BAETSELIER, P., URBAIN, J., LEO, O. & MOSER, M. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med,* 184**,** 1413-24.

DELEMARRE, F. G., CLAASSEN, E. & VAN ROOIJEN, N. (1989) Primary in situ immune response in popliteal lymph nodes and spleen of mice after subcutaneous immunization with thymus-dependent or thymus-independent (type 1 and 2) antigens. *Anat Rec,* 223**,** 152-7.

DELVES, P. J. & ROITT, I. M. (2000) The immune system. First of two parts. *N Engl J Med,* 343**,** 37-49.

DEMANGEL, C., BEAN, A. G., MARTIN, E., FENG, C. G., KAMATH, A. T. & BRITTON, W. J. (1999) Protection against aerosol Mycobacterium tuberculosis infection using Mycobacterium bovis Bacillus Calmette Guerin-infected dendritic cells. *Eur J Immunol,* 29**,** 1972-9.

DENSEN, P., WEILER, J. M., GRIFFISS, J. M. & HOFFMANN, L. G. (1987) Familial properdin deficiency and fatal meningococcemia. Correction of the bactericidal defect by vaccination. *N Engl J Med,* 316**,** 922-6.

DIMITROVA, P., IVANOVSKA, N., SCHWAEBLE, W., GYURKOVSKA, V. & STOVER, C. The role of properdin in murine zymosan-induced arthritis. *Mol Immunol,* 47**,** 1458-66.

DUNKELBERGER, J. R. & SONG, W. C. Complement and its role in innate and adaptive immune responses. *Cell Res,* 20**,** 34-50.

DUNKELBERGER, J. R. & SONG, W. C. Role and mechanism of action of complement in regulating T cell immunity. *Mol Immunol,* 47**,** 2176-86.

DUPONT, A. (2008) Properdin in immunity: In vitro and in vivo investigations. *Department of Infection, Immunity & Inflammation.* University of Leicester.

DUVOIX, A., SCHNEKENBURGER, M., DELHALLE, S., BLASIUS, R., BORDE-CHICHE, P., MORCEAU, F., DICATO, M. & DIEDERICH, M. (2004) Expression of glutathione S-transferase P1-1 in leukemic cells is regulated by inducible AP-1 binding. *Cancer Lett,* 216**,** 207-19.

FARRIES, T. C. & ATKINSON, J. P. (1989) Biosynthesis of properdin. *J Immunol,* 142**,** 842-7.

FEARON, D. T. & AUSTEN, K. F. (1975a) Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J Exp Med,* 142**,** 856-63.

FEARON, D. T. & AUSTEN, K. F. (1975b) Properdin: initiation of alternative complement pathway. *Proc Natl Acad Sci U S A,* 72**,** 3220-4.

FEARON, D. T., AUSTEN, K. F. & RUDDY, S. (1975) The properdin pathway: mechanisms of complement activation and analogies to the classical pathway. *Rheumatology,* 6**,** 2-16.

FIJEN, C. A., KUIJPER, E. J., DROGARI-APIRANTHITOU, M., VAN LEEUWEN, Y., DAHA, M. R. & DANKERT, J. (1998) Protection against meningococcal serogroup ACYW disease in complement-deficient individuals vaccinated with the tetravalent meningococcal capsular polysaccharide vaccine. *Clin Exp Immunol,* 114**,** 362-9.

FIJEN, C. A., VAN DEN BOGAARD, R., SCHIPPER, M., MANNENS, M., SCHLESINGER, M., NORDIN, F. G., DANKERT, J., DAHA, M. R., SJOHOLM, A. G., TRUEDSSON, L. & KUIJPER, E. J. (1999) Properdin deficiency: molecular basis and disease association. *Mol Immunol,* 36**,** 863-7.

GOLDBLATT, D., LEVINSKY, R. J. & TURNER, M. W. (1992) Role of cell wall polysaccharide in the assessment of IgG antibodies to the capsular polysaccharides of Streptococcus pneumoniae in childhood. *J Infect Dis,* 166**,** 632-4.

GOUNDIS, D., HOLT, S. M., BOYD, Y. & REID, K. B. (1989) Localization of the properdin structural locus to Xp11.23-Xp21.1. *Genomics,* 5**,** 56-60.

GOUNDIS, D. & REID, K. B. (1988) Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature,* 335**,** 82-5.

GRIFFIOEN, A. W., RIJKERS, G. T., JANSSENS-KORPELA, P. & ZEGERS, B. J. (1991) Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. *Infect Immun,* 59**,** 1839-45.

