The role of properdin in the pathogenesis of murine systemic lupus erythematosus

Thesis submitted for the degree of Doctor of Philosophy

By

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Abstract

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In systemic lupus erythematosus (SLE), complement-activating immune complexes (ICs) are central to the immunopathogenesis of disease. Their deposition in kidney initiates an inflammatory response resulting in glomerulonephritis. Properdin is the only known positive regulator of complement activation, but its role in SLE disease severity has not been studied yet. Properdin-deficient mice were crossed with MRL/lpr mice on C57Bl/6 background. MRL/lpr mice develop lupus-like disease spontaneously. We derived properdin wildtype, properdin deficient, and properdin heterozygous MRL/lpr mice. Compared to MRL/lpr properdin wildtype mice, MRL/lpr properdin-deficient mice developed significantly less severe disease, had lower serum anti-DNA antibody titre, reduced complement activation, less serum pro-inflammatory cytokines and showed less splenic surface expression of CR2 on B220 positive cells. Parallel analysis of properdin heterozygous MRL/lpr mice revealed a gene dose effect in some measures. There was less serum creatinine in MRL/lpr properdin-deficient mice and colocalising activated C3 fragments and immunoglobulins (ICs) in the mesangium, and less SMA-α and nephrin expression in MRL/lpr properdin-deficient mice compared to MRL/lpr properdin wildtype mice. These findings demonstrate that properdin plays a significant role in the severity of lupus and involvement of kidney in MRL/lpr mice. Because MRL/lpr properdin deficient mice had lower levels of anti-DNA Abs, inflammatory mediators and marker of renal impairment, the study implies that properdin could constitute a novel therapy target.
Statement

This accompanying thesis submitted for the degree of PhD entitled “The role of properdin in the pathogenesis of murine systemic lupus erythematosus” based on work performed by the author at the University of Leicester mainly during the period between July 2015 and June 2018.

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

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

Signed: ______________ Date: ___________________
Dedicate

To My…

Mother Fatemah,

Father Yassen,

Sisters Heba & Amna,

Brother Mustafa

&

My wife Ting
Acknowledgement

‘For his mercy and blessing, all praise and gratitude goes to the Almighty God’

Writing of thesis including two subjects has not been easy work. That required a lot of hard work, concentration, patience and time. I would like to mention that this work could have never been written without guidance and support from others. I am extremely grateful to my supervisors Dr Stover for her guidance, support, advice and patience through this research, and to give me the opportunity to undertake this higher research degree. I am definitely blessed I got the best mentor, and without her, this work would never happen. Furthermore, I would like to present my thanks to my second supervisor Professor Jonathan Barratt for his encouragement, expert supervision and guidance throughout this work. Also, I would like to thank my progress review panel members, Dr Michael Browning and Dr Yassine Amrani for their advice, support, encouragement and positive feedback.

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Publications

One paper is written now and it is on way to publish:

- Complement Factor P Determines Disease Activity in MRL/lpr Mice.

- Vitamin D3 ameliorates bone mineral density reduction associated with LDLR deficiency. *(Submitted and under revision)*
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LIST OF ABBREVIATIONS

TNF-alpha: Tumour Necrosis Factor-alpha
ICs: Immune Complexes
IL-6: Interleukin-6
C3: Complement component 3
CP: Classical Pathway
LP: Lectin Pathway
AP: Alternative Pathway
MBL: Mannan-Binding Lectin
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
MASP1, 2 or 3: MBL-associated serine protease-1,2 or 3
C3c: Complement Component 3, c Subcomponent
C3b: Complement Component 3, b Subcomponent
C3d: Complement Component 3, d Subcomponent
C3dg: Complement Component 3 degradation products
iC3b: inactivated C3b
TCC: Terminal complement complex
fD: Facto D
fB: Facto B
TSRs: Thrombospondin Repeats
Fc: Fragment crystallizable region
M1: Macrophage type 1
M2: Macrophage type 2
CR 1, 2, 3 or 4: Complement Receptor 1, 2, 3 or 4
CD: Cluster of Differentiation
Th1 or 2: T helper cell 1 or 2
Bcl-2: B-cell lymphoma 2
BAFF: B-cell activating factor
WT: Wildtype
KO: Knockout
TLRs: Toll-Like Receptors
IMS: Industrial Methylated Spirit
DPX: Dibutyl Phthalate Xylene
PBS: Phosphate Buffered Saline
CNMS: Commercial Normal Mouse Serum
ELISA: The enzyme-linked immunosorbent assay
IHC: Immunohistochemistry
IF: Immunofluorescence
FITC: Fluorescein Isothiocyanate
TR: Texas Red
DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
AOBS: Acousto-Optical Beam Splitter
SMA-α: Smooth Muscle Actin
TRI: Trizol
DEPC: Diethyl Pyrocarbonate
cDNA: Complementary DNA
PCR: Polymerase Chain Reaction
qPCR: Quantitative/Real-Time Polymerase Chain Reaction
dH₂O: distilled dH₂O
HRP: Horse Radish Peroxidase
mRNA: messenger RNA
dNTP: Nucleoside triphosphate containing deoxyribose
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
EGTA: Ethylene glycol-bis(2-aminoethylether)-N N'N'-tetraacetic acid
UV: Ultraviolet
EDTA: Ethylene diaminetetraacetic acid
RT-qPCR: Reverse Transcription Quantitative PCR
TdT: Terminal deoxynucleotidyl transferase
NMS: Normal mouse serum
C9: Complement Component 9
Tm: Temperature
Ct: Cycle threshold
C4: Complement Component 4
ES: Embryonic Stem
kDa: Kilodalton
EMT: Epithelial-to-Mesenchymal Transition
C2: Complement Component 2
Ig: Immunoglobulin
H₂O: Dihydrogen Monoxide
MpJ: Metatarsophalangeal joint
%: Per cent
°C: Degree Celsius
D.W: Distilled Water
PT buffer: PBS+Tween20
PB buffer: PBS+BSA
MAC: Membrane Attack Complex
AAA: Abdominal aortic aneurysm
LN: Lupus nephritis
1 Chapter one: Introduction
1.1 Introduction:

1.2 Clinical presentation of SLE:

Systemic lupus erythematosus (SLE), also known as lupus, affects approximately 1–8 per 100,000 people globally (Lupus UK, 2018). Loss of self-tolerance leads to disease, which varies in its manifestation and severity. Common, unspecific, symptoms in SLE may include: painful and swollen joints, chest pain, hair loss, mouth ulcers, swollen lymph nodes, red rash on the face (giving the disease its name, lupus, Latin for “wolf”), fever, headache, and appetite and weight loss. Lupus could appear at the all ages, though symptoms, in most patients, appear between 15-40 years, with a mean between 29-32 years. Conversely, this disease may appear before 15 years of age in a similar percentage (8-15%) to older people (above 55 years of age). Recent studies suggested that the age at the symptom onset could modify clinical and immunological SLE characteristics: in the ‘Euro-Lupus cohort’, it was found that 76 out of 1,000 SLE patients (8%) developed this disease before 14 years of age, with female to male ratio (10 to1). Additionally, in childhood onset patients, the clinical and immunological SLE patterns could slightly differ from SLE in the other patients. Childhood onset patients are likely to develop severe organ involvement, in particular nephritis. However, progression of SLE is quite similar in the adult patients and the childhood onset group. Although explanation of the age-related variability in the disease expression is still unclear, environmental factors and differences in genetic predisposition/responsiveness of the aging immune system could be implicated (Cervera et al. 1993). Women (of childbearing age) are affected more than men. Many women develop more lupus symptoms before menstrual periods or during pregnancy, when there is high estrogen production. Hormone replacement therapy has been found to be associated with an increased risk of developing SLE (Batool et al. 2016). Clinical studies consistently demonstrated female predominance: it was found among the SLE patients, in America 88% were females and in Europe 91% were females, with a female/male ratio (approximately 10:1). This females excess is observed in the 15 to 64 year of age group. These differences in the age-related disease between females and males could well be related to the changes in hormones. The immunological SLE features in males and females, both at the
disease onset and also during the follow up period showed interesting clinical
differences. There is a higher serositis prevalence in males, and less commonly
arthritis. In contrast, during disease progression, development of nephritis and
serositis are similar in both sexes (Johnson et al. 1995).

SLE is a heterogeneous autoimmune disease associated with a wide range of
clinical manifestations which can affect different organs such as skin, lungs,
nervous system, bones and joints, blood, muscles, digestive system and
kidneys. More than 70% of the SLE patients have renal involvement at some
stage of disease. Life expectancy is lower for patients with SLE due to renal
failure, but about 80% of patients survive more than 15 years with treatment
(Batool et al. 2016). Diffuse proliferative glomerulonephritis which has the worst
prognosis, and results in 11-48% of the SLE patients with end stage of renal
disease at five years (Cojocaru et al. 2011). There is proteinuria and
haematuria. In addition, hyperlipidaemia, edema and weight gain may be
observed (Cojocaru et al. 2011). The disease is classified using American
College of Rheumatology (ACR) criteria (Edworthy et al. 1988) drawing clinical
presentation, immunological and biochemical measurements.

Genome-wide screening has been used in an attempt to localise susceptibility
genes in lupus. There is an association between HLA-DR2 and HLA-DR3 and
lupus (Batool et al. 2016). Among identical twins, if only one is affected, there is
a 24% chance that the other one may be as well (NIH 2015). Sunlight, smoking,
deficiency of Vitamin D and certain infections, are thought to increase the risk
to develop SLE. A further study found that there are associations between SLE
and the exposure to mercury in the agricultural work and dental clinics (Batool
et al. 2016), pointing to a significant role of environmental factors. Furthermore,
people being treated for a long-term disease could develop lupus. However,
drug-induced lupus symptoms disappear once the medication which triggers the
episode, is stopped (Manson and Isenberg 2003).

Different techniques are used to diagnose this disease: Antinuclear antibody
(ANA) testing and anti-extractable nuclear antigen (anti-ENA) are used to detect
ANAs. However, the most widely used method is IF. The fluorescence pattern
shows antibody present in the serum, deposits of immunoglobulins and complement components in the skin and kidney. A positive IF test is evidence of SLE. However, other tests routinely conducted in SLE are the levels of complement system (consumption of serum components), kidney function, liver enzymes and blood count (Batool et al. 2016).

There is no cure for lupus, but treatment involves corticosteroids and other immunosuppressants. The patients with SLE who require steroids may go on to develop diabetes, obesity, osteoporosis, puffy round face and difficulty in sleeping (Henderson et al. 2013). Mild disease sometimes may be safely left untreated, but if the treatment is required, non-steroidal anti-inflammatory drugs could be used. Since a large percentage of the patients with SLE develop chronic pain, opioids could be used if the counter drugs do not provide effective relief. Kidney transplants is considered a choice for end stage kidney disease requiring dialysis. Flares are common; the full disease spectrum manifests itself in up to 30% of the patients.

1.2.1 Immunopathogenesis of SLE:

Systemic lupus erythematosus is a B-lymphocyte hyper-reactive disease leading to the production of autoantibodies which target more than 2000 antigens in the body (Salmon and Gordon 1999; Pisetsky 2016). These include proteins and nucleic acids in the nucleus of cells, and constituents of mitochondria (cardiolipin). There is increased deposited immune complexes in various organs, including kidney leading to tissue damage. T lymphocytes from patients with SLE overexpress the oncogene bcl-2, which promotes cell survival by decreasing apoptosis. This process could allow autoreactive T-cell to potentially persist, propagating the autoimmune response (Manson and Isenberg 2003). Hyper-reactive lymphocytes are associated with increased anti-DNA Abs (Salmon and Gordon 1999).

Autoreactive antibodies (Antinuclear antibody, ANA and anti-extractable nuclear antigen, anti-ENA) are pathogenic in SLE and can be detected by immune fluorescence of skin and kidney biopsies.
SLE is a chronic inflammatory disease which, immunologically, is a type-III hypersensitivity response with type-II involvement (de Amorim et al. 2019). Type-III hypersensitivity is the inflammatory response to the presence of circulating ICs while Type-II hypersensitivity describes antibody-dependent reaction to antigens on the surfaces of cells. Type-II hypersensitivity engages antibody dependent cell-mediated cytotoxicity; both, type II and type III responses entail involvement of complement activation via the recognition of immune complexes (antigen-antibody complexes) using C1 (Black 1999).

Antinuclear autoantibodies (ANA) presence is the SLE immunological hallmark. A positive ANA test is sensitive and found in 98% of patients but anti-DNA Abs presence is a much more specific finding, which are seen in approximately 60% of the patients. Anti-DNA antibodies are considered one of a group of antinuclear antibodies (ANA) (Pisetsky 2016). Serial serum anti-DNA Abs concentrations reflect the activity of disease in the patients with SLE. anti-DNA Abs are associated with glomerulonephritis. To prove their renal pathogenicity, a study has shown that injecting human hybridoma-derived anti-DNA antibodies into SCID mice (severe combined immunodeficiency) resulted in renal ICs deposition and associated with proteinuria (Manson and Isenberg 2003). Anti-DNA antibodies were first described 50 years ago by Ceppellini in 1957 as markers of lupus nephritis. American College of Rheumatology in 1997 included these antibodies in the classification criteria of systemic lupus erythematosus. Detection of these antibodies in the serum could occur for up to 9 years before the diagnosis of lupus nephritis. There are different methods to measure anti-DNA Abs, including ELISA and Crithidia luciliae (immunofluorescence) (Almeida Gonzalez et al. 2015).

Recently, there has been growing interest to study the apoptosis role and suggested that it is an important player in the autoimmunity development. It has been demonstrated that the intracellular components make up target autoantigens clustered in blebs on the apoptotic cells surface which are presented as antigens. Essentially, apoptotic cells are normally rapidly cleared.
However, if the apoptosis rate overwhelms this function, or impaired clearance, immunogenicity will be increased. Therefore, apoptosis could provide a central pivot for the development of disease. UV light, drugs or infections could cause increased apoptosis. Alternatively, these factors could induce dysfunctional clearance of apoptotic cells, and deficiency of apoptotic cells clearance could lead to accumulated apoptotic bodies in the body which provide signals for autoreactive B lymphocytes, and that could explain why B-cells lose their tolerance and induce T-cells to help against autoantigens (Munoz et al. 2010). This this in turn will result in increasing the target antigen exposure leading to subsequent production of the autoantibodies (Manson and Isenberg 2003).

The apoptosis process can maintain an appropriate number of cells, in the early stages, it causes nuclear chromatin margination and cytoplasm condensation, in addition to the presence of nuclear fragmentation, and then it begins by degrading DNA to small oligo nucleosomes. It was suggested that the mechanism responsible for the regulation of excess mesangial cells is the apoptosis processd (Watson et al. 2006). Apoptotic cells must be removed, or that could cause immune response leading to inflammation breakdown of self-tolerance. In human, SLE is believed to be driven by autoantigens. It was suggested that SLE autoimmunity might be related to impaired or delayed clearance of apoptotic cells. Therefore, the daunting challenge to the immune system is how to clear the apoptotic cells. This process could fail under certain circumstances leading to form immunogenic apoptotic cells (Darvill and Ballardie 2006; Jourde-Chiche et al. 2012).

Apoptosis can be distinguished from necrosis. Necrosis is characterised by an unregulated cell membrane integrity loss leading to the cell swelling and cell content leakage causing tissue injury and inflammation. In contrast, programmed cell death typified causes cell shrinkage, nuclear condensation and blebbing of membrane leading to dying cells removal by phagocytes. This process results in intracellular cell signalling, including activation of caspases. Apoptosis may be induced by complement: It has been demonstrated in vitro for the first time that the complement components of the terminal complement pathway can induce caspase-3 dependent apoptosis (Nauta et al. 2002).
Caspases cleave and modify proteins, DNA and RNA in the cells, and normally apoptotic cells will be quickly removed by phagocytes before modified contents have been released by damaged cells (Luan et al. 1995; Wenderfer et al. 2008; Munoz et al. 2008). The complement system plays an important role in apoptotic cell recognition and removal. C1q can bind to apoptotic cells (autoantigens), MBL binds to late-apoptotic and necrotic cells (Nauta et al. 2002).

SLE is aggravated by impaired clearance of apoptotic cells by C1q because of three reasons:

- Hereditary C1q (genetic) deficiency which causes SLE.
- SLE leads to the consumption of C1q.
- SLE causes the development of anti-C1q antibodies (Nash et al. 2001), and these autoantibodies bind to (neo-epitope) in the free C1q only which cause hypocomplementemia.

However, it was shown that properdin also plays a significant role in apoptosis by binding to glycosaminoglycans on early apoptotic T cells (Kemper and Hourcade 2008). Properdin binding to dying cells does not compete with C1q and MBL because both of these C1q and MBL interact with structures of DNA in a different way from those that properdin recognises. Additionally, P binds to the surface of eukaryotic cell via glycosaminoglycans and to the altered apoptotic cells surfaces, but the physiological relevance is uncertain (Xu et al. 2008).

1.2.2 The role of complement in immune complex deposition:

Low serum C3 levels due to consumption and increased renal C3 deposition are found in systemic lupus erythematosus. Elevated production of anti-DNA Abs results in increased ICs deposits in lupus nephritis. Complexes of antigen-antibody (immune complexes) are responsible for the glomerulonephritis and a variety of other SLE manifestations, especially vasculitis, an inflammation of
blood vessel. Immune complexes are the end-organ damage cause in lupus because they initiate local inflammation (Figure 1-1).

ICs are removed by binding to Fcγ receptors on macrophages/Kupffer cells (in the case of circulating IC which get retained by the monocyte/macrophage system of the liver) by a mechanism called phagocytosis which is a cellular event where the cells have the ability to engulf and ingest the large molecules after recognising them via the surface receptors (Flannagan et al. 2012). Soluble ICs activate the complement by binding to C1q, and this activation leads to binding of C4b and C3b to the ICs which will be cleared by binding of C4b and C3b to CR1 on the erythrocytes surface. Then, the erythrocytes transport ICs and complement to the spleen and liver, macrophages have CR1 and Fc
receptors, remove ICs from the erythrocytes surface and degrade them. This may be qualitatively different in rodents where there is greater importance for FcγR mediated removal (Hyams et al. 2010; Ehrnthaller et al. 2011; Rojko et al. 2014).

In addition, modulation of molecules on the apoptotic cells cellular membrane releases CD46 and CD59 which can downregulate the complement system and bind to C3b and C4b to promote phagocytosis to apoptotic cells elimination (Ricklin et al. 2010; Noris and Remuzzi 2013).

Immunoglobulin Fc receptors (FcR) are considered to be a key player in the innate and adaptive immune responses regulation. They provide a link between cellular effector machinery and antibody-antigen complexes to lead to endocytosis and phagocytosis of IgG opsonised particles leading to release of inflammatory mediators. There is a distinct receptor for the Fc portion of the IgG molecule which are called FcγRs. These receptors expressed by immune cells to transmit activation or inhibition the signals to cells. Structurally, there are four different classes of FcγRs, recognised in mice which are varying from human, and known as FcγRI (CD64), FcγRIIb (CD32), FcγRIII (CD16) and FcγRIV (CD16-2) (Gerard and Gerard 1994; Smith and Clatworthy 2010; Karsten et al. 2012).

FcγRII and FcγRIII are low-affinity receptors for IgG molecules, expressed on hemopoietic cells. Only mature B lymphocytes can express FcγRII (CD32) that functions as a B-cell activation inhibitor. C3b and complement receptors are effective in immune adherence (Gerard and Gerard 1994).

FcγRIIB is considered the only inhibitory Fc receptor that controls many immune and inflammatory responses aspects, and importantly, its changed expression level is associated with autoimmune disease susceptibility, particularly systemic lupus erythematosus (SLE) (Huang et al. 2011).

ICs are considered one of the causes of the glomerular injury in SLE. These immune complexes could activate the mesangial cells through Fc receptors on
the surface leading to mesangial damage, causing the production of different chemo-attracts for the inflammatory cells. Immune complexes deposit along the basement membrane of glomeruli to develop glomerulonephritis (Scindia et al. 2010).

1.2.3 The role of complement in pro-inflammatory cytokines production:

Cytokines are proteins of low-molecular-weight which act as chemical modulators in the immune system. Some have been identified as therapeutic targets in SLE (BAFF, TNF-α). The cytokines B-lymphocyte stimulator (BLys), interleukin 6, interleukin 17, interleukin 18, type I interferons, and tumor necrosis factor α (TNFα) are involved in the inflammatory process (Danchenko et al. 2006). IL-6, TNF-α and BAFF are the main pro-inflammatory cytokines involved in the immune disorder in human SLE (Scheller et al. 2011). They are secreted by macrophages, monocytes and T cell in addition to mesangial cells in the presence of inflammation, and act to stimulate proliferation of B-cell and production of antibody. Serum levels of IL-6 and BAFF in lupus patients were significantly higher than in healthy controls. Furthermore, high abnormal IL-6 production has been described in MRL/lpr mice leading to develop hyper-reactive B-cells and abnormal T-cells in this model causing increased IgG production (Tackey et al. 2004). In addition, there is an association between high serum TNF-α level and increased lupus nephritis (Muraoka et al. 2006). TNF-α plays an important role at the injury sites by activation of the inflammatory cells or regulation of the immune response in SLE. Elevated TNF-α levels are typically found in SLE patients with progressive kidney damage (Aringer and Smolen 2008). Moreover, B-cell activating factor (BAFF) plays a significant role in B-cell proliferation and differentiation (Steri et al. 2017) suggesting it regulates B cell hyperactivity in SLE.

The complement system plays an important role in the inflammatory reaction and pro-inflammatory cytokines production in SLE. Activation of the complement cascade leads to release of key anaphylatoxins (C5a and C3a) which function as chemo-attractants for macrophages, monocytes, eosinophils, and T lymphocytes, and modulate complement mediated tissue injury through cytokine release. An increase in serum IL-6, TNF-α and BAFF production and
activated complement system is markedly correlated with high serum creatinine protein level, a marker of glomerular/tubular dysfunction in kidneys (Mäkelä et al. 2004; Aringer and Smolen 2008). In addition, deposition of ICs causes renal infiltration involving direct interaction between the infiltrating cells and ICs leading to activate the complement system resulting in activated inflammatory cascade and increased production of proinflammatory cytokines (Leung et al. 2003; Moura et al. 2001).

Macrophages are activated in SLE. Fc and C3b surface receptors on macrophages allow binding to the ICs and C3b to activate the macrophages. Therefore, complement can play an important role in the stimulation of macrophages. The Fc fragments on immunoglobulins can bind to specific Fc receptors on macrophages. Usually, that activates the inflammatory pathway because the macrophages are involved in recognising and digesting the immune complexes (Gronwall et al. 2012). In addition, the macrophages control the repair of kidney tissue by replacing the damaged cells (Silva et al. 2012).

It can be concluded that the role of complement in various inflammatory disorders is multifaceted; for example, the activation of complement can significantly contribute to enhance an inflammation.

1.2.4 The role of complement activation in lupus nephritis:

In systemic lupus erythematosus (SLE), the deposition of complement-containing ICs in the kidney initiates an inflammatory response resulting in glomerulonephritis. CP activation with C3 deposition is considered pathogenic in lupus nephritis (Abrera-Abeleda et al. 2007).

The mechanism of complement activation in SLE is thought to occur as follows: CP activation is initiated when C1q subunit binds with the high avidity to Fc portion of immunoglobulins, especially IgG in ICs. Besides immunoglobulins, C1q also can bind to apoptotic cells to facilitate the removal of apoptotic debris, which affords the complement a significant role in maintaining immune tolerance in SLE. The AP is spontaneously activated by change in C3 conformation to generate hydrolysed C3 (C3(H2O)) leading to activated C3-convertase, or by properdin binding to ICs/apoptotic cells. Furthermore, the MBL-ICs binding to
MASPs activates the LP (Bao et al. 2015). Links between the complement system and SLE were first identified since it was discovered that complement components levels are decreased in patients with SLE. The decrease was shown to be due to consumption of complement proteins due to overactivation of the system in most patients, not absence of complement proteins (though genetic deficiencies can carry an increased risk to develop SLE. In addition, it was noticed that autoantibodies and complement system components are deposited in inflammatory lesions in the glomeruli in the patients with renal diseases. The original models, developed to explain these observations was that ICs containing autoantigens and autoantibodies activate the complement system, and these ICs deposition within kidney causes inflammatory response. The animal models imply that targeting complement would protect the patients from the renal injury development in SLE (Bao et al. 2015).

While the laboratory tests for lupus includes anti-DNA Abs among the other auto-antibodies, activation of complement has proven pivotal in the SLE pathogenesis related ICs damage. For years, complement proteins in serum, specifically, C3 and C4 have been used to gauge the activity of SLE disease (SLEDAI), so much so that the recent suggestions have included the reduction of serum C3 and C4 levels in the SLE classification criteria. In the patients with SLE, complement levels could be low in the serum due to complement components deposition in the damaged kidney (Sandhu and Quan 2017). Deposited C3, C4, and other complement proteins can be easily detected in biopsies (kidney) from patients with SLE. In addition, complement system activity associated with protein levels of CP are generally reduced in relation to the activity of the disease. In SLE, the initial cause of the activation of complement is thought to be the high levels formation of ICs which in turn activate the complement system via CP. Consistent with this, different studies on SLE patients demonstrated that the circulating complement components levels provide a rough guide to the activity of disease.

A significant function for the complement system is to remove ICs from the circulation. This is achieved by ICs binding to the CR1 expressed on erythrocytes. In lupus nephritis (LN), ICs deposits in glomeruli are found
regularly and these contribute to activate the complement system in the kidneys. As such, the pathologic finding C4d/C3+ICs in the kidney biopsies is considered a strong evidence for SLE diagnosis. In LN, deposition of C3/C4d is common, however, there is little correlation of deposition of C3 in kidney and activity of SLE. This is not surprising since the immune complexes are found in the glomeruli in the patients with SLE even without any manifestations which indicate lupus nephritis. Deposition of C3b in kidneys is considered a good marker for LN. An MRI based study suggested that there is possibility to detect deposition of C3b in the kidneys to monitor progression of LN in mice. Furthermore, auto-antibodies against DNA and the C1q are found regularly in the glomerular ICs deposition and these auto-antibodies colocalise with complement components, observed in the recent studies on the kidney biopsies (Leffler et al. 2014).

Therefore, it is thought that complement system is a double-edged sword, also with regard to this disease. On the one hand, it provides significant protective roles against the SLE development, shown by ELISA, where it was found that typically, low levels of the CP components, particularly, C1q, C2, C4 are associated especially with the patients with severe disease (Pickering and Walport 2000). Although the direct pathogenic role of the complement system may still influence the expression of disease (Bao et al. 2002; Bao et al. 2015), the complement system has an adverse effect on LN, however, as complement activation induces LN by an increase in C3 production which may lead to an increase in ICs deposition in the kidney. Since the ICs activate the complement system via CP, and the classical pathway components function is to activate C3; thus, C3 fragments (activated) can deposit on the immune complexes, however, the expectation is that deficiency of C3 would be ‘an even greater predisposing deficiency state for SLE’ (Aharon et al. 1997).

1.3 Murine model of systemic lupus erythematosus:

One very suitable mouse model to study SLE is the MRL/lpr strain, which has been used by other groups who tried to dissect the relative importance of certain complement proteins within their interests.
The spontaneous mutation of *lpr* (lymphoproliferation) is located on chromosome 19 and alters *Fas* receptor transcription (Nagata 1994). *Fas* is a surface-bound receptor that belongs to the tumour necrosis factor receptor (TNF-R) family, inducing apoptosis process by interacting with *Fas* ligand (*FasL*) (Hassin *et al.* 2011). *Fas gene* is essential in the development of T cells, in addition to its role in killing tumour cells (Figure 1-2) (Andersen *et al.* 2006). Precursor T cells (more than 95%) die by programmed cell death when T cells develop in the thymus. Because *Fas gene* has a significant role in apoptosis, a mutation could mainly cause autoimmune disease and lymphoproliferation in *lpr* mice. *Fas* transcription defect is caused by inserting ETn (retrotransposon) leading to aberrant, non-functional transcripts (Figure 1-3). MRL/*lpr* mice showed that lack of functional FasR causes a defect in apoptosis process (Nagata 1994). This recessive mutation located on chromosome 19 causes a change in the single amino acid (Leu > Phe) leading to prevent FasL binding to FasR. Therefore, *Fas* signaling deficiency results in an SLE-like phenotype because of a defect in *Fas*-mediated apoptosis (Itoh and Nagata 1993; Reap *et al.* 1995; Teachey *et al.* 2010). The symptoms of lupus are attributed to the deficiency of *Fas* in MRL/*lpr* mouse model which are CD4+ T-cells and B-cells dependent. High levels of IgG and IgM, in particular, anti-DNA antibodies are detected. *Fas* deletion in B cells and/or T cells leads to higher levels of autoantibodies (Reap *et al.* 1995).

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Figure 1-2: Fas-mediated programmed cell death showing schematically the regions (signal transducing and inhibitory domains).
Figure 1-3: Developing of a murine model of systemic lupus erythematosus (MRL/MpJ-Faslpr/J).

An early transposable element inserting in FasR intron2. FasR gene intron2 restriction map of WT and lpr of MRL strain. Exon3 is represented by the shaded boxes. In lpr gene, LTR of ETn is represented by the open boxes. Major restriction enzymes are indicated (E, EcoRI; B, BamHI and HindIII).

The MRL-lpr or lpr mutant mouse model is homozygous for the lymphoproliferation spontaneous mutation (Fas/lpr) and develops systemic autoimmunity associated with massive lymphadenopathy, splenomegaly, vasculitis, arthritis and glomerulonephritis (Hewicker et al. 1990), although the penetrance of each can vary between lines which are studied by various groups.

Several crosses of inbred strains could be used to develop MRL strain such as C3H/Di, C57BL/6, LG/J and AKR/J. In this study, the MRL/lpr mouse model used was on C57BL/6 background because this was the genetic background of the properdin deficient mouse line.

Manifestation of SLE disease could be starting in MRL/lpr at three months of age and is associated with high levels of circulating immune complexes (Hewicker et al. 1990). This mouse model shows accelerated mortality rate, high levels of immunoglobulins, including anti-DNA antibodies and anti-Sm resulting in large amounts of ICs (Watson et al. 1992). At 4-7 months of age, MRL/lpr mice develop extensive renal disease. Skin lesions associated with loss of hair
and formation of scab are one of the characteristic manifestations in this mouse model (McBrearty *et al.* 1998). It was reported also that 14-20 weeks old MRL/\(^{lpr}\) mice show increased body weight that could be due to impaired carbohydrate and lipid metabolism (Andrews *et al.* 1978, Clark *et al.* 1998; Choi *et al.* 2006; Schaevitz *et al.* 2017).

### 1.3.1 How does SLE in humans differ from MRL mice?

This mouse model develops lupus-like diseases associated with a subset of symptoms akin to human lupus such as the production of anti-DNA Abs, lymphoid hyperplasia, increased apoptosis, activated inflammatory and complement cascades and nephritis associated with IC deposits. In general, in human SLE and this disease model, females are found to be more susceptible. However, although massive lymphadenopathy is not the typical of SLE in human, recent studies reported that there is an association of *FasR* and *Fas* ligand polymorphisms with the SLE susceptibility and increased double-negative T lymphocytes in the kidneys of the patients with SLE pointing to failed thymic deletion.

The mouse model cannot encompass all aspects of human disease. Disease progression is considered one of the main differences between human and this mouse model where no external effector breaks peripheral tolerance. MRL/\(^{lpr}\) mice fail to inactivate or delete autoreactive B lymphocytes with specificity for DNA/chromatin and RNA, suggesting a central tolerance induction defect (Perry *et al.* 2011). Moreover, contrasting with this disease model, in human SLE, no single gene defect is responsible for SLE. Even a genetic deficiency in C1q does not give 100% penetrance to develop SLE (Van Schaarenburg *et al.* 2016).

As for many models of chronic inflammation, protracted disease in human is studied in a short time frame governed by welfare considerations but with the benefit of studying the effect of a deleted candidate gene of interest.
1.4 The complement system:

Because properdin-deficient mice were used in this work, an expanded overview of the complement system follows, before a detailed summary of work achieved so far by crossing MRL/lpr mice with existing complement deficient mice.

The complement system bridges innate and adaptive immunity (Menges et al. 2005). Complement is a cascade of carefully regulated enzymatic reactions. Complement proteins are found in blood and other body fluids, and specific complement receptors mediate cellular effects, including complement receptor 2 (CR2) on B cells (Dempsey et al. 1996) and complement C5a receptors (C5aR) on immune cells such as neutrophils, macrophages, and dendritic cells (Gutzmer et al. 2006; Connelly et al. 2007), in addition to CR3 on monocytes and macrophages (Ross and Vetvicka 1993).

The complement system has a pathogenic role during autoimmune diseases, where inflammation is non-resolving. Inflammation in general may be initiated by infection, changes in cell surface due to ischemia, or injury. The function of inflammation is to protect the body against microbes, in addition to repair injuries, and to contribute to apoptotic cells or immune complexes removal (Zipfel and Skerka, 2009). Complement activation promotes inflammation by generating anaphylatoxins (C3a and C5a) which facilitate cell recruitment, phagocytosis, B cell activation. Non-enzymatic formation of MAC (membrane attack complex) leads to pore formation causing rapid lytic destruction (Zipfel and Skerka, 2009) but is also detectable in a soluble form in SLE (Chauhan and Moore 2006).

A deficiency in complement system components, such as C2 and C4 deficiency, could result in tissue injury, as was observed in autoimmune diseases due to immune complexes are not solubilised and removed leading to to FcyR-mediated macrophage activation (Walport 2001).
Furthermore, alterations in the complement regulatory proteins expression that leads to the excessive activation of the complement system can also contribute to tissue injury because they no longer protect the body cells from self-attack. Complement activation occurs via three pathways, the classical pathway, the lectin pathway and alternative pathway (Figure 1-4). Central to each pathway is formation of C3 convertases, which cleave C3 to C3a and C3b. The C3 split products effects are as follows: C3a mediates mast cells degranulation, vasodilatation, and bronchial constriction, while C3b has a role in opsonisation. In addition, C3dg binds to B cells (CR2) leading to lower the threshold of activation to produce antibodies. However, during complement activation, C3b binds to altered self-target cells to initiate assembly of the C3 convertase. Furthermore, C3b can bind directly to C3 convertase forming C5 convertase, which in turn will be cleaved into C5a and C5b. The C5a fragment acts as a chemo-attractant for leukocytes. C5a binds to C5aR and C5L2 receptors. C5L2 receptor (previously known as GPR77) is activated after binding to the C5a. C5aR is a classical G protein-coupled receptor, but C5L2 is structurally homologous and does not link to G protein coupling (Li et al. 2012). The complement convertases are assembled on target surfaces that activate their proteolytic potential to mark the target for phagocytosis or lysis. In addition, complement-dependent recognition of apoptotic cells can take a place via the CP, LP and AP (Mevorach et al. 1998; Ogden et al. 2001).

1.4.1 The classical pathway:
The classical pathway (CP) activation occurs by antigen-antibody complexes and, importantly for the immunopathogenesis of SLE, other molecular patterns such as DNA and histones. CP was the first complement pathway discovered and it is initiated by the recognition of IgG and IgM bound to antigen, complement fixing isotypes of IgG, in addition to several other proteins, via the C1 complex which is formed by C1q, and dimeric C1r and C1s respectively. The C1 complex is activated subsequently and cleaves complement C4 into C4a and C4b. The C4b cleaves bound complement component C2a, to form enzymatically active of C4bC2a complex that cleaves complement C3 and is the C3 convertase of classical pathway. C4bC2a cleaves C3 into C3a and C3b.
This C3 convertase promotes generation of further C3b molecules that bind to the surface of cells leading to the C5 convertase C4b2aC3b formation which cleaves C5 into C5a and C5b. C5b initiates the non-enzymatic formation of MAC (Li et al. 2012).

1.4.2 The lectin pathway:

The lectin pathway (LP) activation occurs by binding of pathogen-associated molecular patterns with lectin binding proteins, such as ficolins and mannose-binding lectin (MBL) which are found complexed with three serine proteases MASP (mannose-binding protein-associated serine protease) called MASP-1, MASP-2, and MASP-3. Furthermore, the MASP-2 gene encodes a non-enzymatic protein (MAp19). MBL-MASP-2 and ficolin-MASP-2 complexes bind to the surface of pathogens to activate MASP-2. Activated MASP-2 cleaves complement C4 and C2. C4 complement will be cleaved into C4a and C4b, C4b bind to the surface of cell. C2 complement will be cleaved into C2a and C2b. As with the CP, the C3 and C5 convertases formation is via binding of C4b and C2a, with the final stages of complement, concluding again with the MAC. MAp1 (also known as MAp44) (Degn et al. 2009) inhibits the lectin complement pathway activation (Pavlov et al. 2012). MASP-1 is involved in both the lectin and the alternative pathways activation, because MASP-1 and MASP-3 deficient mice showed a lack of the activation of alternative pathway (Iwaki et al. 2011).

1.4.3 The alternative pathway

The alternative pathway (AP) forms a complement activation amplification loop. It is initiated by C3 spontaneous hydrolysis with the Factor B to form C3(H2O)B complex caused to constant low-level auto-activation in the fluid phase (so-called tickover). Factor D cleaves factor B into Ba and Bb. Its activity is stabilised by properdin binding to the C3bBb complex, thereby stabilising the C3 convertase. It was shown that the alternative pathway could be activated without any antibody participation. Properdin contributes to the alternative pathway activation by C3 and C5 convertases stabilisation, and the complement activation amplifying ongoing by any of the three pathways (Li et al. 2012).
Figure 1-4: An overview of complement system activation cascade.
1.4.4 Complement factor P (Properdin):

Properdin is a soluble, plasma glycoprotein found in blood, and is secreted by leukocytes, macrophages, monocytes, T cell and activated endothelial cells, and has a designated role in the alternative complement pathway. Properdin was first discovered by Dr Louis Pillemer and collaborators as an important complement component of an antibody-independent complement activation pathway in 1954 (Pillemer et al. 1954), and then in the 1970s it was shown as a stabilising component of C3 convertase of the alternative pathway. The name properdin is derived from a Latin word (perdere) which means to destroy (Kemper and Hourcade 2008). In addition, properdin is necessary in the classical and mannose-binding lectin pathways amplification (Kimura et al. 2010).

The gene that encodes properdin (termed PFC/Pfc) is localised on the chromosome X, in human and mouse (Evans et al. 1990; Nolan et al. 1992). Properdin exists as a basic glycoprotein in plasma at a concentration of about 15-25μg/ml, which is formed rod-like by head-to-tail associations of identical monomers. Complement factor P is released from stimulated neutrophil granules and consists of an oligomers mixture of a 53-kDa monomer, which are mostly as dimer, trimer, or tetramer, in a constant ratio of 1:2:1. Each properdin monomer is composed of an N-terminal domain and seven thrombospondin repeats (TSRs) of type one domain, and each about 60 amino acids long, representing independently folded modules (Figure 1-5). The TSRs are numbered in order from the N-terminus from TSR-0 until TSR-6. TSR-4 and TSR-5 are involved in the C3bBb complex stabilisation (Higgins et al. 1995). It was shown that myeloid cells could be the main source of properdin in the blood that was released from activated neutrophils (Kimura et al. 2010).
Figure 1-5: Schematic representation showing the structure of properdin. Dimer, trimer and tetramer of properdin (From head-to-tail the binding of monomeric properdin).
Properdin is the only positive regulator of AP and acts by binding and stabilising the inherently labile C3 convertases C3bBb and C3bBbC3b. Properdin is able to bind to a surface ligand of C3b alone or to iC3b, C3bB or C3bBb complex by mean of one of its subunits. Because of its oligomeric structure, properdin which is bound to C3bB and C3bBb enables more C3b to bind. C3bBb is a short-lived complex (t½ about 90 seconds), that is cleaved by Factor D in Mg²⁺ presence at a single site in the Factor B subunit, leading to the release of the Factor B amino-terminal fragment (Ba) and the activation of the serine protease domain (Bb). Substantially properdin extends their half-lives and protects the convertases of alternative pathway (C3bBb and C3bBb5b) from rapid inactivation (Fearon and Austen 1975). Properdin attains its full stabilising effect on C3bBb by binding to more than one C3b or more ligands at a time on the surface (Hourcade 2006). In addition, it stabilises the C5 convertases by binding to C3b in C3bnBb and C3b2b4b, then inhibits the C3 convertase deactivation by Factors I and H. Properdin amplifies more C3b deposition on the pathogens activating surfaces to generate more of anaphylatoxins C3a and C5a, thus the AP is called an amplification loop of complement activation. The alternative pathway amplifies the complement system effector functions (Lutz et al. 2007). It has been shown that blocking the AP via neutralising factor D by using human serum (diluted 1:2), was shown to inhibits more than 80% of C5a and terminal complement complex (TCC) formation (Figure 1-6). This has been demonstrated using anti-factor D mAb (clone 166–32, IgG1) and block the classical pathway by anti-C2 mAb (clone 175–62, IgG1). Factor D is the rate-limiting serine protease in an amplification loop of AP. C5a and the TCC formation were induced by IgM in solid phase and IgG solid- and human aggregated IgG in fluid-phase (Harboe et al. 2004).