GUTTORMSEN, H. K., STUART, L. M., SHI, L., CARROLL, M. C., CHEN, J., KASPER, D. L., EZEKOWITZ, R. A. & TAKAHASHI, K. (2009) Deficiency of mannose-binding lectin greatly increases antibody response in a mouse model of vaccination. *Clin Immunol,* 130**,** 264-71.

GUZMAN, C. A., DOMANN, E., ROHDE, M., BRUDER, D., DARJI, A., WEISS, S., WEHLAND, J., CHAKRABORTY, T. & TIMMIS, K. N. (1996) Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of Listeria monocytogenes. *Mol Microbiol,* 20**,** 119-26.

HARBOE, M. & MOLLNES, T. E. (2008) The alternative complement pathway revisited. *J Cell Mol Med,* 12**,** 1074-84.

HARRIS, S. L., FINN, A. & GRANOFF, D. M. (2003) Disparity in functional activity between serum anticapsular antibodies induced in adults by immunization with an investigational group A and C Neisseria meningitidis-diphtheria toxoid conjugate vaccine and by a polysaccharide vaccine. *Infect Immun,* 71**,** 3402-8.

HARTMANN, S. & HOFSTEENGE, J. (2000) Properdin, the positive regulator of complement, is highly C-mannosylated. *J Biol Chem,* 275**,** 28569-74.

HAZLEWOOD, M. A., KUMARARATNE, D. S., WEBSTER, A. D., GOODALL, M., BIRD, P. & DAHA, M. (1992) An association between homozygous C3 deficiency

and low levels of anti-pneumococcal capsular polysaccharide antibodies. *Clin Exp Immunol,* 87**,** 404-9.

HEIDELBERGER, M. (1984) Cross-reactions of polysaccharides of staphylococci and streptococci in antipneumococcal and other antisera. *Mol Immunol,* 21**,** 1011-3.

HENDERSON, R. A., WATKINS, S. C. & FLYNN, J. L. (1997) Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol,* 159**,** 635-43.

HICKMAN, S. P., CHAN, J. & SALGAME, P. (2002) Mycobacterium tuberculosis induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization. *J Immunol,* 168**,** 4636-42.

HIGGINS, J. M., WIEDEMANN, H., TIMPL, R. & REID, K. B. (1995) Characterization of mutant forms of recombinant human properdin lacking single thrombospondin type I repeats. Identification of modules important for function. *J Immunol,* 155**,** 5777-85.

HOURCADE, D. E. (2006) The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem,* 281**,** 2128-32.

HU, C., MAYADAS-NORTON, T., TANAKA, K., CHAN, J. & SALGAME, P. (2000) Mycobacterium tuberculosis infection in complement receptor 3-deficient mice. *J Immunol,* 165**,** 2596-602.

HU, Y. & TEST, S. T. (2004) Functional differences in IgG anti-polysaccharide antibodies elicited by immunization of mice with C3d versus ovalbumin conjugates of pneumococcal serotype 14 capsular polysaccharide. *Vaccine,* 23**,** 21-8.

HUMPHREYS, I. R., STEWART, G. R., TURNER, D. J., PATEL, J., KARAMANOU, D., SNELGROVE, R. J. & YOUNG, D. B. (2006) A role for dendritic cells in the dissemination of mycobacterial infection. *Microbes Infect,* 8**,** 1339-46.

INABA, K., INABA, M., NAITO, M. & STEINMAN, R. M. (1993) Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. *J Exp Med,* 178**,** 479-88.

INABA, K., INABA, M., ROMANI, N., AYA, H., DEGUCHI, M., IKEHARA, S., MURAMATSU, S. & STEINMAN, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med,* 176**,** 1693-702.

IVANOVSKA, N. D., DIMITROVA, P. A., LUCKETT, J. C., EL-RACHKIDY LONNEN, R., SCHWAEBLE, W. J. & STOVER, C. M. (2008) Properdin deficiency in murine models of nonseptic shock. *J Immunol,* 180**,** 6962-9.

JARVA, H., JOKIRANTA, T. S., WURZNER, R. & MERI, S. (2003) Complement resistance mechanisms of streptococci. *Mol Immunol,* 40**,** 95-107.

JIAO, X., LO-MAN, R., GUERMONPREZ, P., FIETTE, L., DERIAUD, E., BURGAUD, S., GICQUEL, B., WINTER, N. & LECLERC, C. (2002) Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J Immunol,* 168**,** 1294-301.