Properdin which is released from activated or degranulated neutrophils may differently respond comparing to neutrophils in blood which release properdin in serum. This thus provides a platform for new convertase assembly and function of the alternative pathway C3 convertase. Convertase assembly begins with properdin binding to a target surface. This directs complement activation of the alternative pathway because properdin, via its multiple protein subunits is able to potentiate both, the binding to ligands clustered on a microbial surface.
and the binding of C3b molecules. Properdin is bound by one ligand-binding site and provides additional sites for C3b binding via the remaining polypeptide chains (between 1 and 3) and C3bBb assembly. Until now it is shown that alternative pathway could be activated by wherever properdin is bound. Properdin binds differently to different types of LPS and lipooligosaccharide (LOS) modifications (Kemper and Hourcade 2008). In addition, a recent study showed that properdin could interact with IgG-C3 complexes to amplify the complement activation (Jelezarova et al. 2000).

Figure 1-6: Schematic representation showing the role of properdin in stabilising of C3-convertase of the alternative pathway.

Properdin role in the alternative pathway activation is essential for the C3/C5 convertases. Properdin deficiency is a relatively rare event. Properdin has been shown to have a role in the adaptive immune response, results from the cells which express this protein. The neutrophils ability to secrete properdin from their granules upon stimulation by C5a, resulting local inflammation by activation of complement. Thus, it generates the alternative pathway C3 convertase to produce more C3b upon enzymatic cleavage. Another properdin role in the adaptative immune response modulation relies on the properdin synthesis by T cells and macrophages, releasing upon anaphylatoxins (C5a and C3a). However, activated T lymphocytes secrete INFγ, that in turn plays a negative regulatory effect on the macrophages ability to produce more properdin. The anaphylatoxins lead to local inflammation (Harboe et al. 2004).
My group has been instrumental in working out the relative role of complement properdin in a variety of diseases. The picture proved complex: while the presence of properdin was detrimental in models that involved the formation of specific or the presence of natural antibodies (Ivanovska et al. 2008; Dimitrova et al. 2012, Dupont et al. 2014; Zhou et al. 2012), the absence of properdin worsened disease when a skew of macrophage activity away from M1 (typical of the genetic background) was problematic (Ivanovska et al. 2008; Dupont et al. 2014). Others have shown that in the genetic absence of surface bound complement down-regulators as is found in haemolytic uremic syndrome, the blockade of properdin may be of therapeutic benefit (Ueda et al. 2018). Properdin deficient mice had reduced mRNA FcγR2b expression in spleen (Dupont et al. 2014), also FACS data showed that properdin deficient mice had less FcγR2b in splenic B cells from properdin deficient mice compared to wildtype mice, suggesting that properdin deficiency affected FcγR2b protein expression not only mRNA. The analysis of FcgR2b expression in lupus prone mice was added in this work.

1.4.5 Properdin deficiencies:

1.4.5.1 In human:

Data available on deficiencies of properdin are confined to case studies carried out on the patients showing severe microbial infections, in particular meningitis. A deficiency of human properdin was first reported in 1982 in a Swedish family and it was associated with a high susceptibility to *meningococcal meningitis* with high mortality (Sjoholm et al. 1982). Deficiency of properdin is considered the most common genetic defect of the alternative pathway components, and it is inherited in an X-linked manner, with all index cases for deficiency of properdin being male. There are three types of properdin deficiency phenotypes: type I, type II and type III. Type I complete deficiency is the most common type associated with no serum properdin (less than 0.01 mg/l) in spite of a normal mRNA level. Type II is an incomplete deficiency associated with less than 10% of the serum properdin normal level. Type III is characterised by a normal serum properdin concentration but a dysfunction in properdin protein, it was detected in one family only. Individuals have properdin deficiency have several infections
in the early years of life. However, not all of individuals with deficiency of properdin get infections, and it was suggested that could be because of high concentration of subclasses IgG2 as contributory susceptibility factor (Schejbel et al. 2009). However, nearly 50% of people with properdin deficiency remain perfectly healthy and never developed meningitis (Fijen et al. 1998).

1.4.6 Complement system functions:

1.4.6.1 Opsonisation:

Activation of any of three complement pathways results C3-5 cleavage leading to produce C3b, C4b and C5b which can act as the opsonin proteins via binding to the relevant receptors (Lindorfer et al. 2003; Ehrnthaller et al. 2011).

1.4.6.2 Chemotaxis

It is a moving biological molecules/cells process such as phagocytes toward the inflammatory/injured site under the chemical gradients influence which are produced from the chemotactic proteins (Ricklin et al. 2010; Isfort et al. 2011).

1.4.6.3 Activation state of cells:

Complement activity is believed to go well beyond the known classic immune functions, suggesting that this system could be also required for development, activity, integrity and homeostasis of the general cells and tissues. However, it has been discovered that activation of the complement system is not confined to the extracellular space but it could occur within cells, suggesting involvement of the complement system in the basic processes regulation in the cells, particularly metabolism. In fact, in the late 1980s, it was suggested that there is a functional connection between lipid metabolism regulation and complement activity in the blood, when it was shown that C3a (desarginated form) stimulates the accumulation of triglyceride and transportation of glucose in the adipocytes through C3a-C5aR2 interaction (Hess and Kemper 2016; Kolev and Kemper 2017).

Recent researches have been made particularly to understand how the cell metabolic machinery can enable and drive T lymphocyte immunity. However,
normally quiescent T lymphocytes maintain low glycolytic activity and reduced nutrient intake using OXPHOS (oxidative phosphorylation) as a source for energy, while activated T cells will increase both OXPHOS and glycolysis. Differentiation of T lymphocytes into effector T cells can initiate dramatic changes in the cellular metabolic pathway such as aerobic glycolysis upregulation that is required for growth, expansion and acquisition of the effector function. However, it seems that key inflammatory cytokine production requires an exceptionally high glycolytic demand on T lymphocytes. The earlier work established to study the autocrine anaphylatoxin activity role during activation of mouse CD4+/CD8+ T lymphocytes suggested already that there is a direct impact of complement on metabolism of the cells: autocrine engagement of C3aR and C5aR on T lymphocytes can activate the signaling cascades of phosphatidylinositol-4,5-bisphosphate 3-kinase PI(3)K and protein kinase B (PKB) to promote activation, proliferation and survival of CD4+/CD8+ T lymphocytes. However, it was shown that C5ar1 deficient mice have decreased lifespan of circulating T cells, and these cells were unable to produce normal IFN-γ amounts and showed defects in Th1 responses. Furthermore, recent study demonstrated that T lymphocytes harbor an intracellular C5 activation system driven by C5aR1 (intracellularly expressed) which is required for induction of the high oxygen metabolism levels that sustain Th1 responses particularly as augmented generation of C5a (intracellular) from stores of C5 upon costimulation of CD46 and TCR inducing mitochondrial ROS (reactive oxygen species) production. These discoveries allowed us to have a better understanding how tightly the complement system and metabolism of cell are interlinked (Hess and Kemper 2016; Kolev and Kemper 2017).

1.4.7 Crossing of complement deficient mice with MRL/lpr mice:
Some classical components deficiencies (e.g., C4 and C1q) were associated with an increase in lupus incidence in humans and lupus-like disease in murine knockout strains. Lupus-like disease development in C4- and C1q-deficient mice is associated with defects in B lymphocyte tolerance in the former and defects in apoptotic clearance cells in the latter. Thus, the complement cascade could have protective action against the disease. However, previous studies
investigated the role of complement system in the renal disease development in the murine SLE model (MRL/\textit{lp}r mouse) by crossing these mutant mice with genetically engineered complement deficient mice. MRL/\textit{lp}r mouse model succumbs to a lupus-like syndrome, including typically glomerulonephritis which progresses to kidney failure at 4 to 8 months of age.

An important protective activity of Decay-accelerating factor (DAF) was identified in MRL/\textit{lp}r murine model. Therefore, to dissect the protection mechanism in this disease model, the effect of C3 gene ablation was evaluated in MRL/\textit{lp}r-Daf-1 deficient mice. No significant differences were observed in splenomegaly and lymphadenopathy between complement-sufficient mice and complement-deficient mice. However, complement deficiency reduced strikingly severity and incidence of dermatitis in MRL/\textit{lp}r-Daf-1 deficient mice; thus, it was concluded that DAF functions as a complement regulator to protect MRL/\textit{lp}r mice from inflammation in the skin (Miwa \textit{et al.} 2007).

The alternative pathway could enhance the disease by amplifying the classical pathway activation and its pro-inflammatory effects. However, the AP effect on inflammation and destruction of tissue in glomerulonephritis was to this point unknown. Therefore, to determine the alternative complement pathway role in autoimmune nephritis, Bf-deficient mice were crossed to MRL/\textit{lp}r mice for four generations. The results demonstrated that factor B plays a significant role in the glomerulonephritis pathogenesis in MRL/\textit{lp}r mice and that the amplification loop of AP has prominent effects on C3 levels in the serum. There was less proteinuria, less glomerular IgG deposition, lower vasculitis, lower serum levels of ant-DNA antibodies, and mesangial proliferation was mild without fibrosis or inflammation comparing with control mice (Table 1-1) (Watanabe \textit{et al.} 2000). However, Bf deficient mice are different to their wild-type littermates at MHC that has H-2b haplotype rather than H-2k haplotype in WT MRL mice. The gene that encodes Factor B lies within the class III region of MHC locus, and prior gene mapping studies in MRL/\textit{lp}r mouse model showed that there was link between MHC haplotype and renal disease development. Therefore, although it was thought unlikely that MHC was a significant factor, it was nonetheless unclear whether the protective effect which was seen in the Bf-deficient mice was due
to the lack of AP activation or the H-2b haplotype. Thus, another mouse model was developed by crossing factor D deficient mice with MRL/\textit{pr} mice for nine generations. The results demonstrated that pathologic and functional disease in the kidney was significantly reduced in Df-deficient mice compared to controls to confirm that the alternative pathway plays an important role in the proliferative glomerulonephritis in MRL/\textit{pr} mice. There was less glomerular C3 deposition, less serum creatinine levels, Bowman’s capsule and vascular spaces were clear (Table 1-1) (Elliott et al. 2004).

These studies provide evidence for the importance of complement activation in the disease development in MRL/\textit{pr} mice. As properdin is the only known naturally positive regulator of complement activation this thesis studied the role of properdin in the pathogenesis of lupus in MRL/\textit{pr} mice on C57Bl/6 background.

Table 1-1: Summary of recent findings in MRL/\textit{pr} alternative pathway deficient mice

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>KO mice comparing to WT mice</th>
<th>Conclusion</th>
</tr>
</thead>
</table>
| MRL/\textit{pr} BIKO | ❖ Less proteinuria  
❖ Less glomerular IgG deposition  
❖ Lower vasculitis  
❖ Normal serum C3 levels  
❖ Mesangial proliferation was mild without fibrosis or inflammation | Factor B has a significant role in the immunopathogenesis of glomerulonephritis and vasculitis in MRL/\textit{pr} mice |
| MRL/\textit{pr} DIKO | ❖ Glomerular IgG deposition was similar to WT mice  
❖ Similar levels of proteinuria and autoantibody  
❖ Less glomerular C3 deposition  
❖ Less serum creatinine levels  
❖ Bowman’s capsule and vascular spaces were clear  
❖ Normal glomerular structure | Deficiency of fD is considered to be protective against renal diseases development in MRL/\textit{pr} mice. |
1.5 Hypothesis:

The following hypothesis was tested: ‘absence of properdin lessens lupus disease in MRL/lpr mice’.

1.6 Aims and objectives:

This study was to determine whether in the absence of properdin, the systemic and organ-specific inflammation is less severe. A novel mouse model was generated by crossing the lupus-prone MRL/MpJ-\textit{Fas}\textsuperscript{lpr}/J mice with properdin knock-out mice to produce lupus-prone strain of properdin-deficient (MRL/lpr \textsuperscript{P\textsuperscript{KO}}) mice, and lupus-prone properdin-sufficient littermates (MRL/lpr \textsuperscript{P\textsuperscript{WT}}) as controls.

Matched mice were analysed for disease activity markers, such as anti-DNA Abs, CP and LP activity, pro-inflammatory cytokines (TNF-\textgreek{a}, IL-6 and BAFF) and creatinine in the serum using ELISA, CD21/CD35 surface expression in splenic B220 positive cell population using flow cytometry, mRNA expression of splenic Fc\textgamma RIIB and C5aR, and renal SMA-\textgreek{a} and nephrin (inflammatory makers) using normal PCR and qPCR, mesangial immune complexes deposition and F4/80 (macrophage marker') and CD3 (T cell marker) reactivity using IF, and Caspase-3 protein expression in the kidney using WB.
2 Chapter two: Materials and Methods
2.1 Materials and methods:

2.2 Developing of MRL/\textit{pr} properdin-deficient mice:

For the purpose of studying the role of properdin in the immunopathology of systemic lupus erythematosus, a novel mouse model was generated. The commercially available lupus-prone MRL/\textit{pr} mutant mouse line was crossed with the University of Leicester owned properdin knock-out mouse line. They were both on the C57Bl/6 background.

Properdin-deficient mice were genetically engineered by specifically targeting the mouse properdin gene (described in Stover \textit{et al.} 2008) and have been extensively researched by my group and collaborators who received the line. MRL/\textit{pr} embryos were obtained from European Mouse Mutant Archive (EMMA, France. EM:00037). The first rederivation unexpectedly produced pups of white coat colour, presumably arising from a BALB/c derivative of MRL/\textit{pr}, so a replacement straw of embryos was requested and sent with delay. Rederivation then produced pups of black coat colour as expected. These were used for inter-crossing and confirmed to be \textit{Fas} mutant by PCR. Next, properdin deficient male or properdin heterozygous female mice were crossed with \textit{Fas} mutant mice. This step introduced the properdin deficient allele but produced \textit{Fas} heterozygous mice. Further pairs were set up to achieve \textit{Fas} mutant and properdin wildtype or knockout mice, bearing in mind x-chromosomal linkage of the properdin gene.

When required, WT mice from the properdin deficient colony (bred as female heterozygous with wildtype male) were taken as controls.

2.3 Maintenance of mice:

Animal experimentation was performed in accordance with UK Home Office regulations and institutional guidelines. Mice were housed in a specific pathogen-free barrier facility in groups in ventilated cages at 21°C, 50% humidity, with 12/12h light/dark cycle, and had ad libitum access to food and water. Mice were maintained on 5LF2 (TestDiet). The cage floor was covered
with corn cob as bedding material; nesting material (sizzle pet) was made from recycled paper. There was equal environmental enhancement.

2.4 Genotyping:

2.4.1 Mouse-ear snips:

Polymerase chain reaction (PCR) was used for amplification of target genes (Fas and properdin) (Table 2-1) using genomic DNA prepared from ear snips. The reactions were performed in a total volume of 25 µl containing 14.8 µl PCR-grade dH2O, 2.5µl (10x) reaction buffer, 2µl MgCl2 (25mM), 4µl dNTP mix (1.25 mM), 0.2µl Taq polymerase (5 U/µL), 0.5µl of forward primer (200ng/ml), 0.5µl of reverse primer (200ng/ml) and 0.5µl gDNA in PCR Eppendorf tubes. The PCR tubes were loaded in the (Bio-Rad Thermocycler) PCR machine. The program started at 94°C for 3 min. Next, the reaction was cycled to 35 times including denaturation (94°C for 30sec), annealing: 55°C (properdin) 65°C (Fas WT) and 58°C (Fas mut) for 30sec, and elongation (72°C for 45sec). Finally, the temperature was kept at 72°C for 5min for the final extension and then the reaction stopped and stabilized at 4°C. Fas primers were combined as follows: Fas1/2= to amplify the wildtype allele, Fas1/3= for the mutant allele.

Table 2-1: The set of primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence of primers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas1</td>
<td>5’ ACAGCATAAAGATTCCATTTGCTGCT 3’</td>
<td>Ma et al. (1996)</td>
</tr>
<tr>
<td>Fas2</td>
<td>5’ TGAGTAATGGGCTCAGTGCAGCA 3’</td>
<td></td>
</tr>
<tr>
<td>Fas3</td>
<td>5’ CAAATTTTATTGGTTCGAGCACCCA 3’</td>
<td></td>
</tr>
<tr>
<td>Properdin</td>
<td><strong>For WT mice</strong>&lt;br&gt;5’-GGATTATCACATACATCGTGACGG-3’&lt;br&gt;5’-CTCTTGAAGTGGCAGCTACAG-3’&lt;br&gt;<strong>For KO mice</strong>&lt;br&gt;5’-CGTGCAATCCATCTTGTCA-3’&lt;br&gt;5’-CAAGGGCAGTCTGAGCATGC-3’</td>
<td>Stover</td>
</tr>
</tbody>
</table>

2.4.2 Agarose Gel Electrophoresis:

1% agarose gel was prepared (1g agarose in 100 ml of 0.5x Tris-Borate-EDTA buffer), heated for 3min in a microwave. 10µg/ml ethidium bromide was added. Then, 2µl of loading dye (10x DNA gel loading dye, 15% (w/v) BP blue, 50%
(v/v) glycerol and 0.5mM EDTA) was added to the PCR product. 7µl of each sample and 5µl of GeneRuler 50bp DNA Ladder, (0.5μg/μL) were loaded on the gel. The electrophoresis was performed at a constant voltage of 90V for 60 minutes. Finally, DNA on the gel was visualized using UV illumination.

2.5 Blue Native Gel Protocol:

2.5.1 Technique:

Blue native gel was done to detect oligomer formation of properdin protein according to the manufacturer’s protocol (NativePAGE™ Novex® Bis-Tris Gel System, Cat. No. BN1001BOX). The gel cassette was prepared: the gel cassette was opened, then pouched and drained away the gel packaging buffer. The gel was removed from the pouch. Then, the gel cassette was rinsed with deionised water. Then, in one smooth motion, gently the comb was pulled out of the cassette. The sample wells were rinsed with 1X NativePAGE™ Cathode Buffer. The gel was inverted and shacked to remove the buffer. The wells were filled with 1X NativePAGE™ Cathode Buffer. Procedure using XCell™ SureLock™ Mini-Cell: XCell™ SureLock™ Mini-Cell requires ~200 mL buffer for the Upper Buffer Chamber and ~600 mL buffer for the Lower Buffer Chamber. The two gels were oriented in the Mini-Cell such that the notched “well” side of the cassette faces inwards toward the Buffer Core. The gels were seated on the bottom of the Mini-Cell and locked into place with the Gel Tension Wedge. 10 µl of the sample were loaded onto the gel. Samples are loaded before filling the Upper Buffer chamber to provide easy visualisation of the sample wells containing the blue Cathode Buffer. The Upper Buffer Chamber was filled with a small amount of the running buffer to check for tightness of seal. Once the seal is tight, the Upper Buffer Chamber (inner) was filled with ~200 mL of the appropriate 1X Cathode buffer. The Lower (outer) Buffer Chamber was filled with ~600 mL of the 1X Anode Buffer. The lid was placed on the assembled Mini-Cell. The samples were run: 150 V, 90–115 minutes, Start: 12–16 mA, End: 2–4 mA. Equilibrate the Gel Strip: After electrophoresis, the lane was marked on the cassette that will be excised prior to opening the cassette. The gel was removed from the cassette. With the gel adhering to one plate, the desired gel strip (lane) was excised along the markings on the
cassette using a Gel Knife. **Loading and running the gel:** Carefully each gel strip was transferred to the SDS-PAGE gel, then, the gel was run for 30 mins at 90 V Am400, then 120V, 400Am.