KADIOGLU, A. & ANDREW, P. W. (2005) Susceptibility and resistance to pneumococcal disease in mice. *Brief Funct Genomic Proteomic,* 4**,** 241-7.

KAHLER, C. M., KAUFMANN, G., HOGUE-ANGELETTI, R., FISCHER-COLBRIE, R., DUNZENDORFER, S., REINISCH, N. & WIEDERMANN, C. J. (1999) A

soluble gradient of the neuropeptide secretoneurin promotes the transendothelial migration of monocytes in vitro. *Eur J Pharmacol,* 365**,** 65-75.

KANG, J. H., CHANG, S. Y., YEOM, D. H., KIM, S. A., UM, S. J. & HONG, K. J. (2004) Weakening of the repressive YY-1 site on the thrombospondin-1 promoter via c-Jun/YY-1 interaction. *Exp Mol Med,* 36**,** 300-10.

KELLY, D. F., SNAPE, M. D., CLUTTERBUCK, E. A., GREEN, S., SNOWDEN, C., DIGGLE, L., YU, L. M., BORKOWSKI, A., MOXON, E. R. & POLLARD, A. J. (2006) CRM197-conjugated serogroup C meningococcal capsular polysaccharide, but not the native polysaccharide, induces persistent antigen-specific memory B cells. *Blood,* 108**,** 2642-7.

KERR, A. R., PATERSON, G. K., RIBOLDI-TUNNICLIFFE, A. & MITCHELL, T. J. (2005) Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. *Infect Immun,* 73**,** 4245-52.

KIM, S. A. & HONG, K. J. (2000) Responsive site on the thrombospondin-1 promotor to down-regulation by phorbol 12-myristate 13-acetate in porcine aortic endothelial cells. *Exp Mol Med,* 32**,** 135-40.

KIM, S. A., KANG, J. H., CHO, I., BAE, S. W. & HONG, K. J. (2001) Cell-type specific regulation of thrombospondin-1 expression and its promoter activity by regulatory agents. *Exp Mol Med,* 33**,** 117-23.

KIMURA, Y., MIWA, T., ZHOU, L. & SONG, W. C. (2008) Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood,* 111**,** 732-40.

KLEIN, E., SMITH, D. L. & LAXMINARAYAN, R. (2007) Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005. *Emerg Infect Dis,* 13**,** 1840-6.

LEE, C. J., BANKS, S. D. & LI, J. P. (1991) Virulence, immunity, and vaccine related to Streptococcus pneumoniae. *Crit Rev Microbiol,* 18**,** 89-114.

LEE, C. J., LEE, L. H., LU, C. S. & WU, A. (2001) Bacterial polysaccharides as vaccines--immunity and chemical characterization. *Adv Exp Med Biol,* 491**,** 453-71.

LEENEN, P. J., RADOSEVIC, K., VOERMAN, J. S., SALOMON, B., VAN ROOIJEN, N., KLATZMANN, D. & VAN EWIJK, W. (1998) Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J Immunol,* 160**,** 2166-73.

LENTZ, V. M. & MANSER, T. (2001) Cutting edge: germinal centers can be induced in the absence of T cells. *J Immunol,* 167**,** 15-20.

LEPOW, I. H. (1980) Presidential address to American Association of Immunologists in Anaheim, California, April 16, 1980. Louis Pillemer, Properdin, and scientific controversy. *J Immunol,* 125**,** 471-5.

LESLIE, R. G. & NIELSEN, C. H. (2004) The classical and alternative pathways of complement activation play distinct roles in spontaneous C3 fragment deposition and membrane attack complex (MAC) formation on human B lymphocytes. *Immunology,* 111**,** 86-90.

LINTON, S. M. & MORGAN, B. P. (1999) Properdin deficiency and meningococcal disease--identifying those most at risk. *Clin Exp Immunol,* 118**,** 189-91.

LUE, C., VAN DEN WALL BAKE, A. W., PRINCE, S. J., JULIAN, B. A., TSENG, M. L., RADL, J., ELSON, C. O. & MESTECKY, J. (1994) Intraperitoneal

immunization of human subjects with tetanus toxoid induces specific antibody-secreting cells in the peritoneal cavity and in the circulation, but fails to elicit a secretory IgA response. *Clin Exp Immunol,* 96**,** 356-63.

LUTZ, M. B., KUKUTSCH, N., OGILVIE, A. L., ROSSNER, S., KOCH, F., ROMANI, N. & SCHULER, G. (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods,* 223**,** 77-92.