Transferring the protein from the gel to the membrane and antibody staining were done as explained in section 2.12.3 using polyclonal sheep anti-human/mouse/rat properdin protein (AF8216-SP, diluted 1/1000; NovusBio) as a primary antibody and polyclonal donkey anti-sheep IgG, HRP conjugated antibody (HAF016, diluted 1/4000; NovusBio) as a secondary antibody.

### 2.6 Samples:

Blood samples were collected by cardiac puncture of terminally anaesthetised mice. Kidneys were harvested and cut longitudinally. These were embedded in OCT (Tissue-Tek OCT, Sakura, Netherlands) using a cryomold, and snap-frozen in an isopentane bath cooled with liquid nitrogen and stored at -80°C for IF studies. For RNA and protein analysis, the remainder was dissected and snap frozen in liquid nitrogen and then stored at -80°C (**Table 2-2**).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mice number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td></td>
<td>frozen</td>
</tr>
<tr>
<td>WT mice</td>
<td>6</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;WT&lt;/sup&gt; mice</td>
<td>9</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;MO&lt;/sup&gt; mice</td>
<td>8</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;Het&lt;/sup&gt; mice</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mice number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>WT mice</td>
<td>6</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;WT&lt;/sup&gt; mice</td>
<td>7</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;MO&lt;/sup&gt; mice</td>
<td>8</td>
</tr>
<tr>
<td>MRL/prP&lt;sup&gt;Het&lt;/sup&gt; mice</td>
<td>3</td>
</tr>
</tbody>
</table>

* The different colours mean that these samples were taken from different mice.
* RNA was isolated from the kidney.

### 2.7 Enzyme-linked immunosorbent assay (ELISA):

#### 2.7.1 Anti-DNA Abs ELISA:

This study was performed according to a recent study (Seery et al. 1997). The plates were coated with 100μl poly-L-lysine (Sigma, Cat. No. P2636, 5.0 μg/ml
/dilution 1:5000) for 1.5 h at 37°C. Then, the wells were washed with 200 μl per well of washing buffer 3 times, 5 minutes each. After washing, 100μl ‘Deoxyribonucleic acid sodium salt from calf thymus’ (Sigma, Cat. No. D1501, mixed dsDNA and ssDNA) were added at 5.0 μg/ml in 1xPBS and incubated overnight at 4°C. After washing, the serum samples were diluted (1:4000, 1:2000, 1:1000, 1:500 and 1:250) with (0.1% Tween-20 and 0.5% BSA) and incubated for 1.5h at room temperature. After washing,100μl of polyclonal goat anti-mouse IgG immunoglobulins HRP conjugated (Dako, Cat. No. P0447, diluted 1:200, 1.5 μg/ml) were added and incubated at room temperature for 1.5h. After washing, 100μl TMB were added and incubated at room temperature for 5-10 minutes, and then, 50μl stop solution were added. Finally, the result was read immediately using ELISA reader at 492 nm (TECAN Magelian for F50).

2.7.2 Functional complement assays:

ELISA was used to quantitatively measure the activity of three complement pathways at the level of C5b-9 formation in serum samples from experimental mice following a published method (Kotimaa et al. 2015).

The plates were coated with Human IgM purified immunoglobulin (BIO RAD, Cat. No. OBT1524) at 2μg/ml in CB buffer (Table 2-3) (diluted 1:1000) to activate the classical pathway (CP), Mannan from Saccharomyces cerevisiae (Sigma, Cat. No. M7504l) at 10μg/ml in coating buffer (CB) (diluted 1:1000) to activate the lectin pathway (LP) and LPS (Lipopolysaccharide from Salmonella enteriditis S form, concentration 1mg/ml, HyCult Biotechnology, Cat. No.HC4059) at 1μg/ml in PBS/10 mM MgCl2 (Table 2-3) (diluted 1:1000) to activate the alternative pathway (AP). The plates were incubated for 16h at 4°C overnight, then were washed with 200μl of PT buffer (Table 2-3) 3 times, 5 minutes each. The three complement pathways were blocked with 150μl of PB buffer for 90 minutes at 37°C (Table 2-3). After washing, the serum samples were added in triplicate and incubated for 1h at 37°C using BVB++ for CP&LP and BVB++/MgEGTA for AP. Serial dilutions (0.5, 1, 2.5, 5, 10 and 20%) of commercial normal mouse serum (CNMS) were used, heat inactivated CNMS (heated at 56°C for 30 minutes) were used as controls. Heat-inactivated serum was used in this study to demonstrate
heat liability of the activity. After washing, 100μl polyclonal rabbit complement component 9 antibody (MyBioSource, Cat. No. MBS2027772; diluted 1:200 /1 µg/ml) were added and incubated at 37°C for 2h. After washing, 100μl polyclonal swine anti-rabbit HRP antibody (Dako, Cat. No. P0399, diluted 1:200/1.5 µg/ml) were added and incubated at 37°C for 1h. After washing, 100μl coloured substrate 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) were added per well and left at room temperature for 5-10 minutes, and then 50μl stop solution (Table 2-3) were added. Finally, the result was read immediately using ELISA reader at 405 nm (TECAN Magelian for F50).

Table 2-3: Preparation of ELISA buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating buffer</td>
<td>1.5g Na2CO3 2.9g NaHCO3 dH2O 1litre pH 9.6 Then autoclaved</td>
</tr>
<tr>
<td>PBS/10 mM MgCl₂</td>
<td>1ml MgCl₂ (1M) 99ml dH₂O</td>
</tr>
<tr>
<td>0.5 mM MgCl₂</td>
<td>500µl MgCl₂ 1M 999.5ml dH₂O</td>
</tr>
<tr>
<td>PT (Washing buffer)</td>
<td>400ml 1xPBS 200µl Tween-20 (0.05%)</td>
</tr>
<tr>
<td>PB (Blocking buffer)</td>
<td>400ml 1xPBS 4g BSA</td>
</tr>
<tr>
<td>Stop solution</td>
<td>1.11ml H₂SO₄ (Sigma) 8.89ml dH₂O</td>
</tr>
<tr>
<td>BVB++</td>
<td>2ml Veronal buffer (1X) 20µl 2mM CaCl₂ 5µl 0.5mM MgCl₂ 100g BSA (Thermo Fisher Scientific) 50µl Tween-20 (Chem) pH 7.5 Then complete the volume to 10ml with dH₂O</td>
</tr>
<tr>
<td>BVB++/MgEGTA</td>
<td>200µl 10 mM EGTA 50µl 5mM MgCl₂ The volume was completed to 10ml with BVB++</td>
</tr>
</tbody>
</table>

2.7.3 Aggregated immunoglobulins ELISAs:

This study was conducted to measure the activity of three complement pathways in the commercial NMS using aggregated immunoglobulins according to recent studies and published papers (Gadd and Reid 1981, Kotimaa et al. 2015) who showed that C3 can bind as C3b to IgG in aggregates. The results were compared to heat-inactivated NMS and background absorbance.
ELISA plates were coated with 100μl serial dilutions (1:1000 (20μg/ml), 1:5000 (4μg/ml) and 1:10000 (2μg/ml)) of aggregated mouse gamma globulin fraction (Rockland Cat. No D6090100), heated at 63°C for 90 minutes, using CB buffer for CP and LP and PBS/10 mM MgCl₂ for AP, and incubated for 16h at 4°C overnight. After washing, the plates were blocked with 150μl of PB buffer for 90 minutes at 37°C. 20% of NMS was used. The protocol was performed as shown in section 2.7.2.

2.7.4 BAFF ELISA:

The BAFF development kit (R&D Systems, MBLYS0) was used to quantify the level of BAFF in the serum samples from the experimental mice.

Following the manufacturer’s protocol, mouse BAFF/BLyS Standard was reconstituted with Calibrator Diluent RD6-12 to prepare a stock solution (3000 pg/mL). Serial concentrations were prepared (3000, 1500, 750, 375, 188, 93.8, 46.9 and 0) pg/mL (Figure 2-1) by pipetting 200 μL of Calibrator Diluent RD6-12 into each tube. Mouse BAFF/BLyS Standard (3000 pg/mL) was used as the high standard, and Calibrator Diluent RD6-12 was used as the zero standard (0 pg/mL). The samples were diluted (1:100) with Calibrator Diluent RD6-12. 80 μL of Assay Diluent RD1N were added to each well. 40 μL of standard or sample were added, then covered with the adhesive strip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. Each well was aspirated and washed four times with Wash Buffer (400 μL) 5 minutes each. 120 μL of Mouse BAFF/BLyS Conjugate were added to each well, then covered with a new adhesive strip and incubated for 2 hours at room temperature on the shaker, then aspirated and washed. 120 μL of Substrate Solution were added to each well, then incubated for 30 minutes at room temperature on the benchtop. 120 μL of Stop Solution were added to each well. Finally, the result was read immediately using ELISA reader at 450nm (TECAN Magelian for F50). The concentration of BAFF in the samples was multiplied x 100 (dilution factor 1:100).
Figure 2-1: An example of BAFF standard curve. Absorbance was measured at 450nm.

2.7.5IL-6 ELISA:

The murine IL-6 ELISA development kit (PeproTech, 900-TM50) was used to quantify the level of IL-6 in the serum samples from the experimental mice.

Following the manufacturer’s protocol, the capture antibody (diluted 1:100 at 1µg/ml) immediately was added and incubated overnight at room temperature. The plate was washed (4times, 5 minutes each) with 300µl of washing buffer. Then, 300µl of the blocking buffer were added and incubated for 1h at room temperature. After washing, the standard was diluted with the diluent (diluted 1:250 at 2500pg/ml), then the standard was prepared using serial concentrations with the diluent (4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.6 and zero) pg/ml (Figure 2-2). The serum samples were diluted further (1:100) with the diluent. Then, 100µl from the diluted standard/serum samples were added and incubated at room temperature for 2h. After washing, 100µl per well of the biotin detection antibody (diluted 1:1000 at 0.1µg/ml) were added and incubated at room temperature for 2h. After washing, 100µl of the Streptavidin-HRP Conjugate (diluted 1:666 at 0.15µg/ml) were added and incubated at room temperature for 30 minutes. After washing, 100µl per well of substrate solution (TMB) were added and incubated at room temperature for 15-20 minutes, and then 50µl of the stop solution were added. Finally, the result was read immediately using ELISA reader at 450nm (TECAN Magelian for F50). The concentration of IL-6 in the samples was multiplied x 100 (dilution factor 1:100).
The Murine TNF-α ELISA development kit (PeproTech 900-TM54) was used to quantify the level of TNF-alpha in the serum samples from the experimental mice.

Following the manufacturer’s protocol, the capture antibody was diluted (diluted 1:200 at 0.50µg/ml) and incubated overnight at room temperature. The plate was washed (4times, 5 minutes each) with 300µl per well of washing buffer. Then, 300µl of the blocking buffer were added and incubated for 1h at room temperature. After washing, the standard was diluted with the diluent (diluted 1:400 at 2500pg/ml), then prepared using serial concentrations (2500, 1250, 625, 312.5, 156.25, 78.125, 39.6, 19.5, 9.7 and zero) pg/ml (Figure 2-3). The serum samples were diluted further (1:100) with the diluent. Then, 100µl from the diluted standard/serum samples were added and incubated at room temperature for 2h. After washing, 100µl per well of the biotin detection antibody (diluted 1:400 at 0.25µg/ml) were added and incubated at room temperature for 2h. After washing, 100µl per well of the Streptavidin-HRP Conjugate (diluted 1:4000 at 0.025µg/ml) were added and incubated at room temperature for 30 minutes. After washing, 100µl of TMB were added and incubated at room temperature for 15-20 minutes, and then 50µl of the stop solution were added. Finally, the result was read immediately using ELISA reader at 450nm (TECAN
Magelian for F50). The concentration of TNF-α in the samples was multiplied x 100 (dilution factor 1:100).

![Graph of TNF-α standard curve](image)

**Figure 2-3:** An example of TNF-α standard curve. Absorbance was measured at 450nm.

### 2.8 Cell Proliferation Assay (MTS):

#### 2.8.1 Principle:

This colourimetric test is widely used for the sensitive quantification to assess the effect of various effectors on different living cells to measure cell proliferation (cells could respond to cytokines or growth factors), viability or cytotoxicity. This technique is usually based on the ability of NAD (P) H-dependent dehydrogenase enzyme to reduce (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) to form coloured formazan (soluble in cell culture media) by the living cells, not in the dead cells (Riss et al. 2004).

#### 2.8.2 Technique:

MTS Assay Kit (Cell Proliferation Cat. No. ab197010) was used to measure the splenocytes proliferation from wt mouse spleen stimulated with the serum samples from the experimental mice.

The protocol was adopted from Zhu et al. (2012). The splenocytes were prepared as shown in (section 2.13.1), and the number of living solenocytes was adjusted to 2x10^4 cell/50µl in RPMI-1640 media. The serum samples were filtered using
syringe filters (0.2 µm) (PALL) with RPMI-1640 media. 50µl of RPMI-1640 media containing 2x10⁴ cell were added to 50µl of RPMI-1640 media containing serum per well in 96 Well Cell Culture Cluster (Costar 3596’) to adjust the final volume to 100µl per well containing 1x10⁴ splenocytes/2% serum. The samples were incubated for 24h at 37°C. After incubation, 10µl of MTS reagent were added per well and incubated for 4h at 37°C. The optical density was measured using the ELISA reader (TECAN Magelian for F50) at 492nm wavelength.

2.9 Serum creatinine:

2.9.1 Technique:

This colourimetric assay was performed according to the manufacturer’s protocol (QuantiChromTM Creatinine Assay Kit, BioAssay Systems (DICT-500)) to measure the creatinine level in the serum samples from the experimental mice, also, to use commercial normal mouse serum (CNMS) and heat inactivated CNMS as controls.

The standard was diluted with dH₂O (diluted 1:25/working concentration 2mg/dL (for blood samples)). The serum samples were diluted (1:100) with dH₂O. 30µl per well in triplicate of the diluted standard and were added to 96 well microplates (Nunc Maxisorp). The working reagent was prepared by mixing per well reaction at least 100µl from reagent A, and 100 µl from reagent B. 200µl of working reagent were added quickly per well. The plate was tapped briefly to mix the reagents. The optical density was read immediately (OD0) and then at 5 min (OD5) at 492nm using (TECAN Magelian for F50). The data were exported to Excel file, and the creatinine concentration of each sample was calculated as follows:

\[
\text{Serum creatinine} = \frac{\text{OD5 samples} - \text{OD0 samples}}{\text{OD5 standard} - \text{OD0 standard}} \times [\text{STD}] \text{ (mg/dl)}
\]

- STD is 2mg/dL for the serum samples.
- The concentration of creatinine in the samples was multiplied x 100.
2.10 Immunofluorescence (IF):

2.10.1 Technique:

Frozen kidney sections (5μm) were fixed with acetone and methanol (1:1) for 10 minutes at room temperature, then washed with 1xPBS 3 times, 3 minutes each. The sections were blocked with 5% BSA for 30 minutes at room temperature. 30 μl of the primary antibody solution (Figure 2-4) were added to each section; then were incubated at 4°C overnight. The negative controls (no primary antibody) were used in this study. After washing, 30 μl of the secondary antibody solution (Figure 2-4) were added to each section, then were incubated at room temperature for 1h. After washing, coverslipped in mountant. Images were acquired with a confocal microscope (OLYMPUS 1X70) using FLUOVIEW software, five non-overlapping fields at a magnification of x60 were captured per section. The confocal microscope used does not detect blue colour; thus, there was no DAPI reactivity to show.

Figure 2-4: Experimental design for IF analysis of mouse kidneys.
2.10.2 Optimisation:

Optimisation was performed with different dilutions of antibodies. In bold, the dilutions that were chosen for the study.

- Activated C3 fragments primary antibody: 1: 100, 1:50 and 1:25.
- Immunoglobulins secondary antibody: 1: 100, 1:50 and 1:25.
- Rat anti-Mouse F4/80 primary antibody: 1: 200, 1:100 and 1:50.
- FITC Rat anti-mouse CD3 secondary antibody: 1: 200, 1:100 and 1:50.
- Polyclonal FITC labelled rabbit anti-rat IgG secondary antibody: 1: 200, 1:100 and 1:50.
- Rabbit anti-Rat IgG (H+L) Secondary antibody: 1: 300, 1:200 and 1:100.

A negative control (no primary antibody) was used in order to ensure there is no specific background staining.

2.10.3 IF-data acquisition:

All the data matched the same conditions and were quantified using exposure times between 25-30 seconds. Absorption wavelength FITC (494-518nm), TR (589-617nm). The confocal microscope channels (red and green lasers) were used in parallel to detect the yellow colour which represents ICs.

Thresholding of images by ImageJ was used to split IC images into three segments using green/red or yellow scale value to define their boundary. The advantage is that it reduces the complexity of the data and simplifies the process of recognition and measuring the intensity of staining.

2.11 Reverse Transcriptase Polymerase Chain reaction (RT-PCR):

2.11.1 RNA preparation:

Total RNA was isolated from the kidneys, using TRI reagent, and the manufacturer’s instructions were followed (Thermo SCIENTIFIC Cat. No. #K1632). The samples were added to 1 ml of TRI (Sigma), each sample when homogenised was transferred to 5ml reaction tubes. To guarantee that nucleoprotein complexes dissociation will be complete, the samples were left at
room temperature for 5 minutes, then, 0.2 ml of chloroform (Fisher Scientific, Loughborough, UK) were added to per ml of used TRI reagent, then, were shaken for 15 seconds using vortex mixer (Stuart), and left at room temperature for 10–15 minutes. Later, the samples were centrifuged (GenFuge 24D) at 12,000 x g for 15 minutes to separate the mixture into 3 phases: a red organic phase (containing protein), interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). 0.5 ml of 2-propanol (Fisher Scientific, Loughborough, UK) per ml of TRI reagent was added to each sample, then were left for 5–10 minutes at room temperature. Later, the samples were centrifuged (GenFuge 24D) at 12,000 x g for 10 minutes. In the bottom of the tubes, the RNA formed a visible yellowish pellet. Then, the supernatant was removed and 1 ml of 75% (v/v) ethanol was added to per 1 ml of used TRI reagent for washing the pellet. The samples were shaken in a vortex mixer and then were centrifuged for 5 minutes at 7,500 x g. RNA pellet was left for 5–10 minutes at room temperature, and then 50µl of DEPC-treated dH2O (0.02% (v/v) Diethylpyrocarbonate) was mixed with the RNA pellet. To make a complete dissolution, the samples were mixed by repeated pipetting for 10–15 minutes at 55–60 °C using a hot plate (Grant). The concentration of the extracted RNA was measured using NanoDrop One (Thermo scientific) in triplicate (the mean of three readings was taken). Then, the RNA samples were kept at -20°C.