MACALLAN, D. C., WALLACE, D. L., ZHANG, Y., GHATTAS, H., ASQUITH, B., DE LARA, C., WORTH, A., PANAYIOTAKOPOULOS, G., GRIFFIN, G. E., TOUGH, D. F. & BEVERLEY, P. C. (2005) B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. *Blood,* 105**,** 3633-40.

MARKHAM, R. B., NICHOLSON-WELLER, A., SCHIFFMAN, G. & KASPER, D. L. (1982) The presence of sialic acid on two related bacterial polysaccharides determines the site of the primary immune response and the effect of complement depletion on the response in mice. *J Immunol,* 128**,** 2731-3.

MARTINO, A. (2008) Mycobacteria and innate cells: critical encounter for immunogenicity. *J Biosci,* 33**,** 137-44.

MASCANFRONI, I., MONTESINOS MDEL, M., SUSPERREGUY, S., CERVI, L., ILARREGUI, J. M., RAMSEYER, V. D., MASINI-REPISO, A. M., TARGOVNIK, H. M., RABINOVICH, G. A. & PELLIZAS, C. G. (2008) Control of dendritic cell maturation and function by triiodothyronine. *Faseb J,* 22**,** 1032-42.

MATSUNO, K., EZAKI, T., KUDO, S. & UEHARA, Y. (1996) A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J Exp Med,* 183**,** 1865-78.

MATSUSHITA, M., ENDO, Y. & FUJITA, T. (2000) Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. *J Immunol,* 164**,** 2281-4.

MATSUSHITA, M., TAKAHASHI, A., HATSUSE, H., KAWAKAMI, M. & FUJITA, T. (1992) Human mannose-binding protein is identical to a component of Ra-reactive factor. *Biochem Biophys Res Commun,* 183**,** 645-51.

MAVES, K. K., GUENTHNER, S. T., DENSEN, P., MOSER, D. R. & WEILER, J. M. (1995) Cloning and characterization of the cDNA encoding guinea-pig properdin: a comparison of properdin from three species. *Immunology,* 86**,** 475-9.

MAVES, K. K. & WEILER, J. M. (1994) Human liver-derived HEP G2 cells produce functional properdin. *J Lab Clin Med,* 124**,** 837-42.

MCCOOL, T. L., HARDING, C. V., GREENSPAN, N. S. & SCHREIBER, J. R. (1999) B- and T-cell immune responses to pneumococcal conjugate vaccines: divergence between carrier- and polysaccharide-specific immunogenicity. *Infect Immun,* 67**,** 4862-9.

MCLAY, J., LEONARD, E., PETERSEN, S., SHAPIRO, D., GREENSPAN, N. S. & SCHREIBER, J. R. (2002) Gamma 3 gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti-polysaccharide IgG3 is corrected by induction of anti-polysaccharide IgG1. *J Immunol,* 168**,** 3437-43.

MELO, M. D. & STOKES, R. W. (2000) Interaction of Mycobacterium tuberculosis with MH-S, an immortalized murine alveolar macrophage cell line: a comparison with primary murine macrophages. *Tuber Lung Dis,* 80**,** 35-46.

METLAY, J. P., WITMER-PACK, M. D., AGGER, R., CROWLEY, M. T., LAWLESS, D. & STEINMAN, R. M. (1990) The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med,* 171**,** 1753-71.

MINTA, J. O. (1988) Biosynthesis of complement factor P (properdin) by the human pre-monocyte cell line (U-937). *Mol Immunol,* 25**,** 1363-70.

MINTA, J. O. & LEPOW, I. H. (1974) Studies on the sub-unit structure of human properdin. *Immunochemistry,* 11**,** 361-8.

MIYAGOE, Y., GALIBERT, M. D., GEORGATSOU, E., FOUREL, G. & MEO, T. (1994) Promoter elements of the mouse complement C4 gene critical for transcription activation and start site location. *J Biol Chem,* 269**,** 8268-79.

MORGAN, B. P. & WALPORT, M. J. (1991) Complement deficiency and disease. *Immunol Today,* 12**,** 301-6.

MORTON, D. B. & GRIFFITHS, P. H. (1985) Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Rec,* 116**,** 431-6.

MOSER, M. & MURPHY, K. M. (2000) Dendritic cell regulation of TH1-TH2 development. *Nat Immunol,* 1**,** 199-205.

MOULTON, R. A., MASHRUWALA, M. A., SMITH, A. K., LINDSEY, D. R., WETSEL, R. A., HAVILAND, D. L., HUNTER, R. L. & JAGANNATH, C. (2007) Complement C5a anaphylatoxin is an innate determinant of dendritic cell-induced Th1 immunity to Mycobacterium bovis BCG infection in mice. *J Leukoc Biol,* 82**,** 956-67.

NIELSEN, H. E. & KOCH, C. (1987) Congenital properdin deficiency and meningococcal infection. *Clin Immunol Immunopathol,* 44**,** 134-9.

NOLAN, K. F., KALUZ, S., HIGGINS, J. M., GOUNDIS, D. & REID, K. B. (1992) Characterization of the human properdin gene. *Biochem J,* 287 (Pt 1)**,** 291-7.

NOLAN, K. F. & REID, K. B. (1993) Properdin. *Methods Enzymol,* 223**,** 35-46.

NOLAN, K. F., STRONG, V., SOLER, D., FAIRCHILD, P. J., COBBOLD, S. P., CROXTON, R., GONZALO, J. A., RUBIO, A., WELLS, M. & WALDMANN, H. (2004) IL-10-conditioned dendritic cells, decommissioned for recruitment of adaptive immunity, elicit innate inflammatory gene products in response to danger signals. *J Immunol,* 172**,** 2201-9.

OBUKHANYCH, T. V. & NUSSENZWEIG, M. C. (2006) T-independent type II immune responses generate memory B cells. *J Exp Med,* 203**,** 305-10.

OCHSENBEIN, A. F. & ZINKERNAGEL, R. M. (2000) Natural antibodies and complement link innate and acquired immunity. *Immunol Today,* 21**,** 624-30.

PANGBURN, M. K. (1983) Activation of complement via the alternative pathway. *Fed Proc,* 42**,** 139-43.

PANGBURN, M. K. (1989) Analysis of the natural polymeric forms of human properdin and their functions in complement activation. *J Immunol,* 142**,** 202-7.

PANGBURN, M. K. & MULLER-EBERHARD, H. J. (1980) Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. *J Exp Med,* 152**,** 1102-14.

PANGBURN, M. K., SCHREIBER, R. D. & MULLER-EBERHARD, H. J. (1981) Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med,* 154**,** 856-67.

PANGBURN, M. K., SCHREIBER, R. D. & MULLER-EBERHARD, H. J. (1983) C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. *J Immunol,* 131**,** 1930-5.

PARK, S., PARAMESWAR, A. R., DEMCHENKO, A. V. & NAHM, M. H. (2009) Identification of a simple chemical structure associated with protective human antibodies against multiple pneumococcal serogroups. *Infect Immun,* 77**,** 3374-9.

PARKIN, J. & COHEN, B. (2001) An overview of the immune system. *Lancet,* 357**,** 1777-89.

PASARE, C. & MEDZHITOV, R. (2003a) Toll-like receptors: balancing host resistance with immune tolerance. *Curr Opin Immunol,* 15**,** 677-82.

PASARE, C. & MEDZHITOV, R. (2003b) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science,* 299**,** 1033-6.

PENG, Q., LI, K., PATEL, H., SACKS, S. H. & ZHOU, W. (2006) Dendritic cell synthesis of C3 is required for full T cell activation and development of a Th1 phenotype. *J Immunol,* 176**,** 3330-41.

PERDIKOULIS, M. V., KISHORE, U. & REID, K. B. (2001) Expression and characterisation of the thrombospondin type I repeats of human properdin. *Biochim Biophys Acta,* 1548**,** 265-77.

PILLEMER, L., BLUM, L., LEPOW, I. H., ROSS, O. A., TODD, E. W. & WARDLAW, A. C. (1954) The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science,* 120**,** 279-85.

PROMEGA.

REIS, E. S., BARBUTO, J. A. & ISAAC, L. (2006) Human monocyte-derived dendritic cells are a source of several complement proteins. *Inflamm Res,* 55**,** 179-84.

REIS, E. S., BARBUTO, J. A. & ISAAC, L. (2007) Complement components, regulators and receptors are produced by human monocyte-derived dendritic cells. *Immunobiology,* 212**,** 151-7.

REIS, E. S., BARBUTO, J. A., KOHL, J. & ISAAC, L. (2008) Impaired dendritic cell differentiation and maturation in the absence of C3. *Mol Immunol,* 45**,** 1952-62.

RESCIGNO, M., WINZLER, C., DELIA, D., MUTINI, C., LUTZ, M. & RICCIARDI-CASTAGNOLI, P. (1997) Dendritic cell maturation is required for initiation of the immune response. *J Leukoc Biol,* 61**,** 415-21.