2.11.2 First strand cDNA synthesis:

To remove contaminating genomic DNA from RNA samples, DNase (RNase free) digestion (Thermo SCIENTIFIC Cat. No. #K1632) was done using 2 µl RNA sample, 10 µl Reaction buffer 10 X, 5 µl DNase, then incubation for 30 minutes at 37 °C, and then adding 1µl EDTA 0.5 mM and 82µl PCR – grade dH2O, and then incubated for 10 minutes at 65 °C, then kept at -20 °C. The concentration of the extracted RNA after was measured in triplicate using NanoDrop One (Thermo scientific). The RNA volume needed was calculated by the following equation (RNA concentration/1000, then (RNA con./1000 x µl)/10µg/ml).
First strand cDNA was synthesized from total extracted RNA using RevertAid H Minus Kit (Thermo scientific K1632) for RT-PCR (Invitrogen) following the manufacturer’s instructions. 0.1ng-5000ng of poly (A) total RNA was used to generate first strand cDNA as the initial step of a two-step RT-PCR protocol. The RNA volume needed was calculated by the following equation ($RNA\ quantity = \frac{5000ng}{total\ RNA}$). Tube A mix was prepared using 5 µg of total RNA, 1 µl Oligo dT 18 primer (0.5 µg/µL), then the total volume was made up to till 12 µl with water (nuclease free). Tube B of the MasterMix was prepared containing 4 µl of 5x reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl2, 50 mM DTT), 1 µl of Ribolock RNase inhibitor (20 U/µL), and 2 µl of dNTP Mix (10mM) and 1 µl of Revert Aid H Minus reverse transcriptase (200 U/µL). The tube A was run for 3 minutes for initial denaturation at 65.0°C. Then, 8 µl of tube B Master Mix was added to tube A for a total volume of 20 µl then, samples were initially denatured at 65.0°C for a further 5 minutes. The mixture was incubated at 42°C for 1hr, and the final extension was carried out for 5min at 70°C. The cDNA was kept at -20°C until further use.

2.11.3 Polymerase chain reaction (PCR): 
Polymerase chain reaction (PCR) was used to amplify GAPDH, nephrin, SMA-α, FcγRIIB and C5aR (Table 2-5). The reaction was performed in a total volume of 25 µl containing 10.8 µl PCR-grade dH2O, 2.5µl (10x) reaction buffer, 2.5µl MgCl2 (25mM), 4µl dNTP mix (1.25 mM), 0.2µl Taq polymerase (5 U/µL), 2µl of forward primer (5 µM), 2µl of reverse primer (5 µM) and 1µl cDNA in PCR tubes. The PCR tubes were loaded in the (Bio-Rad Thermocycler) PCR machine and the program started at 94°C for 3 min. Next, the reaction was cycled up to 30 times including denaturation (94°C for 30sec), annealing (53-66°C depending on primers annealing temperature for 30sec), and elongation (72°C for 45sec) steps. Finally, the temperature was kept at 72°C for 5min for the final extension and then the reaction stopped and stabilized at 4°C.

2.11.4 Real-Time Quantitative polymerase chain reaction (RT-qPCR): 
The RT-qPCR technique using SYBR Green I dye (SensiMix™ SYBR Kit, Cat.QT605) was used to quantify different target genes expression. SYBR
Green dye is a high-performance reagent designed for superior sensitivity and specificity on various real-time instruments. The qPCR reactions were performed using 20μl total volume strip tubes and caps. Each tube contains 3μl of cDNA (previously diluted 1:4 in dH2O), 10μl SYBRGreen (containing MgCl₂) and 2μl of diluted forward primer (5μM), 2μl of diluted reverse primer (5μM) for genes of interest (1μl of stock primer (100μM) in 19μl dH2O for each forward and reverse primer) and made up to 20μl by adding 3μl dH2O. In parallel, water rather than cDNA template was used as a negative control (NTC). The gene expression was quantified and normalised to GAPDH expression. GAPDH was previously used as a housekeeping gene in qPCR for MRL/lpr mice and properdin knockout mice because it is stable (Shiozawa et al. 2004; Mohamed 2013).

The samples were run on the Corbett: Rotor-Gene™ 6000 machines and software using the RT-qPCR reaction program in (Table 2-4). The temperature cycling conditions and primers sequences are shown in (Table 2-5).

### Table 2-4: The temperature and conditions of cycling of qRT-PCR

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Cycle Point for targeted gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>Hold @ 95°C, 10min 0s</td>
</tr>
<tr>
<td>Cycling (40 repeats)</td>
<td>Step 1: Hold @ 95°C, 15s</td>
</tr>
<tr>
<td></td>
<td>Step 2: Hold @ X°C, 60s, acquiring to Cycling A</td>
</tr>
<tr>
<td></td>
<td>Step 3: Hold @ 72°C, 15s</td>
</tr>
<tr>
<td>Melting</td>
<td>Ramp from 55°C to 95°C</td>
</tr>
<tr>
<td></td>
<td>Hold for 90s on the 1st step</td>
</tr>
<tr>
<td></td>
<td>Hold for 5s on next steps, Melt A</td>
</tr>
</tbody>
</table>

At the end of each extension step, the signal of fluorescence was measured. It was checked the occurrence of one distinct peak for each sample for each gene in a melting curve, to ensure that only one product was amplified and contaminants absence or dimer of primer products.

The amplification of each sample is tracked in real time and the machine can calculate the threshold of cycles (CT) value for each sample. In RT-qPCR, a positive reaction is detected by a fluorescent signal accumulation.
The CT is defined as the cycle number which are required for the fluorescent signal to cross the threshold. The relative position of the crossing of this CT line with the threshold against the cycle numbers (on the x-axis) gives an indication of the gene expression abundance. Therefore, when CT < 29, it means on abundant amount of the cDNA target; CT between 30-37 means indicative of moderate amounts of target, however, if CT is 38-40 there are likely to be negligible amounts of target and that the amplification may have occurred because of some environmental contamination. The threshold of each target gene product was set manually and CT under the threshold through all 40 cycles means a negative result or that the expression of the target gene is very low or undetectable.

The ΔCT method was used to calculate the relative expression target gene in each sample.

\[
\Delta CT \text{ (test)} = CT \text{ (target gene)} - CT \text{ (ref gene)}
\]

\[
\Delta CT \text{ (calibrator)} = CT \text{ (target gene)} - CT \text{ (ref gene)}
\]

\[
2^{\Delta CT} = \text{the level of the gene expression}
\]

CT: Cycle number at which detectable signal is achieved.

Calibrator: The control sample, meaning an untreated sample.

Test: Test sample means treated.

Reference gene (ref): The reference gene is one that is expressed at a constant level in all test and control samples without being affected by the experimental treatment in the study.

**Table 2-5: The set of primers used in qPCR.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence of primers</th>
<th>Annealing Temp. °C</th>
<th>Reference sequence accession number (NCBI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’ CCTGGAGAAAACCTGCCAAGTATG-3’ 5’ AGAGTTGGAGTCTTGTTGAGTC-3’</td>
<td>60</td>
<td>NM-001289745.1</td>
</tr>
<tr>
<td>Nephrin</td>
<td>5’ CCAACACTGGAAGAGGTGT-3’ 5’ CTGGTCGTAGATTCCCTTG-3’</td>
<td>60</td>
<td>NM-019459.2</td>
</tr>
<tr>
<td>SMA-α</td>
<td>5’ GATCCACGAAACCACCTA-3’ 5’ CACGAGTAACAAATCAAAGC-3’</td>
<td>55</td>
<td>NM_007392</td>
</tr>
<tr>
<td>FcyRIIB</td>
<td>5’ CTGAGGCTGAGAATACGATC-3’ 5’ GTGGATCGATAGCAGAAGAG-3’</td>
<td>60</td>
<td>NM_001077.189.1</td>
</tr>
<tr>
<td>C5aR</td>
<td>5’ GGTATTAACTATGGTGGGGGTAGC-3’ 5’ GCAGCCAGAAGATAAAAGAAAAAGA-3’</td>
<td>60</td>
<td>NM_007577.4</td>
</tr>
</tbody>
</table>
2.12 Protein immunoblot (Western blot):

2.12.1 Preparation of cell lysate:

ACK lysing buffer (GIBCO, Cat. No. A10492) containing phenyl Methyl sulfonyl fluoride (PMSF) was added at 9 times the kidney weight in lysing matrix D-tubes (Cat. No. 9613-050). The tubes were homogenised using Fast Prep®-24 machine for 20 minutes; then were placed in ice for 5 minutes. This process repeated 3 times. The tubes then were centrifuged at 13,000xg for 15 minutes at 4°C (Sigma 1-14 K), then, the supernatants were transferred to new reaction tubes and kept at -80°C.

2.12.2 Protein Assay:

Protein assay was done according to the manufacturer’s protocol (Pierce™ 660nm Protein Assay Reagent, Cat. No. 22660) (Figure 2-5). A range of bovine serum albumin (BSA) (Thermo Fisher Scientific) concentrations was prepared (standard curve) (0, 25, 50, 125, 250, 500, 750, 1000, 1500 and 2000) µg/ml diluted with 1xPBS. For the kidney lysates, protein concentration was measured first using NanoDrop One (Thermo scientific), then the average of three readings was taken. Each sample was diluted with 1xPBS 1:15. 10µl of each BSA concentration and diluted kidney lysates were added per well in triplicate in 96 well microplates (Nunc Maxisorp), and then it was added 150µl of the protein assay reagent per well and incubated for 5 minutes at room temperature, and the colour was changed from yellow to blue, and that change was proportional to the BSA and kidney lysates concentrations. The plate was spectrophotometrically read using (TECAN Magelian for F50) at 660nm.
2.12.3 Caspase-3 Western Blot Protocol:

20μg total protein was subjected to the Western blotting under reducing conditions using 2X Laemmli-10mM DTT sample buffer (Table 2-6) in a ratio 1:1 with the sample. The mixture was boiled at 95-100°C for 5 minutes then it was ready to run on the SDS-PAGE.

<table>
<thead>
<tr>
<th>Table 2-6: Preparation 10ml of 2X Laemmli sample buffer.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>1 M Tris-Cl (pH 6.8)</td>
</tr>
<tr>
<td>H2O</td>
</tr>
<tr>
<td>Bromophenol blue was added to a final concentration of 0.02% (w/v) and the 2X Laemmli sample buffer was stored at room temperature.</td>
</tr>
</tbody>
</table>

The electrophoresis under reducing conditions was done by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as mentioned in (Table 2-7). The 15% SDS-PAGE was used to increase the chance of active caspase-3 detection which is a small polypeptide protein of 17&23 kDa. The samples were loaded in running buffer (also called standard migration 62 buffer) 1x Tris-glycine (Table 2-8) for 1 hour at 90 V. Then, the gel was blotted overnight at 30V as shown in (Figure 2-6) for protein transfer onto a nitrocellulose membrane (Thermo Fisher.com) using 1x transfer buffer (Table 2-9). The membrane was blocked with blocking buffer (5% skimmed milk in PBS) for 1 hour at room temperature, washed with 1X Tris Buffered Saline-Tween-20 (TBST) buffer.
(Table 2-10) 3 times, 15 minutes each, and incubated with Monoclonal Mouse Anti-Mouse Caspase-3 Antibody (Santa Cruz, Cat. No. Sc-56053, concentration 1mg/ml/diluted 1:400/working concentration 2.5µg/ml) overnight at 4°C. After washing 3 times 15 minutes each by washing step (with TBS buffer containing 0.05% Tween 20), Goat Anti-Mouse IgG H&L (HRP) (Abcam, Cat. No. ab6789) was used as a secondary antibody at a final concentration of 2.5 µg/ml (1:400 dilution) at room temperature for 1 hour, followed by an additional washing step (3 times 15 minutes each with TBS buffer containing 0.05% Tween 20). Immunoreactive proteins were visualized using Western blot chemiluminescence reagents (Amersham Biosciences) in accordance with the instructions of the manufacturer. The protein bands on the blot membrane were detected using a Bio-Rad ChemDoc Touch imager.

2.12.4 β-actin Western Blot Protocol:

To assess whether equal amounts of protein were loaded, β-actin was used. For stripping and reprobing, the stripping buffer (20ml 10% SDS, 12.5ml of 1.5M Tris pH 6.8 and 67.5ml ddH2O) was warmed to 50°C, then was incubated with the blot for 45 minutes at 50°C. The blot was then rinsed under running water for 1h, and then was washed with washing buffer 5 times, 5 minutes each, then blocked using 5% skimmed milk. The blot was incubated overnight at 4°C with 10ml of Monoclonal Anti-β-Actin antibody produced in mouse (Sigma, Cat. No. 5316, diluted 1:400/ 3.75µg/ml), then was washed with washing buffer 3 times, 5 minutes each, then was incubated at room temperature for 2h with 10ml of Donkey Anti Mouse IgG H&L (HRP) (Dako, Cat. No. P0447, diluted 1:400/ 2.5µg/ml). After washing with washing buffer 3 times, 5 minutes each, protein bands were detected as described above.
Table 2-7: Preparation 15% sodium dodecyl sulfate-polyacrylamide gel for western blotting.

<table>
<thead>
<tr>
<th>Resolving Gel (15% Acrylamide)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>3ml</td>
</tr>
<tr>
<td>ddH2O</td>
<td>2.8ml</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>2ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>80μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>80μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>8ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gel (6% Acrylamide)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>0.75ml</td>
</tr>
<tr>
<td>ddH2O</td>
<td>2.9ml</td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td>1.25ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>5ml</td>
</tr>
</tbody>
</table>

Table 2-8: Preparation of 1x running buffer for western blotting

<table>
<thead>
<tr>
<th>1X Running buffer pH 8.3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris base</td>
<td></td>
</tr>
<tr>
<td>190 mM glycine</td>
<td></td>
</tr>
<tr>
<td>0.1% SDS</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-9: Preparation of 1x Transfer buffer for western blotting

<table>
<thead>
<tr>
<th>1X Transfer buffer pH 8.3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris base</td>
<td></td>
</tr>
<tr>
<td>190 mM glycine</td>
<td></td>
</tr>
<tr>
<td>20% methanol</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2-6: A scheme representing the order of layers of blotting gel in western blot procedure.
2.13 Flow Cytometry:

2.13.1 Preparation of a suspension of single cells from spleen:

The spleen was placed on the 40µM strainer, on 50ml tubes. 0.5 ml of media (RPMI-1640, with L-glutamine and sodium bicarbonate, liquid, sterile-filtered, Cat. No. R8758-500ml) was added. The spleen was mashed against the cell strainer using the rubber end of a plunger from a 2.5 ml syringe to make single splenocytes. This was repeated 4 times. The splenocytes were spun down (CR422, Jouan) centrifuge at 270xg for 5 minutes at 4°C. The supernatant was removed, then the cells were washed again using RPMI media. The splenocytes were spun down again at 270xg for 5 minutes at 4°C. The supernatant was removed, then 1 ml of lysing buffer (to remove red blood cells) (Table 2-11) was added for 5 minutes on ice, then 15 ml of FACS buffer (Table 2-11) were added to dilute the lysing buffer. The splenocytes were spun down again at 270xg for 5 minutes at 4°C. The supernatant was removed, and 1 ml of FACS buffer was added. The number of splenocytes was counted using hemocytometer. 10 µl of trypan blue stain were added to 10 µl of splenocytes. 10 µl of stained splenocytes were placed on the hemocytometer to count the living cells under the light microscope (Nikon Eclipse TS100, magnification 10x). The number of living solenocytes was adjusted to 1x10^6 cell/100µl in FACS buffer.

2.13.2 Splenocytes staining:

Flow cytometry was used to analyse splenocytes populations, including B220, CD21/35 and double positive staining B220+CD21/35+ in the experimental mice according to recent study and published papers (Axcrona et al. 1996; Takahashi et al. 1997). CD21 (Complement receptor 2; CR2) and CD35 (Complement receptor 1; CR1) are alternative products of one Cr2 gene, which the antibody cannot distinguish).

Table 2-10: Preparation of 1x washing buffer for western blotting

<table>
<thead>
<tr>
<th>1X Tris Buffered Saline-Tween-20 (TBST) pH (7.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.42 g Tris base</td>
</tr>
<tr>
<td>8 g NaCl</td>
</tr>
<tr>
<td>0.1% Tween-20</td>
</tr>
<tr>
<td>Complete to 1L dH2O</td>
</tr>
</tbody>
</table>

53
FACS buffer (100µl) containing 1x10^6 cell was added to each FACS tube (6 in total for each sample). 0.5 µl of Purified anti-mouse CD16/32 Antibody (Biolegend, Cat. No. 101302, diluted 1:200 at 2.5 µg/ml) was added per 100 µl of splenocytes and incubated on ice for 30 minutes. After incubation, the splenocytes were centrifuged using Sigma 1-14 K centrifuge at 4°C for 5 minutes at 7,500xg. The supernatant was removed, then 100µl of FACS buffer were added to the pellet, and cells were centrifuged again at 4°C for 5 minutes at 7,500xg. The supernatant was removed, then, 100µl of FACS buffer were added to the pellet, then staining was done as shown in (Figure 2-7). The tubes were covered with foil and incubated in ice for 30 minutes. After incubation, the splenocytes were centrifuged using Sigma 1-14 K centrifuge at 4°C for 5 minutes at 7,500 g. The supernatant was removed, 100µl of FACS buffer were added to wash the pellet, and then the splenocytes were centrifuged at 4°C for 5 minutes at 7,500 g, the supernatant was removed again. Then, 100µl of 4% Paraformaldehyde (Table 2-11) were added to the pellet. The splenocytes were analysed using BD FACSCANTO II machine, software FACSDiva Version 6.1.3, and event: 10000. Voltage (V) was: 429 (FSC), 511 (SSC), 269 (FITC) and 264 (PE).

![Figure 2-7: Splenocytes staining, experimental design.](image)
Table 2-11: Preparation of FACS buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NaN₃</td>
<td>0.65mg NaN₃ (Fisher Chemical) 10ml H₂O Add 1ml from the first step to 9ml of H₂O to make 10% NaN₃</td>
</tr>
<tr>
<td>10x PBS</td>
<td>25.6g Na₂HPO₄-7H₂O (Fisher Chemical) 80g NaCl (Fisher Chemical) 2g KCl (Fisher Chemical) 2g KH₂PO₄ (Fisher Chemical) Added to 1 litre with H₂O. Autoclaved at 121°C for 40 minutes</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>7.5ml Fetal Calf Serum (Thermo Scientific) 2.5ml 10 NaNaN₃ (Fisher Chemical) 25ml 10x PBS 215ml H₂O</td>
</tr>
<tr>
<td>Lysing buffer</td>
<td>802mg 150mM NH₄Cl (Fisher Chemical) 100mg of 10 mM KHCO₃ (Fisher Chemical) 20µl of 0.5M EDTA (Thermo Scientific) Make up to 100ml, autoclave or sterile in the filter</td>
</tr>
<tr>
<td>4% Paraformaldehyde</td>
<td>10ml 10% PFA 2.5ml 10X PBS 12ml H₂O.</td>
</tr>
</tbody>
</table>

2.13.3 Optimisation:

Optimisation was performed with different dilution factors to get the right dilution:

- Purified anti-mouse CD16/32 antibody was optimised by the other students in our lab, and 1:200 was chosen.
- For the other antibodies, three sequential dilutions were tested: 1:300, 1:200 and 1:100, and 1:200 was adopted.

2.14 Adherent splenocytes/ Real-Time Polymerase Chain Reaction (qPCR):

Splenocytes were prepared as shown in (section 2.13.1) and adjusted to 1x10³ cell/1ml in RPMI media. 12ml of RPMI media containing 12x10³ cell were spun down using (CR422, Jouan) centrifuge at 270xg for 5 minutes at 4°C, then, The supernatant was removed, then 1ml of TRI reagent was added to the pellet and the manufacturer’s instructions were followed (Thermo SCIENTIFIC Cat. No. #K1632) (section 2.11).
To study adherent splenocytes, cells were adjusted to $1\times10^3$ cell/1ml in RPMI media. 2ml of RPMI media containing $2\times10^3$ cell were added per well in 6 Well Cell Culture Plate sterile (Cellstar®, Cat. No. 657160). The plate then was incubated at 37°C for 72h to allow macrophages and monocytes to adhere to the plate (Figure 2-8). Then, the supernatant was removed and 1ml of 1xPBS was added per well, and the plate was shaken for few seconds to wash the adherent splenocytes. Then, the supernatant was removed and 200μl of TRI reagent were added and scratched using cell scraper (Costar®3010) for few seconds, and the supernatant was centrifuged at 270xg for 5 minutes at 4°C, then, 1ml of TRI reagent was added to the pellet and the manufacturer’s instructions were followed (Thermo SCIENTIFIC Cat. No. #K1632) (section 2.11). qPCR was done as described in section 2.11 using specific primers.

Figure 2-8: Adherent splenocytes.