RICKERT, R. C. (2005) Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex. *Curr Opin Immunol,* 17**,** 237-43.

ROSICA, V., GIANNINI, E. & CURCIO, C. G. (1965) [On the use of yeasts in chronic bronchial inflammations]. *Clin Ter,* 34**,** 42-6.

ROSSBACHER, J. & SHLOMCHIK, M. J. (2003) The B cell receptor itself can activate complement to provide the complement receptor 1/2 ligand required to enhance B cell immune responses in vivo. *J Exp Med,* 198**,** 591-602.

RUSSELL, M. S., ISKANDAR, M., MYKYTCZUK, O. L., NASH, J. H., KRISHNAN, L. & SAD, S. (2007) A reduced antigen load in vivo, rather than weak inflammation, causes a substantial delay in CD8+ T cell priming against Mycobacterium bovis (bacillus Calmette-Guerin). *J Immunol,* 179**,** 211-20.

RUSSELL, N. D., CORVALAN, J. R., GALLO, M. L., DAVIS, C. G. & PIROFSKI, L. (2000) Production of protective human antipneumococcal antibodies by transgenic mice with human immunoglobulin loci. *Infect Immun,* 68**,** 1820-6.

SALEHEN, N. (2006) Differentiation and 3D migration of the human myeloid leukaemia cell line U937 and primary monocytic cells by endogenous protein factors. *Department of Cancer Studies and Molecular Medicine* University of Leicester.

SALEHEN, N. & STOVER, C. (2008) The role of complement in the success of vaccination with conjugated vs. unconjugated polysaccharide antigen. *Vaccine,* 26**,** 451-9.

SALLUSTO, F. & LANZAVECCHIA, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med,* 179**,** 1109-18.

SATTHAPORN, S. & EREMIN, O. (2001) Dendritic cells (I): Biological functions. *J R Coll Surg Edinb,* 46**,** 9-19.

SCHLESINGER, L. S. (1993) Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. *J Immunol,* 150**,** 2920-30.

SCHLESINGER, L. S. (1998) Mycobacterium tuberculosis and the complement system. *Trends Microbiol,* 6**,** 47-9; discussion 49-50.

SCHLESINGER, L. S., BELLINGER-KAWAHARA, C. G., PAYNE, N. R. & HORWITZ, M. A. (1990) Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3. *J Immunol,* 144**,** 2771-80.

SCHWAEBLE, W., DIPPOLD, W. G., SCHAFER, M. K., POHLA, H., JONAS, D., LUTTIG, B., WEIHE, E., HUEMER, H. P., DIERICH, M. P. & REID, K. B. (1993) Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells. *J Immunol,* 151**,** 2521-8.

SCHWAEBLE, W. J. & REID, K. B. (1999) Does properdin crosslink the cellular and the humoral immune response? *Immunol Today,* 20**,** 17-21.

SEN, G., KHAN, A. Q., CHEN, Q. & SNAPPER, C. M. (2005) In vivo humoral immune responses to isolated pneumococcal polysaccharides are dependent on the presence of associated TLR ligands. *J Immunol,* 175**,** 3084-91.

SHARBATI-TEHRANI, S., STEPHAN, J., HOLLAND, G., APPEL, B., NIEDERWEIS, M. & LEWIN, A. (2005) Porins limit the intracellular persistence of Mycobacterium smegmatis. *Microbiology,* 151**,** 2403-10.

SHORTMAN, K. & LIU, Y. J. (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol,* 2**,** 151-61.

SIM, R. B., DAY, A. J., MOFFATT, B. E. & FONTAINE, M. (1993) Complement factor I and cofactors in control of complement system convertase enzymes. *Methods Enzymol,* 223**,** 13-35.

SJOHOLM, A. G., BRACONIER, J. H. & SODERSTROM, C. (1982) Properdin deficiency in a family with fulminant meningococcal infections. *Clin Exp Immunol,* 50**,** 291-7.

SJOHOLM, A. G., SELANDER, B., OSTENSON, S., HOLMSTROM, E. & SODERSTROM, C. (1991) Normal human serum depleted of C1q, factor D and properdin: its use in studies of complement activation. *Apmis,* 99**,** 1120-8.

SJOHOLM, A. G., SODERSTROM, C. & NILSSON, L. A. (1988) A second variant of properdin deficiency: the detection of properdin at low concentrations in affected males. *Complement,* 5**,** 130-40.

SMITH, C. A., PANGBURN, M. K., VOGEL, C. W. & MULLER-EBERHARD, H. J. (1984) Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. *J Biol Chem,* 259**,** 4582-8.

SNAPPER, C. M., MCINTYRE, T. M., MANDLER, R., PECANHA, L. M., FINKELMAN, F. D., LEES, A. & MOND, J. J. (1992) Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J Exp Med,* 175**,** 1367-71.

SODERSTROM, C., BRACONIER, J. H., KAYHTY, H., SJOHOLM, A. G. & THURESSON, B. (1989) Immune response to tetravalent meningococcal vaccine: opsonic and bactericidal functions of normal and properdin deficient sera. *Eur J Clin Microbiol Infect Dis,* 8**,** 220-4.

SODERSTROM, C., BRACONIER, J. H., SJOHOLM, A. G. & THURESSON, B. (1991) Granulocyte functions and Neisseria meningitidis: influence of properdin-deficient serum. *Apmis,* 99**,** 965-71.

SONG, W. C., DENG, C., RASZMANN, K., MOORE, R., NEWBOLD, R., MCLACHLAN, J. A. & NEGISHI, M. (1996) Mouse decay-accelerating factor: selective and tissue-specific induction by estrogen of the gene encoding the glycosylphosphatidylinositol-anchored form. *J Immunol,* 157**,** 4166-72.

SPATH, P. J., SJOHOLM, A. G., FREDRIKSON, G. N., MISIANO, G., SCHERZ, R., SCHAAD, U. B., UHRING-LAMBERT, B., HAUPTMANN, G., WESTBERG, J., UHLEN, M., WADELIUS, C. & TRUEDSSON, L. (1999) Properdin deficiency in a large Swiss family: identification of a stop codon in the properdin gene, and association of meningococcal disease with lack of the IgG2 allotype marker G2m(n). *Clin Exp Immunol,* 118**,** 278-84.

SPITZER, D., MITCHELL, L. M., ATKINSON, J. P. & HOURCADE, D. E. (2007) Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol,* 179**,** 2600-8.

STEINMAN, R. M. (2007) Dendritic cells: understanding immunogenicity. *Eur J Immunol,* 37 Suppl 1**,** S53-60.

STEINMAN, R. M., PACK, M. & INABA, K. (1997) Dendritic cell development and maturation. *Adv Exp Med Biol,* 417**,** 1-6.

STEINMAN, R. M. & YOUNG, J. W. (1991) Signals arising from antigen-presenting cells. *Curr Opin Immunol,* 3**,** 361-72.

STOVER, C. M., LUCKETT, J. C., ECHTENACHER, B., DUPONT, A., FIGGITT, S. E., BROWN, J., MANNEL, D. N. & SCHWAEBLE, W. J. (2008) Properdin plays a protective role in polymicrobial septic peritonitis. *J Immunol,* 180**,** 3313-8.

SULLIVAN, K. E., WU, L. C., CAMPBELL, R. D., VALLE, D. & WINKELSTEIN, J. A. (1994) Transcriptional regulation of the gene for the second component of human complement: promoter analysis. *Eur J Immunol,* 24**,** 393-400.

SUN, Z., REID, K. B. & PERKINS, S. J. (2004) The dimeric and trimeric solution structures of the multidomain complement protein properdin by X-ray scattering, analytical ultracentrifugation and constrained modelling. *J Mol Biol,* 343**,** 1327-43.

TAILLEUX, L., NEYROLLES, O., HONORE-BOUAKLINE, S., PERRET, E., SANCHEZ, F., ABASTADO, J. P., LAGRANGE, P. H., GLUCKMAN, J. C., ROSENZWAJG, M. & HERRMANN, J. L. (2003) Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells. *J Immunol,* 170**,** 1939-48.

TAN, J. K. & O'NEILL, H. C. (2005) Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J Leukoc Biol,* 78**,** 319-24.

TANG, L., JENNINGS, T. A. & EATON, J. W. (1998) Mast cells mediate acute inflammatory responses to implanted biomaterials. *Proc Natl Acad Sci U S A,* 95**,** 8841-6.

TIAN, H., GRONER, A., BOES, M. & PIROFSKI, L. A. (2007) Pneumococcal capsular polysaccharide vaccine-mediated protection against serotype 3 Streptococcus pneumoniae in immunodeficient mice. *Infect Immun,* 75**,** 1643-50.

TIAN, T., WOODWORTH, J., SKOLD, M. & BEHAR, S. M. (2005) In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. *J Immunol,* 175**,** 3268-72.