2.15 Statistics and Data analysis:

I was blinded to the genotypes in my analysis. Results are expressed as mean ± standard error of the mean (SEM) and standard deviation (SD). Statistical advice was obtained from two statisticians: Raeed Altaee (graduate of department of Neuro', Psych & Behaviour) and Salim Kadhim (graduate of department of Cardiovascular Sciences) respectively, on criteria for normality of
distribution and appropriateness of statistical tests. The statistical analysis was performed using Graph Pad Prism 7 (Graph Pad, San Diego California, USA). The normality test used was Shapiro-Wilk normality test. Three tests were used: One-way ANOVA Tukey’s multiple comparisons test (for normally distributed data/parametric), One-way ANOVA Dunn’s multiple comparisons test (for not normally distributed data/non-parametric) and Two-way ANOVA Sidak’s multiple comparisons test.

The density of the stained area of IF was measured by ImageJ2 software. The mean of bands intensity (PCR and western blot) was measured by Image Lab software.
3 Chapter three: Results
3.1 Results:

3.2 Properdin-dependent phenotype of MRL/lpr mice.

This project aimed to investigate the importance of the only positive complement regulator properdin in the pathogenesis of systemic lupus erythematosus. We used MRL/lpr mouse model which is an excellent model to study SLE disease. For the first time, we crossed properdin deficient mice with MRL/lpr mice to generate MRL/lpr properdin-wildtype (PWT)/properdin-deficient (PKO) mice. Our study shows that properdin could be used as a novel therapy target. This deduction comes from the results we achieved in this project. As this mouse model is characterised by glomerulonephritis, lymphadenopathy, increased ICs deposition and anti-DNA Abs production and inflammation, and end-up with renal failure, so we hypothesised that properdin deficiency could reduce these measurements. We found for the first time in in vivo studies that properdin deletion is associated with less anti-DNA Abs production, less splenic surface expression of CR2 on B220 positive cells, less serum creatinine and reduced IC-deposits and inflammation. We suggest that this is due to properdin gene deletion, thus, in this future, (non-complement activating) anti-properdin antibodies could be used as a treatment.

3.2.1 Pilot studies for evaluating the complement system activity in mouse serum samples.

Typically, papers use plasma to measure the activity of complement. The following experiments were set up to determine the usefulness of serum (which was routinely obtained as part of this and other studies in the lab) to evaluate complement activity in mouse. A method published by Kotimaa et al. (2015) was followed which measures the (heat-labile) activity of the individual complement pathways separately, depending on coating of the plate and buffer conditions. It uses purified human IgM to activate the classical pathway, mannan from Saccharomyces cerevisiae to activate the lectin pathway and LPS (Lipopolysaccharide) from Salmonella enteriditis S form, to activate the alternative pathway. Standardised conditions were provided by using BVB++ buffer containing calcium for CP and LP, and BVB++/MgEGTA containing
magnesium for AP. These conditions define the activities of the different pathways that are selectively investigated. To be able to compare activities across batches of analyses, a reference serum (Normal mouse serum, NMS) was purchased and its activity set at 100%.

The aim of this part of the study was to investigate whether the complement activation that occurs on the plate is sufficient to detect a reduction of activity in the supernatant that is transferred to a separate activation plate. If this is the case, this would demonstrate that serum is a suitable medium in which to analyse complement activity and indicate that the ELISA is a sensitive method to study depletion of complement factors as we would find in the case of “consumption” described in clinical chemistry. This is important because the intended experimental samples from lupus prone mice are likely to reflect varying activities of complement depending on the extent of in vivo activation.

The activities of three complement system pathways were dilution dependent, and CP activity was relatively stronger than LP and AP (Figure 3-1(A)). The supernatant from this experiment was saved and used to evaluate on a separate plate, coated identically to study the consumption of the complement components, the hypothesised reduced activities of three complement pathways in activated serum (Figure 3-1 (B)). There was an apparent reduction of absorbance compared to the original plate Figure 3-1 (A). This means that mouse serum contains significant residual complement activation ability after coagulation that retained further activity after one incubation, although at a reduced level, as was hypothesised. This is associated with occurring consumption.
Figure 3-1: The activity of CP, LP and AP in NMS.
The activity was measured to the formation of C9. The average of heat-inactivated CNMS ODs (CP = 0.0280, LP = 0.0231 and AP = 0.0191). Data points represent each concentration.

These measurements revealed that consumption affected the activities of all three pathways. It was concluded that serum samples could be used to analyse in vitro the extent of complement activation and consumption.

Next, aggregated immunoglobulins were used as a substrate to trigger complement activation. Gamma globulins were heated at 63°C for 90 minutes. The results show that aggregated immunoglobulins activated classical, lectin and alternative pathways, and these activities were concentration dependent (Figure 3-2).
Figure 3-2: The contribution of aggregated immunoglobulins to activation of CP, LP and AP.
20% of NMS was used. The average of heat-inactivated CNMS ODs (CP = 0.0207, LP = 0.0182 and AP = 0.0169). Data points represent each concentration.

Complement activation on aggregated immunoglobulins was further increased when additional properdin protein was added (Figure 3-3).

Figure 3-3: The effect of properdin protein on the activation of CP/LP/AP in CNMS.
The plat was coated with 20μg/ml of aggregated immunoglobulins, and serial concentrations of properdin protein (kindly donated by Professor Sim/University of Oxford) (0, 75, 150, 240 and 300) μg/ml were added to NMS. Increasing the properdin protein concentration led to increase the activity of CP, LP and AP. The average of heat-inactivated CNMS ODs (CP = 0.0182, LP = 0.0170 and AP = 0.0129). Data points represent each concentration.

Finally, the extent of contribution of the intact amplification pathway to CP and LP was tested by measuring the activities using serum from properdin-wildtype and properdin-deficient mice taken from the breeding colony. Properdin-deficient mice showed reduced absorbance of the CP and LP compared to properdin wildtype mice. As expected, AP activation was absent in properdin
deficient mice and conforms with the rabbit red cell lysis assay conducted in the first description of the mice (Stover et al. 2008) (Table 3-1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CP</th>
<th>LP</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Properdin-wildtype mice</td>
<td>0.54 ±0.02</td>
<td>0.43 ±0.048</td>
<td>0.40 ±0.05</td>
</tr>
<tr>
<td>Properdin-deficient mice</td>
<td>0.37 ±0.032</td>
<td>0.34 ±0.02</td>
<td>0.02 ±0.018</td>
</tr>
</tbody>
</table>

-Absorbance (OD 492). Mean ± SEM (the experiment was repeated three times in triplicate each), n=3 mice per group.

In conclusion, our pilot work established the suitability of serum to detect complement activation.

3.2.2 Phenotype of MRL/lpr mice.

Systemic lupus erythematosus is an autoimmune disease characterised by an expansion of autoreactive lymphocytes, anti-DNA antibodies production, complement C3 deposits, activated the components of the classical complement pathway and elevated serum creatinine levels as kidney function declines (Lichtnekert et al. 2019). Therefore, these features were used to determine severity of the mouse model and from there, an acceptable (humane) endpoint. MRL/lpr mice lifespan is 17 wks in the females and 22 wks in the males (Hewicker et al. 1990). This indicates that the disease progression is different between males and females; thus, lupus nephritis was studied in both males and females in this thesis.

We first had to find evidence of the phenotype of the Fas mutant mice. There are many phenotypes and time points of analyses in the literature that may exceed the allowed severity under the Home Office license that pertained to this work.

Using MRL/lpr mouse model in the present study, we show three time points (9-12, 15-17 and 19-22) wks (Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7 and Figure 3-8). I studied these as the rederived mice were being intercrossed (MRL/lpr het x MRL/lpr het) to obtain a stable line, from which to take mice for breeding to the properdin deficient line. Anti-DNA Abs, glomerular C3
deposition and classical pathway activity were early disease activity markers in MRL/\textit{pr} mice at 9-12 wks of age, while spleen weight and serum creatinine were not significantly different to wildtype. MRL/\textit{pr} mice showed disease progression between 9-12 and 15-17 wks of age; there was a significant difference in spleen weight, serum creatinine, anti-DNA Abs, glomerular C3 deposition and classical pathway activity, measurements at late timepoint are different from WT control. Reduced residual activity of the CP in the diseased mice compared to WT mice is likely to be due to consumption of the complement components, as explained in 3.2.1. No significant difference was observed between MRL/\textit{pr} mice at 15-17 and 19-22 wks in these markers.

Mice at 9-12 wks of age showed no lymph node enlargement and increased abdominal fats compared to MRL/\textit{pr} mice at 15-17 and 19-22 wks of age which showed increased abdominal fats and enlarged lymphoid organs.

Figure 3-4: The differences in the spleen weight at three-time points of age in MRL/\textit{pr} mice.

The spleen weight was measured in the experimental mice. 10-14 wks males and females (wildtype mice n=4, MRL/\textit{pr} mice n=4). 15-17 wks in males and females (wildtype mice n=3, MRL/\textit{pr} mice n=5). 19-22 wks in males and females (wildtype mice n=4, MRL/\textit{pr} mice n=4). Data represent the Mean ±SD of Two-way ANOVA Sidak’s multiple comparisons test; ***P<0.001, ****P<0.0001; ns= no significant differences.
Figure 3-5: The differences in the serum anti-DNA Abs at three-time points of age in MRL/lpr mice.
10-14 wks males and females (wildtype mice n=4, MRL/lpr mice n=4). 15-17 wks in males and females (wildtype mice n=3, MRL/lpr mice n=5). 19-22 wks in males and females (wildtype mice n=4, MRL/lpr mice n=4). Data represent the Mean ±SEM of Two-way ANOVA Sidak’s multiple comparisons test; *P <0.05, **P<0.01, ****P<0.0001; ns= no significant differences. The experiment was repeated three times in triplicate each.

Figure 3-6: The differences in the glomerular activated C3 fragments deposition at three-time points of age in MRL/lpr mice.
This figure represents the statistical analysis of stained areas measured by Image J. 10-14 wks males and females (wildtype mice n=4, MRL/lpr mice n=4). 15-17 wks in males and females (wildtype mice n=3, MRL/lpr mice n=5). 19-22 wks in males and females (wildtype mice n=4, MRL/lpr mice n=4). Data represent the Mean ±SEM of Two-way ANOVA Sidak’s multiple comparisons test; ****P<0.0001; ns= no significant differences. The experiment was repeated three times.
Figure 3-7: The differences in the serum creatinine at three-time points of age in MRL/lpr mice.
The serum creatinine level was tested using QuantiChromTM Creatinine Assay Kit. 10-14 wks males and females (wildtype mice n=4, MRL/lpr mice n=4), 15-17 wks in males and females (wildtype mice n=3, MRL/lpr mice n=5), 19-22 wks in males and females (wildtype mice n=4, MRL/lpr mice n=4). Data represent the Mean ±SEM of Two-way ANOVA Sidak's multiple comparisons test; ****P<0.0001; ns= no significant differences. The experiment was repeated three times in triplicate each.

Figure 3-8: The differences in the residual activity of the classical pathway at three-time points of age in MRL/lpr mice.
The activity was measured to the formation of C9. Relevant activity to normal mouse serum is shown, absorbance (OD405nm). 10-14 wks males and females (wildtype mice n=4, MRL/lpr mice n=4), 15-17 wks in males and females (wildtype mice n=3, MRL/lpr mice n=5), 19-22 wks in males and females (wildtype mice n=4, MRL/lpr mice n=4). Data represent the Mean ±SEM of Two-way ANOVA Sidak's multiple comparisons test; ****P<0.0001; ns= no significant differences. The experiment was repeated three times in triplicate each.

3.2.3 Generation of properdin deficient MRL/lpr mice.

When offspring became available from the cross between the properdin deficient line and MRL/lpr mice, the following groups were studied. Mice at 15-17 wks of age were used in this study: MRL/lpr PKO (males n=15, females n=16 and MRL/lpr PWT (males n=17, females n=15), MRL/lpr Phet (females n=9). We used heterozygous mice in this study as conventionally they should have 50% less properdin compared to wildtype mice due to X-chromosome linkage of the properdin gene and random in activation of X-chromosomes in females.
Firstly, to screen for the mutant alleles for Fas and properdin, ear snips were taken from mice, for genomic DNA extraction, and these samples were then analysed by PCR (Figure 3-9).

**Figure 3-9:** Genomic DNA was prepared from mouse ear snips of the experimental mice and then subjected to PCR. Representative bands are shown. D.W was used as a negative control.

For further confirmation, Blue Native Gel was performed for properdin absence verification in the serum. The result showed absence of properdin protein expression in the serum from MRL /lpr properdin deficient mice (Figure 3-10). Importantly, it shows oligomerisation of properdin (as shown in Figure 1-5).

**Figure 3-10:** Absence of properdin in serum of properdin deficient lupus prone mouse Blue Native Gel. Representative bands are shown. Polyclonal sheep anti-human/mouse/rat properdin protein was used as a primary antibody and polyclonal donkey anti-sheep IgG, HRP conjugated antibody was used as a secondary antibody.

To verify functionally the lack of the AP activity in MRL/lpr PKO mice, activation of the AP was quantified in the serum from MRL/lpr PWT/KO/het mice. Sera
was tested for poly C9 formation by coating the plate with LPS from *Salmonella enteriditis* which was shown to bind to properdin inducing the AP activation (Kimura *et al.* 2008; Kouser *et al.* 2013). The result demonstrated that the MRL/lpr mice deficient in properdin lacked activation of the alternative pathway, as expected (Figure 3-11). In females, MRL/lpr P\textsuperscript{het} mice showed intermediate activity: AP activation was more than MRL/lpr P\textsuperscript{KO} mice and less than MRL/lpr P\textsuperscript{WT} mice.

Figure 3-11: Properdin absence and activation of the alternative complement pathway (AP) in MRL/lpr properdin-deficient mice. The activity was measured to the formation of C9. Relevant activity to normal mouse serum is shown, absorbance (OD405nm). Data represent the Mean ±SEM of One-way ANOVA Tukey's multiple comparisons test; ****P<0.0001. The experiment was repeated three times in triplicate each.

### 3.2.4 Phenotype of MRL/lpr PWT/KO mice.

Litters of breeding pairs seemed normal, so an obvious impairment in fertility was not noted.

We next investigated whether properdin deficiency had an effect on the splenomegaly, lymphoproliferation and abdominal fats, since MRL/lpr mice at sixteen-weeks of age develop lymphoproliferation (females 4.5 and male 10.1 times), splenomegaly (3 to 6) times larger compared with the controls and increased body weight associated with activated complement system (Andrews *et al.* 1978; Levy *et al.* 1992; Clark *et al.* 1998; Choi *et al.* 2006; McAleer and Vella 2008; Schaevitz *et al.* 2017), and since these features are
considered the main features of SLE disease in MRL/lpr mice at 16 wks of age (Schaevitz et al. 2017).

The spleen weight of all experimental mice was measured. The spleen weight was significantly increased in MRL/lpr P^{WT} mice compared to MRL/lpr P^{KO} mice and WT mice, in both males and females. Additionally, no significant difference was observed between MRL/lpr P^{KO} mice compared to WT mice, this means that these two groups had comparable spleen weight (Figure 3-12). Moreover, in females, spleen weight in MRL/lpr P^{het} mice was dramatically more than MRL/lpr P^{KO} mice and less than MRL/lpr P^{WT} mice, pointing to a gene dose effect.

Furthermore, lymph nodes and abdominal fats were evaluated. Our data showed increased lymph nodes size and abdominal fats in MRL/lpr P^{WT} mice (17 males and 15 females) compared to MRL/lpr P^{KO} mice (15 males and 16 females) which were comparable to WT mice (Figure 3-13 and Figure 3-14). The abdominal fats are likely to reflect the extent of lymphoproliferation as no difference in food intake was observed.
Figure 3-12: The spleen weight in MRL/lpr PWT/KO (splenomegaly).
The spleen weight was measured in the experimental mice. Data represent the Mean ±SD of One-way ANOVA Tukey’s multiple comparisons test, **P<0.01, ***P < 0.001; ****P<0.0001; ns= no significant differences).
Figure 3-13: Extent of lymphoproliferative disease in MRL/\textit{ipr} PWT/KO (lymphadenopathy).

Figure 3-14: Abdominal fats in MRL/\textit{ipr} PWT/KO.
3.2.5 Disease activity markers in serum in MRL/\textit{lpr} PWT/KO mice.

In this section the analysis of serum samples is presented.

3.2.5.1 Anti-DNA Abs.

Since we want to investigate the effect of properdin deficiency on the hallmark of SLE disease in MRL/\textit{lpr} mice, thus; we coated the plates with Deoxyribonucleic acid sodium salt from calf thymus, consisting of dsDNA and ssDNA to measure serum anti-DNA Abs levels by ELISA in MRL/\textit{lpr} P\textsuperscript{WT}/P\textsuperscript{KO} mice.

In males, the result shows that development of serum anti-DNA Abs levels was significantly higher in MRL/\textit{lpr} P\textsuperscript{WT} mice compared to MRL/\textit{lpr} P\textsuperscript{KO} mice. Anti-DNA Abs were detectable in WT mice at a low level as expected (Figure 3-15).

In females, the result shows markedly higher serum anti-DNA Abs levels in MRL/\textit{lpr} P\textsuperscript{WT} mice compared to MRL/\textit{lpr} P\textsuperscript{KO} mice. In addition, MRL/\textit{lpr} P\textsuperscript{het} mice showed higher serum anti-DNA Abs levels compared with MRL/\textit{lpr} P\textsuperscript{KO} mice and lower than MRL/\textit{lpr} P\textsuperscript{WT} mice. Anti-DNA Abs were also detectable in WT mice at a low level as expected (Figure 3-15).
Figure 3-15: Serum anti-DNA Abs in MRL/lpr PWT/KO.
The serum anti-DNA Abs were measured in the experimental mice to estimate the effect of properdin absence. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; *P <0.05, **P<0.01, ***P<0.001, ****P<0.0001). The experiment was repeated three times in triplicate each.
3.2.5.2 Activity of CP and LP.

Since we found increased serum level of anti-DNA Abs in MRL/pr properdin-wildtype mice, we proposed that these auto-antibodies could bind to complement components to form circulating ICs, so we wanted to detect consumption of complement; thus, ELISAs were performed to quantify the activity of the classical pathway and lectin pathway in MRL/pr P\textsuperscript{WT}/P\textsuperscript{KO} mice.

MRL/pr P\textsuperscript{WT} mice are expected to have stronger activation compared to MRL/pr P\textsuperscript{KO} mice due to properdin presence that increases AP C3 convertase amplification leading to raise the complement activation.

For the classical and lectin pathways, in males, the activity was significantly lower in MRL/pr P\textsuperscript{WT} mice compared to MRL/pr P\textsuperscript{KO} mice which showed markedly lower activity compared to WT mice. This is interpreted to mean that there was relatively more activation \textit{in vivo} for MRL/pr P\textsuperscript{WT} mice and the residual activity for the assay \textit{ex vivo} is less. Alternatively, more complement proteins are synthesised in MRL/pr P\textsuperscript{WT} mice, but the control serum (WT) has much higher activity \textit{ex vivo}. In females, MRL/pr P\textsuperscript{WT} mice showed lower activity compared to MRL/pr P\textsuperscript{KO} mice which dramatically have lower activity compared to WT mice. In keeping with a dose effect of the properdin gene, MRL/pr P\textsuperscript{het} mice showed lower activity compared to MRL/pr P\textsuperscript{KO} mice and higher than MRL/pr P\textsuperscript{WT} mice (Figure 3-16).
Figure 3-16: The serum activity of classical and lectin pathways in MRL/lpr PWT/KO. The activity was measured to the formation of C9. Relevant activity to normal mouse serum is shown, absorbance (OD405nm). Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; *P <0.05, **P<0.01, ***P<0.001, ****P<0.0001). The experiment was repeated three times in triplicate each.

3.2.5.3 Pro-inflammatory cytokines (BAFF, TNF-alpha and IL-6).

Since we found increased serum anti-DNA-Abs and complement activation in MRL/lpr PWT mice, so we hypothesised that there is increased inflammatory cytokines level in the serum. Thus, to assess the effect of properdin deficiency on the serum pro-inflammatory cytokines levels in MRL/lpr mice, ELISAs were used to measure the serum levels of BAFF, TNF-alpha and IL-6 in MRL/lpr PWT/PKO mice.