TIMENS, W., BOES, A. & POPPEMA, S. (1989) Human marginal zone B cells are not an activated B cell subset: strong expression of CD21 as a putative mediator for rapid B cell activation. *Eur J Immunol,* 19**,** 2163-6.

TJIAN, R. & MANIATIS, T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell,* 77**,** 5-8.

TOELLNER, K. M., KHAN, M. & SZE, D. M. (2004) Analysis of the germinal center reaction and in vivo long-lived plasma cells. *Methods Mol Biol,* 271**,** 111-25.

TRUEDSSON, L., WESTBERG, J., FREDRIKSON, G. N., SJOHOLM, A. G., KUIJPER, E. J., FIJEN, C. A., SPATH, P. J. & UHLEN, M. (1997) Human properdin deficiency has a heterogeneous genetic background. *Immunopharmacology,* 38**,** 203-6.

TUFARIELLO, J. M., CHAN, J. & FLYNN, J. L. (2003) Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis,* 3**,** 578-90.

VELLA, P. P., MARBURG, S., STAUB, J. M., KNISKERN, P. J., MILLER, W., HAGOPIAN, A., IP, C., TOLMAN, R. L., RUSK, C. M., CHUPAK, L. S. & ET AL. (1992) Immunogenicity of conjugate vaccines consisting of pneumococcal capsular polysaccharide types 6B, 14, 19F, and 23F and a meningococcal outer membrane protein complex. *Infect Immun,* 60**,** 4977-83.

VIAU, M. & ZOUALI, M. (2005) B-lymphocytes, innate immunity, and autoimmunity. *Clin Immunol,* 114**,** 17-26.

VIK, D. P., AMIGUET, P., MOFFAT, G. J., FEY, M., AMIGUET-BARRAS, F., WETSEL, R. A. & TACK, B. F. (1991) Structural features of the human C3 gene: intron/exon organization, transcriptional start site, and promoter region sequence. *Biochemistry,* 30**,** 1080-5.

VOLANAKIS, J. E. (1995) Transcriptional regulation of complement genes. *Annu Rev Immunol,* 13**,** 277-305.

VOS, Q., LEES, A., WU, Z. Q., SNAPPER, C. M. & MOND, J. J. (2000) B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol Rev,* 176**,** 154-70.

WALPORT, M. J. (2001) Complement. First of two parts. *N Engl J Med,* 344**,** 1058-66.

WARDEMANN, H., BOEHM, T., DEAR, N. & CARSETTI, R. (2002) B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med,* 195**,** 771-80.

WERNETTE, C. M., FRASCH, C. E., MADORE, D., CARLONE, G., GOLDBLATT, D., PLIKAYTIS, B., BENJAMIN, W., QUATAERT, S. A., HILDRETH, S., SIKKEMA, D. J., KAYHTY, H., JONSDOTTIR, I. & NAHM, M. H. (2003) Enzyme-linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. *Clin Diagn Lab Immunol,* 10**,** 514-9.

WILLIAMS, S. A. & VIK, D. P. (1997) Characterization of the 5' flanking region of the human complement factor H gene. *Scand J Immunol,* 45**,** 7-15.

WILLS-KARP, M. (2007) Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. *Proc Am Thorac Soc,* 4**,** 247-51.

WINZLER, C., ROVERE, P., RESCIGNO, M., GRANUCCI, F., PENNA, G., ADORINI, L., ZIMMERMANN, V. S., DAVOUST, J. & RICCIARDI-CASTAGNOLI, P. (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med,* 185**,** 317-28.

WOLF, A. J., LINAS, B., TREVEJO-NUNEZ, G. J., KINCAID, E., TAMURA, T., TAKATSU, K. & ERNST, J. D. (2007) Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J Immunol,* 179**,** 2509-19.

WURZNER, R. (2003) Complement and infectious diseases. *Contrib Microbiol,* 10**,** 1-17.

YAMAUCHI, Y., KUROKI, M., IMAKIIRE, T., ABE, H., UCHIDA, H., BEPPU, R., YAMASHITA, Y., KUROKI, M. & SHIRAKUSA, T. (2002) Thrombospondin-1 differentially regulates release of IL-6 and IL-10 by human monocytic cell line U937. *Biochem Biophys Res Commun,* 290**,** 1551-7.

YUE, L. (2005) *Department of Infection, Immunity & Inflammation-Bioinformatics.* University of Leicester.

ZHOU, W., PENG, Q., LI, K. & SACKS, S. H. (2007) Role of dendritic cell synthesis of complement in the allospecific T cell response. *Mol Immunol,* 44**,** 57-63.