The concentrations of serum BAFF, TNF-alpha and IL-6 were dramatically elevated in both male and female MRL/lpr PWT mice compared to MRL/lpr PKO mice which displayed dramatically increased levels compared to WT mice. This suggests that there were changes inducing inflammation during properdin
presence which increased the systemic levels of BAFF, TNF-α and IL-6 compared to properdin-deficient lupus prone mice (Figure 3-17).

Figure 3-17: Serum pro-inflammatory cytokines in MRL/lpr PWT/KO.
The serum levels of pro-inflammatory cytokines (BAFF, IL-6 and TNF-alpha) were measured in the experimental mice to estimate the effect of properdin absence. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; *P <0.05, **P<0.01, ****P<0.0001). The experiment was repeated three times in triplicate each.

3.2.5.3.1 Splenocytes proliferation.
Since we found increased pro-inflammatory cytokines level in the serum in MRL/lpr properdin-wildtype mice, so we hypothesised that the humoral components of the serum, including cytokines (BAFF, TNF-alpha and IL-6) could have an effect on the lymphoproliferation observed in section 3.2.4, thus,
to replicate lymphoproliferative disease (splenomegaly) \textit{in vitro}, MTS assay was used to analyse \textit{ex vivo} proliferation of splenocytes in the presence of autologous serum (incubation the splenocytes from wildtype mouse with the serum from MRL/\textit{lpr} P\textsuperscript{WT} mice and MRL/\textit{lpr} P\textsuperscript{KO} mice.

The increase in optical density in the MTS assay signifies increased proliferation. However, the results showed that there was a noticeable reduction in MTS ratio in MRL/\textit{lpr} P\textsuperscript{KO} mice compared MRL/\textit{lpr} P\textsuperscript{WT} mice, in both males and females (Figure 3-18).

![Figure 3-18: Splenocytes proliferation by MTS assay in MRL/\textit{lpr} P\textsuperscript{WT/KO.}](image)

Quantitative measurements of \textit{ex vivo} splenocytes proliferation from wildtype mouse using the serum from the experimental mice. Using MTS cell viability test in comparison to controls. Data represent the Mean ±SEM of One-way ANOVA Dunn’s multiple comparisons test; *$P$ <0.05). The experiment was repeated three times in triplicate each.

\subsection*{3.2.5.4 Serum creatinine.}

Since we found in MRL/\textit{lpr} P\textsuperscript{WT} mice increased serum anti-DNA Abs, complement activation and pro-inflammatory cytokines, as well as a cell stimulatory effect of serum, so we proposed that there could be a more detrimental outcome in renal function for MRL/\textit{lpr} P\textsuperscript{WT} mice. Thus, the level of serum creatinine was measured in the serum from MRL/\textit{lpr} P\textsuperscript{WT/PKO} mice using a colourimetric assay (QuantiChromTM Creatinine Assay Kit).

The concentration of serum creatinine in both males and females was dramatically elevated in MRL/\textit{lpr} P\textsuperscript{WT} mice compared to MRL/\textit{lpr} P\textsuperscript{KO} mice which
showed obviously increased creatinine compared to wildtype mice. In females, the level of serum creatinine was markedly more in MRL/lpr P\textsuperscript{het} mice compared to MRL/lpr P\textsuperscript{KO} mice and less compared to MRL/lpr P\textsuperscript{WT} mice (Figure 3-19).

Figure 3-19: Serum creatinine level in MRL/lpr PWT/KO.
The effect of properdin absence on the serum creatinine level was tested using QuantiChrom\textsuperscript{TM} Creatinine Assay Kit. Data represent the Mean ± SEM of One-way ANOVA Tukey's multiple comparisons test; *P <0.05, **P<0.01, ***P<0.001, ****P<0.0001). The experiment was repeated three times in triplicate each.

3.2.6 Renal inflammation in MRL/lpr PWT/KO mice.
In this section the analysis of kidney samples is presented.

3.2.6.1 Mesangial F4/80 and CD3 reactivity.
Since we found increased serum creatinine level, so we hypothesised that the kidney is impaired. We first measured the reactivity of F4/80 and CD3 in the kidney as initial analysis to investigate whether properdin absence has an effect on the inflammatory burden locally.

3.2.6.1.1 Macrophage marker (F4/80).
In males, there was a significant difference observed between MRL/lpr P\textsuperscript{WT} mice and MRL/lpr P\textsuperscript{KO} mice which were comparable to WT mice (Figure 3-20).

In females, WT mice were markedly comparable to MRL/lpr P\textsuperscript{KO} mice which displayed apparently less staining compared to MRL/lpr P\textsuperscript{WT} mice. Additionally,
MRL/\textit{pr} P_{\text{het}} mice were dramatically more than MRL/\textit{pr} P_{\text{KO}} mice and less than MRL/\textit{pr} P_{\text{WT}} mice (Figure 3-20).

In panel B, F4/80 staining shows a circular area of cells that appear dendritic or cells that have processes as shown in the closed-up view.

\textbf{Figure 3-20: Mesangial F4/80 reactivity in MRL/\textit{pr} PWT/KO.}
Representative images are shown, the magnification of ×60 (A-E). (F-G) represent the statistical analysis of stained areas measured by Image J. The arrows indicate the positive signal. Exposure times were kept constant for all images. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; 
\(* * * * P<0.0001; \text{ns= no significant differences). The experiment was repeated three times in triplicate each.\)
3.2.6.1.2 T cell marker (CD3).

There is noticeable presence of punctuate reactivity in panels B, C and D, that have not been seen in Figure 3-20.

In males, MRL/irpr $^{^P_{^W^T}}$ mice showed significantly increased staining compared to MRL/irpr $^{^P_{^K^O}}$ mice which displayed markedly more staining compared to WT mice (Figure 3-21).

In females, MRL/irpr $^{^P_{^K^O}}$ mice showed obviously less staining compared to MRL/irpr $^{^P_{^W^T}}$ mice and more than WT mice. In addition, MRL/irpr $^{^P_{^h^e^t}}$ mice showed apparently the same level of staining with MRL/irpr $^{^P_{^K^O}}$ mice and less than MRL/irpr $^{^P_{^W^T}}$ mice (Figure 3-21).
Figure 3-21: Mesangial CD3 reactivity in MRL/lpr PWT/KO.
Representative images are shown, the magnification of ×60 (A-E). (F-G) represent the statistical analysis of stained areas measured by Image J. The arrows indicate the positive signal. Exposure times were kept constant for all images. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; ***P<0.001 and ****P<0.0001). The experiment was repeated three times in triplicate each.
3.2.6.2 Immune complexes (ICs) deposition.

We then moved to analyse the kidney sections for detecting the deposition of ICs since we found increased circulating anti-DNA Abs and consumption of the complement components in the above measurements.

Our results showed that MRL/lpr properdin heterozygotes are indiscriminate compared to other groups especially properdin-deficient lupus prone mice. This means that for these analyses, one copy of the properdin gene is sufficient to participate in renal pathology.

3.2.6.2.1 Immunoglobulins (IgG, IgM and IgA).

IF staining was performed to assess glomerular presence of immunoglobulins using an anti IgG, IgA and IgM antibody in MRL/lpr PWT/KO mice.

In males, MRL/lpr PWT mice have significantly increased staining compared to MRL/lpr PKO mice. WT mice were included in the analysis to show baseline staining (Figure 3-22).

In females, there was markedly increased staining in MRL/lpr PWT mice compared to MRL/lpr PKO mice (Figure 3-22).
Figure 3-22: Mesangial IgG, IgA and IgM in MRL/pr PWT/KO.
Representative images are shown, the magnification of ×60 (A-D). (E-F) represent the statistical analysis of stained areas measured by Image J. The arrows indicate the positive signal. Exposure times were kept constant for all images. Data represent the Mean ± SEM of One-way ANOVA Dunn's multiple comparisons test; *P < 0.05, **P<0.01, ***P<0.001 and ****P<0.0001). The experiment was repeated three times in triplicate each.

3.2.6.2.2 Activated C3 fragments (C3c, C3b and iC3b).
The glomerular presence of C3c, C3b and iC3b in MRL/pr PKO/WT mice was tested using an antibody that specifically detects complement activation fragments not whole C3. The choice of this particular antibody was important because it shows that the complement system is activated.

In males, MRL/pr PWT mice showed significantly increased staining compared to MRL/pr PKO mice (Figure 3-23).
In females, there was more staining in MRL/\textit{pr} P\textsuperscript{WT} mice for the activated C3 fragments compared to MRL/\textit{pr} P\textsuperscript{KO} mice (Figure 3-23).

![Image of MRL/\textit{pr} C3c, C3b and iC3b in MRL/\textit{pr} PWT/KO](image)

\textbf{Figure 3-23: Mesangial C3c, C3b and iC3b in MRL/\textit{pr} PWT/KO.}
Representative images are shown, the magnification of ×60 (A-D). (E-F) represent the statistical analysis of stained areas measured by Image J. The arrows indicate the positive signal. Exposure times were kept constant for all images. Data represent the Mean ± SEM of One-way ANOVA Dunn’s multiple comparisons test; *P <0.05 and ***P<0.001). The experiment was repeated three times in triplicate each.

\subsection{3.2.6.2.3 Merged Igs and activated C3 fragments.}
The activated C3 fragments and immunoglobulins were merged to represent deposited ICs that were engaging complement activation, therefore, this IF was performed to study the role of properdin deficiency on the glomerular ICs.
deposition in MRL/lpr mice, as ICs consist of immunoglobulins bound to antigen which engage complement activation (Jonsson et al. 1987).

In males, MRL/lpr PWT mice showed significantly increased staining compared to MRL/lpr PKO mice (Figure 3-24).

In females, there was the same tendency: MRL/lpr PWT mice had markedly more staining for ICs compared to MRL/lpr PKO mice. MRL/lpr Phet mice in this analysis for deposited immunoglobulins and colocalising activated C3 did not show a gene-dose effect (at this timepoint) (Figure 3-24).

Figure 3-24: Mesangial deposition of ICs in MRL/lpr PWT/KO. Representative images are shown, the magnification of ×60 (A-D). (E-F) represent the statistical analysis of stained areas measured by Image J. The arrows indicate the positive signal (yellow staining). Exposure times were kept constant for all images. Data represent the Mean ±SEM of One-way ANOVA Dunn's multiple comparisons test; *P <0.05 and ***P<0.001). The experiment was repeated three times in triplicate each.
3.2.6.3 mRNA expression of the inflammatory markers.

As we explained that increased serum creatinine could indicate the impairment of the kidney, increased F4/80 and CD3 reactivity and ICs deposition in MRL/lpr *P*<sup>WT</sup> mice could confirm that the kidney is affected, so proposed that the kidney is inflammed. Thus, the mRNA expression of the inflammatory markers SMA-α and nephrin were measured. These were chosen as indicators of mesangial and podocyte reactions, respectively.

The GAPDH gene expression was done as a housekeeping gene for cDNAs of MRL/lpr mice. The results showed that there was one amplification peak of the product for each sample regarding GAPDH expression (Figure 3-25), and comparable bands size (Figure 3-26).

**Figure 3-25:** SYBR Green-based qPCR amplification and melting charts for GAPDH expression from kidney in MRL/lpr *PKO*/WT. Representative curves are shown. The experiment was repeated three times in triplicate each.
3.2.6.3.1 Alpha-Smooth muscle of actin (SMA-α).

We proposed that circulating ICs deposit in the kidney, so these ICs could affect the mesangial cells, and injured mesangial cells express SMA-α, that is not...
expressed in the normal glomeruli (Ichimura et al. 2006); thus, for quantifying SMA-α expression in MRL/\(pr\) \(P^{WT}\)/PKO mice, the gene expression was done using the normal PCR and qPCR technique using SYBR Green I dye (SensiMixTM SYBR).

In both males and females, SMA-α gene expression was dramatically increased in MRL/\(pr\) \(P^{WT}\) mice compared to MRL/\(pr\) \(P^{KO}\) mice in qPCR (Figure 3-27) and endpoint PCR (Figure 3-28). MRL/\(pr\) \(P^{KO}\) mice have very low expression, implying that SMA-α mRNA expression is not a sensitive disease reporter gene. The significant increase by qPCR and normal PCR for MRL/\(pr\) \(P^{WT}\) mice must therefore represent a severe reaction for mesangial cells expressing SMA-α in this group.

Figure 3-27: SYBR Green-based qPCR amplification and melting charts for SMA-α expression from kidney in MRL/\(pr\) \(P^{WT}/KO\).
The qPCR analysis for SMA-α gene expression was done to estimate the effect of properdin deficiency. The gene expression was normalised to GAPDH as reference gene expression. Representative curves are shown. Data represent the Mean ±SEM of One-way ANOVA Dunn's multiple comparisons test; *\(P<0.05\); ns= no significant differences). The experiment was repeated three times in triplicate each.
Figure 3-28: Normal PCR for SMA-α expression in kidney in MRL/\(pr\) PWT/KO.

qPCR samples were subjected to normal PCR. Representative bands are shown. 1 Kb Plus DNA Ladder in 1% agarose gel electrophoresis, size=418bp. Representative bands are shown. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; ****\(P<0.0001\); ns= no significant differences). The experiment was repeated three times in triplicate each.
3.2.6.3.2 Nephrological marker (Nephrin).

Since we showed above that there is increased deposited ICs, so we proposed that they could affect the filtration barrier, so nephrin might be a sensitive marker of podocytes involvement in this model, thus, mRNA expression may specify renal/podocytes stress.

Therefore, for quantifying nephrin expression in MRL/lpr PWT/PKO mice, the gene expression was done by using normal PCR and qPCR technique using SYBR Green I dye (SensiMixTM SYBR).

In both males and females, nephrin gene expression was increased in MRL/lpr PWT mice compared to MRL/lpr PKO mice as seen by qPCR (Figure 3-29), and endpoint PCR (Figure 3-30).

![Figure 3-29: SYBR Green-based qPCR amplification and melting charts for nephrin expression from kidney in MRL/lpr PWT/KO.](image)

The qPCR analysis for nephrin gene expression was done to estimate the effect of properdin deficiency. The gene expression was normalised to GAPDH as reference gene expression. Representative curves are shown. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; **P<0.01, ****P<0.0001; ns= no significant differences). The experiment was repeated three times in triplicate each.
Figure 3-30: Normal PCR for nephrin expression in kidney in MRL/lpr PWT/KO.
qPCR samples were subjected to normal PCR. Representative bands are shown. 1 Kb Plus DNA Ladder in 1% agarose gel electrophoresis, size=211bp. Representative bands are shown. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; ****P<0.0001; ns= no significant differences). The experiment was repeated three times in triplicate each.

3.2.6.4 Protein expression of caspase-3.
Since we found increased pro-inflammatory cytokines MRL/lpr PWT mice, so we proposed that caspase-3 could have a role in cleaving the cytokines resulting in activated inflammatory pathway. In addition, since we found increased inflammatory marker reactivities (F4/80 and CD3) and IC deposits in MRL/lpr PWT mice, so we hypothesised that apoptosis process is activated in the kidney
resulting in increased caspase-3 expression. Analysis of tissue using ApopTag detection kit was inconclusive.

Therefore, caspase-3 protein expression was measured by Western blotting using monoclonal anti-mouse caspase-3 antibody, to quantify the effect of properdin absence on caspase-3 activation in MRL/lpr PWT/PKO mice and using β-actin to equal the amounts of loaded protein.

For pro-caspase-3, caspase-3 23 kDa and 17kDa, in males and females, the expression was dramatically increased in MRL/lpr PWT mice compared to MRL/lpr PKO mice. In addition, WT mice showed comparable pro-caspase-3, caspase-3 23 kDa and 17kDa expression compared to MRL/lpr PKO mice (Figure 3-31 and Figure 3-32).

In females, the expression of pro-caspase-3, caspase-3 23 kDa and 17kDa was obviously more in MRL/lpr P^het mice compared to MRL/lpr PKO mice and less than MRL/lpr P^WT mice (Figure 3-32).
Figure 3-31: The expression of caspase-3 in kidney in males MRL/lpr PWT/KO. Representative bands are shown. The protein expression was normalised to β-actin as reference protein expression. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; *P<0.05, **P<0.01; ns= no significant differences). The experiment was repeated two times.
Figure 3-32: The expression of caspase-3 in kidney in females MRL/lpr PWT/KO.
Panel C: for a technical reason, the samples were run again. Representative bands are shown. The protein expression was normalised to β-actin as reference protein expression. Data represent the Mean ±SEM of One-way ANOVA Tukey's multiple comparisons test; *P <0.05, **P<0.01, ***P<0.001; ns= no significant differences). The experiment was repeated two times.
3.2.7 Splenic response in MRL/lpr PWT/KO.

In this section the analysis of marginal zone B cells which are significantly elevated in autoimmune disease, is presented.

3.2.7.1 Cell surface bound disease markers.

Since we found increased C3 activation in MRL/lpr PWT mice, so we hypothesised that different provision of ligands (relatively less C3d in the absence of properdin which was not measured as no mouse specific C3d ELISA was available) would alter expression of complement receptor CR2. In addition, since we found increased anti-DNA Abs in MRL/lpr PWT mice, so we proposed that B cells could be physically increased. Furthermore, since we found increased serum BAFF level in MRL/lpr PWT mice that activates B lymphocytes. Thus, splenocyte suspensions were prepared from MRL/lpr PWT/PKO mice. Cells were stained with PE conjugated rat anti-mouse CD45R/B220 (B cell marker) and FITC conjugated rat anti-mouse CD21/CD35. Cells positive for B200 and CD21/35 were analysed.

The percent of splenic B220 positive cell population was dramatically increased in MRL/lpr PWT mice compared to MRL/lpr PKO mice (Figure 3-35), owing most likely to greater lymphoproliferation in MRL/lpr PWT mice. The abundance of CD21/35 was measurably less in MRL/lpr PKO mice compared MRL/lpr PWT mice (Figure 3-34). The population of splenic double CD21/35-B220 positive cells was more in MRL/lpr PWT mice compared to MRL/lpr PKO mice (Figure 3-36).
Figure 3-33: Flow cytometry analysis showing isotype controls.
The analysed positive stained splenocytes were 10000 in panels A1 and B1. The splenocytes
stained for the isotypes were identified by forward, and side scatter and were gated (Q1) panels
A3 and B3. The histograms show a shift of fluorescence signal (PE in panels A2 and B2, FITC
in panels A4 and B4). Results are representative of two experiments.
Figure 3-34: Splenic CD21/CD35 positive cell population in MRL/lpr PWT/KO.
The analysed positive stained splenocytes were 10000 in panel A1. The splenocytes express CR2 were identified by forward, and side scatter and were gated (Q1) panel A3. These experiments were compiled in panels B and C. The histograms show a shift of fluorescence signal (PE in panel A2, FITC in panel A4). Result is representative of one experiment. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; **P<0.01, ***P<0.001; ns= no significant differences). The experiment was repeated two times.
Figure 3-35: Splenic B220 positive cell population in MRL/\textit{i}pr PWT/KO.
The analysed positive stained splenocytes were 10000 in panel A1. The splenocytes express B220 were identified by forward, and side scatter and were gated (Q4) panel A3. These experiments were compiled in panels B and C. The histograms show a shift of fluorescence signal (PE in panel A2, FITC in panel A4). Result is representative of one experiment. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; ns= no significant differences). The experiment was repeated two times.
Figure 3-36: Splenic double positive CD21/CD35+B220+ positive cell population in MRL/lpr PWT/KO.

The analysed positive stained splenocytes were 10000 in panel A1. The splenocytes express B220 were identified by forward, and side scatter and were gated (Q2) panel A3. These experiments were compiled in panels B and C. The histograms show a shift of fluorescence signal (PE in panel A2, FITC in panel A4). Result is representative of one experiment. Data represent the Mean ±SEM of One-way ANOVA Tukey’s multiple comparisons test; *P <0.05, **P<0.01, ***P<0.001; ns= no significant differences). The experiment was repeated two times.
### 3.2.7.2 mRNA expression of FcγRIIB and C5aR.

Since we found that increased B220 positive cell population in MRL/\textit{lpr} P^WT mice, so we proposed that the B cells are physically increased. Thus, we measured the FcγRIIB mRNA expression as in mice it is an inhibitory receptor which could be changed as a result of a reaction to IC-binding to cells. Then, we measured C5aR mRNA expression as this receptor could be suppressed by FcγRIIB (Karsten \textit{et al.} 2012), in addition, increased complement activation in MRL/\textit{lpr} P^WT mice could result in increased C5aR expression.

Splenocytes from MRL/\textit{lpr} PWT/PKO mice were prepared as described in material and methods to extract the RNA, then were used to measure the splenic FcγRIIB and C5aR expression by using normal PCR and qPCR technique using SYBR Green I dye (SensiMixTM SYBR).

The gene expression of GAPDH was done as a housekeeping gene for MRL/\textit{lpr} mice cDNAs. The results demonstrated that there was one amplification peak of the product for each sample regarding GAPDH expression (Figure 3-37), and comparable bands (Figure 3-38).

**Figure 3-37:** SYBR Green-based qPCR amplification and melting charts for GAPDH expression from spleen in MRL/\textit{lpr} PKO/WT. Representative curves are shown. The experiment was repeated three times in triplicate each.
3.2.7.2.1 B-cell inhibitory receptor (FcγRIIB).

Interestingly, in both males and females, there was decreased FcγRIIB gene expression in MRL/Pr PWT mice compared to MRL/Pr PKO mice (Figure 3-39). An endpoint PCR was run and it showed the FcγRIIB was expressed more in MRL/Pr PKO mice compared to MRL/Pr PWT mice (Figure 3-40).
Figure 3-39: SYBR Green-based qPCR amplification and melting charts for FcγRIIB expression from spleen in MRL/Ip prPWT/KO.

The qPCR analysis for FcγRIIB gene expression was done to estimate the effect of properdin deficiency. The gene expression was normalised to GAPDH as reference gene expression. Representative curves are shown. Data represent the Mean ±SEM of One-way ANOVA Tukey's multiple comparisons test; *P <0.05; ns= no significant differences). The experiment was repeated three times in triplicate each.
Figure 3-40: FcγRIIB expression in spleen in MRL/\textit{Ipr} PWT/KO.
qPCR samples were subjected to normal PCR. Representative bands are shown. 1 Kb Plus DNA Ladder in 1% agarose gel electrophoresis, size=307bp. Representative bands are shown. Data represent the Mean ±SEM of One-way ANOVA Dunn’s multiple comparisons test; * \( P < 0.05 \); ns = no significant differences). The experiment was repeated three times in triplicate each.

3.2.7.2.2 C5a receptor (C5aR).

In both males and females, the results show that the C5aR gene expression was dramatically increased in MRL/\textit{Ipr} \textit{P}^{WT} mice compared to MRL/\textit{Ipr} \textit{P}^{K0} mice (Figure 3-41). An endpoint PCR was run in parallel and showed less C5aR expression in MRL/\textit{Ipr} \textit{P}^{K0} mice compared to MRL/\textit{Ipr} \textit{P}^{WT} mice (Figure 3-42).
Figure 3-41: SYBR Green-based qPCR amplification and melting charts for C5aR expression from spleen in MRL/pr PWT/KO.
The qPCR analysis for C5aR gene expression was done to estimate the effect of properdin deficiency. The gene expression was normalised to GAPDH as reference gene expression. Representative curves are shown. Data represent the Mean ±SEM of One-way ANOVA Dunn’s multiple comparisons test; * $P<0.05$, **$P<0.01$, ***$P<0.001$; ns= no significant differences). The experiment was repeated three times in triplicate each.
Knowing from the previous results that severity of disease is higher in MRL/lpr P<sub>WT</sub> mice, and the decrease of FcγRIIB mRNA expression, and concomitant increase of C5aR mRNA expression in the spleen mean that the complement activation in presence of properdin is higher and that could recruit the inflammatory cells in spleen such as macrophages/monocytes which express C5aR resulting in increased inflammation. Another explanation could be due to increased IC binding to B cells in spleen leasg to downregulate FcγRIIB resulting in increased inflammation and C5aR expression. FcgRIIB functions as
a late checkpoint molecule in the normal maintenance of peripheral tolerance for B cells but is found decreased in human lupus. C5a / C5aR signalling down-regulates FcγRIIB \textit{in vitro} and \textit{in vivo}. There were consistently reversed abundances. However, high levels of BAFF (found in MRL/\textit{pr} \textit{PWT} mice) also decrease FcγRIIB and rescue B cells from apoptosis.

Taken together, the analyses have shown greater disease severity in MRL/\textit{pr} \textit{PWT} mice because of induced complement activation in presence of properdin. A gene-dose effect was seen in measurements of disease activity markers in serum, renal F4/80&CD3 reactivity, activated C3 fragments, immunoglobulins and inflammatory markers, and splenic response analysis.
4 Chapter four: Discussion
4.1 Discussion:

To provide insight into the role of properdin in SLE, we crossed properdin-deficient mice with MRL/\textit{lp}r mice, which were on C57BL/6 background. This was justified because properdin stabilises the amplification loop of C3 convertase of the AP, and using mice engineered genetically to be deficient of selected complement genes, namely Factor D, Factor B and DAF, studies had revealed that the intact complement activation (AP) contributed to severity of SLE (Watanabe \textit{et al.} 2000; Elliott \textit{et al.} 2004; Miwa \textit{et al.} 2007). These studies indicated the importance of the complement system (AP) in the progression of SLE, but the role of the positive regulator (properdin) in the manifestation and progression of SLE has not been studied before, so the data in this thesis present novel findings in the field. MRL/\textit{lp}r properdin-deficient mice had significantly less serum anti-DNA Abs, proinflammatory cytokines, splenic surface expression of CR2 on B220 positive cells, serum creatinine, complement activation, ICs deposition and inflammatory reaction. Thus, these studies demonstrate a key role for properdin in the autoimmune pathogenesis in MRL/\textit{lp}r mice. Therefore, this chapter will discuss the effect of the absence of properdin on lupus nephritis. Disease activity markers will be put in the context of the function of properdin.

4.1.1 Lymphnode assessment was subjective.

MRL/\textit{lp}r mice were evaluated after rederivation. We started with evaluating lymphoproliferation, that was done by observing the enlargement in the lymph nodes. Our study showed for the first time that there is reduced lymphoproliferation (Figure 3-12 and Figure 3-13) observed in MRL/\textit{lp}r properdin-deficient mice, the direct or indirect effect of properdin deficiency on reduction of lymphoproliferation will be discussed in this chapter.

Since we want to evaluate the disease presentation, we evaluated further the effect of properdin deficiency on the abdominal fats. Interestingly, our findings showed for the first time that properdin deficiency was associated with decreased abdominal fats in lupus prone mice (Figure 3-14). Supportive data come from recent observations that the activated complement system has an
effect on the T and B cells activation in lupus-prone MRL/lpr mice resulting in lymphoproliferation, and that was associated with increased autoantibody production, renal disease and body weight (Katzav et al. 2001; Thuret et al. 2012). There may be either simply more lymphoproliferation or: more C3adsarg signalling via C5L2 that transports fatty acids into fat where it is turned into triglycerides (Hess and Kemper 2016; Kolev and Kemper 2017).

4.1.2 Lupus severity is lessened in the absence of properdin.

Anti-DNA Abs are pathogenic in lupus nephritis which are developed in MRL/lpr mice and considered the disease hallmark (Almeida Gonzalez et al. 2015), however, since we found in this project that properdin deficiency had a significant effect on lymphoproliferation in MRL/lpr mice, so we hypothesised that properdin deficiency could be associated with decreased anti-DNA Abs which indicate the disease severity in this disease model. Serum level of anti-DNA Abs were significantly decreased in MRL/lpr properdin deficient mice compared to MRL/lpr properdin wildtype mice (Figure 3-15). This is an important finding because anti-DNA Abs are pathogenic in glomerulonephritis in MRL/lpr mice, associated with activated complement system, particularly, the alternative pathway, however, this study reported that the ICs are not important only because they can facilitate antigen transport to B-lymphocytes, but they can also enhance activation of B-cell (memory and naive B-cells) via C3b and the complement receptors 2 (CD21) resulting in increased production of anti-DNA Abs (Zhang et al. 2016). Watanabe et al. (2000) showed that the complement system, particularly, the AP has an important role in the production of anti-DNA Abs in MRL/lpr mice. That was confirmed by measuring the serum level of anti-DNA Abs in MRL/lpr Factor B deficient mice and MRL/lpr Factor B wildtype mice at 16 wks of age, and the results show that Factor B deficiency was associated with decreased serum anti-DNA Abs level in MRL/lpr mice, suggesting that the complement alternative pathway is a key player in induction of anti-DNA Abs production. These findings raised the possibilities that elevated provision of complement activation split products via the AP was responsible for increased B-cell signalling.
Consumption of the complement components could be due to their binding to anti-DNA Abs to form circulating-ICs that deposit in the kidney, so since we found increased anti-DNA Abs in MRL/\textit{lpr} properdin wildtype mice, thus, we measured the residual activity of CP and LP. Our results showed that there is increased serum residual activity of CP and LP in MRL/\textit{lpr} properdin-deficient mice compared to MRL/\textit{lpr} wildtype-deficient mice (Figure 3-16). Considering the properdin deficiency effect on the activated C3 alone, and using a simplified interpretation, properdin stabilises the amplification loop of AP C3 convertase (Kemper and Hourcade 2008). This result was consistent with a previous study for Harboe et al. (2004) who found that blocking the AP inhibits more than 80% of C3 required in the classical and lectin pathways. Another supporting data demonstrated that the complement system, including the classical and lectin pathways, is increasingly recognised as significant players in the tissue injury following immune complexes disorder. activation of these two pathways results in activation of C3 (Trouw et al. 2005). Giles and Boackle (2013) reported that the decreased serum levels of complement components noted during SLE activity are believed to be secondary to the increased production of autoantibodies and ICs formation which result in the tissue damage; however, it was suggest that activation of complement system (increased C3b production) can drive pathogenic autoantibodies development. Moreover, Perrin et al. (1975) demonstrated the patients with SLE showed elevated anti-DNA Abs level, and decreased serum level of properdin, factor B, C3 and C4d due to local activation of the complement system. It seems that properdin has an effect on anti-DNA Abs production in MRL/\textit{lpr} mice.

Since we found increased anti-DNA Abs and complement activation, so the inflammatory pathway could be activated. Thus, the next step was to measure the serum levels of pro-inflammatory cytokines which are also responsible for B lymphocytes activation and proliferation resulting in increased anti-DNA Abs production. Our results showed for the first time that serum pro-inflammatory cytokines concentrations were significantly reduced in in MRL/\textit{lpr} properdin deficient mice compared to MRL/\textit{lpr} properdin wildtype mice (Figure 3-17). These results were consistent with a recent study that showed that in lupus nephritis, the AP may enhance the inflammatory response via the amplification
loop in which activated C3, in this case, the AP promotes proinflammatory functions of the complement including the C3a and C5a generation resulting in increased pro-inflammatory cytokines production (Miwa et al. 2012). Tackey et al. (2004) demonstrated that activated complement system leads to enhanced inflammation resulting in high IL-6 production in MRL/lpr mice and this cytokine is involved in B-cells hyper-reaction, and that could explain that IL-6 plays a direct role in the production of anti-DNA antibodies. Aringer and Smolen (2008) showed that TNF-α plays an important role in induction of other cytokines release, particularly, IL-6 resulting in increased activation of B-cell and anti-DNA Abs production in MRL/lpr mice. In addition, Steri et al. (2017) demonstrated that there is a correlation between the complement system activation and BAFF production. B-cell activating factor (BAFF), plays an important role in the proliferation, differentiation and activation B-cells and resulting in increased anti-DNA Abs production. Properdin works then via amplification of IC that gives upstream cytokines. It seems that properdin deficiency is likely to affect the production of pro-inflammatory cytokines which may enhance B lymphocytes activation and proliferation resulting in increased anti-DNA Abs production.

Since we found increased serum pro-inflammatory cytokines in MRL/lpr PWT mice, so we proposed that the humoral components of the serum could be sufficient to stimulate the lymphoproliferation. Thus, the next step was to replicate lymphoproliferative disease (splenomegaly) in vitro, and that was done by stimulating the splenocytes from wildtype mouse with the serum from MRL/lpr properdin-wildtype mice and MRL/lpr properdin-deficient mice, then we measured the MTS ration indicating splenocytes proliferation. Our finding showed for the first time that in MRL/lpr properdin-deficient mice, there was reduction in MTS ratio compared to MRL/lpr properdin-wildtype mice (Figure 3-18). However, the majority of studies have agreed that there is association between the complement system activation and increased proinflammatory cytokines production, including BAFF, IL-6 and TNF-α which play a crucial role in the maturation and activation of the T & B-cells resulting in splenocytes proliferation (Li et al. 2002; Tackey et al. 2004; Orme and Mohan 2012), indicating that the complement system components are strongly associated with splenocytes proliferation. This result was consistent further with a recent study
for Sharma and Tiku (2016) who found that IL-6 and IL-17 promotes stimulation of mouse splenocytes proliferation. Another supporting data for Erdei et al. 1984 and Erdei et al. 2009 who reported that increased C3b production enhanced interleukin-2 production via T-cell and that was associated with promoted lymphocytes proliferation and increased antibodies production.

We hypothesised that reduced complement/inflammatory cascades activation and decreased anti-DNA Abs production observed in MRL/\(lpr\) properdin-deficient mice could be associated with less serum creatinine that indicates the changes in renal function, reflecting the effect of the properdin deficiency. We proposed that the kidney is a target organ for IC-mediated damage. Although the direct or indirect effect of properdin gene deletion on the previous measurements in this study is still unknown, we believe the overall effect could result in impaired renal function. Thus, serum creatinine in MRL/\(lpr\) PWT/KO mice was measured. Our result showed that serum creatinine was significantly reduced in MRL/\(lpr\) properdin-deficient mice compared to MRL/\(lpr\) properdin-wildtype mice (Figure 3-19). Low serum C3 levels is associated with reduced kidney damage in lupus nephritis (Watanabe et al. 2000). The previous studies in MRL/\(lpr\) mice deficient in the AP demonstrated that deficiency of the AP, particularly Factor D and Factor B deficiency was associated with less development of renal disease. That was confirmed by measuring the activity of the complement system and creatinine level, showing that reduction of C3 deposition in the kidney and serum level was consistent with less serum creatinine in MRL/\(lpr\) AP deficient mice (Watanabe et al. 2000; Elliott et al. 2004). Mäkelä et al., 2004 demonstrated that that increased activation of the complement system, serum pro-inflammatory cytokines production and ICs deposition was markedly correlated with high serum creatinine associated with glomerular dysfunction in MRL/\(lpr\) mice. Perrin et al. 1974 showed that low serum properdin and C3 levels could be due to local complement activation in the kidney, associated with increased renal disease in the patients with SLE. Recently, renal function was measured by creatinine level in the serum to indicate the critical role of the complement alternative pathway in MRL/\(lpr\) mice in the proliferative renal disease development, It indicates how effectively the kidneys filter the small molecules such as creatinine out of the blood, it was
noted that deficiency of Df in MRL/lpr mice maintained the renal function, it was observed that serum creatinine level at 20 weeks was significantly lower in MRL/lpr fD\textsuperscript{KO} mice compared to MRL/lpr fD\textsuperscript{WT} mice, they compared to WT strain (Elliott \textit{et al.} 2004). This likely resulted in less severe disease in MRL/lpr mice. These data show that MRL/lpr mice without properdin show improved renal function. We decided to end the studies as serum creatinine concentration observed in MRL/lpr properdin-deficient mice at 15-17 wks of age, in both males and females, was decelerated, could be due to reduced ICs deposition in the kidney leading to decrease the inflammatory reaction resulting in less glomerulonephritis; thus, it is likely that properdin deficiency could affect the lupus nephritis outcome in MRL/lpr mice reflected in improved renal function, based on the fact that creatinine level is able to reflect moderately impaired renal function (Levey \textit{et al.} 1988).

Since we found that properdin deficiency was associated with reduced serum concentrations of the pro-inflammatory cytokines and creatinine; thus, we proposed the activation of the inflammatory cascade could be reduced in MRL/lpr properdin-deficient mice, so detected the reactivity of the inflammatory cell marks (F4/80 ‘macrophage marker’ and CD3 ‘T cell marker’) by IF as implications of inflammatory potential which is greater in the presence of properdin but requires further work. Our results showed a novel \textit{in vivo} finding that properdin deficiency was associated with reduction of mesangial F4/80 and CD3 reactivity in MRL/lpr properdin-deficient mice compared to MRL/lpr properdin-wildtype mice (Figure 3-20 and Figure 3-21). It seems that properdin deficiency could have a direct and/or indirect effect on mesangial F4/80 and CD3 reactivity in this disease model, but further analyses are needed to make this point convincing.

Since we found more serum level of anti-DNA Abs and decreased residual activity of the CP and LP in MRL/lpr properdin-wildtype mice, so we hypothesised that these auto-antibodies and complement components (activated C3 fragments) could deposit in the kidney to develop IC deposits. And since we found increased serum creatinine in MRL/lpr properdin-wildtype mice which could confirm that the kidney is a target organ for IC-mediated damage.
Thus, the next step was to detect the deposition of immunoglobulins (IgG, IgA and IgM), activated C3 fragments (C3c, C3b and iC3b) and merged them to detect the ICs deposits in the kidney by IF. Our results showed for the first time that the MRL/lpr properdin-deficient mice had decreased ICs deposition in the kidney compared to MRL/lpr properdin-wildtype mice (Figure 3-24). However, in properdin-producing mice, properdin stabilises the alternative pathway leading to increased C3 activation and C3b production (Walport 2001; Zipfel and Skerka, 2009); which can bind to the immune complexes resulting in increased ICs deposition (Takata et al. 1984). This finding was consistent with a study for Schrager and Rothfield (1976) who demonstrated that in the patients with SLE, it was observed that increased properdin deposition in lesional skin was correlated with the increased presence of C3, C4 and immunoglobulins (IgG IgM and IgA). Another supporting study for Sterner et al (2014) reported that the patients with SLE present increased glomerular properdin deposition and that was associated with increased ICs deposition in the kidney. Elevated anti-DNA Abs production leads to deposition of increased ICs in SLE, and increasingly, ICs interact with Fc-receptors and the complement system receptors on the cells to modulate and upregulate the inflammatory cascade (Watanabe et al. 2000). The inflammation caused by the ICs remains the most significant mechanism for clinical SLE manifestations, particularly, lupus nephritis; thus, ICs are considered one of the fundamental inflammatory causes in autoimmune diseases (Trouw et al. 2004). Thus, it seems that properdin deficiency could diminish the mesangial ICs deposition in this disease model.

Activated inflammatory pathway leads to increased expression of the inflammatory markers in lupus nephritis such as alpha-smooth muscle actin (Abbate et al. 2002). Since in the above studies we found evidence for the induced activation of the inflammatory reaction in the kidney in MRL/lpr properdin-wildtype mice; thus, we aimed to establish more studies to confirm this activation, so we measured the mRNA expression of SMA-α in the kidney. Our finding showed for the first time that there was significantly decreased SMA-α in MRL/lpr properdin-deficient mice compared to MRL/lpr properdin-wildtype mice (Figure 3-27 and Figure 3-28). However, the functional significance of SMA-α in the inflammation is still not fully understood, but its main role perhaps
due to its contribution to monocytes recruitment into the inflamed kidney (Abbate et al. 2002; Lai et al. 2009; Wang et al. 2015). Protein alpha-smooth muscle actin (ASMA) is strongly expressed only in the blood vessel in the healthy kidney, but in pathological conditions, the SMA-α expression is found in the mesangial cells and in cytoplasm of the podocytes to contribute to renal diseases, therefore, elevated expression of SMA-α predicts a progressive decline in renal function (Novakovic et al. 2012). These studies indicate the association between SMA-α and renal disease. This result was consistent with a study conducted by Anders et al. (2004) who showed that MRL/lpr mice have diffuse renal smooth muscle actin staining compared to controls. It has been investigated that C5b-9 colocalised widely with smooth muscle cell α-actin, associated with increased macrophages, therefore, supporting the hypothesis that complement interaction with smooth muscle cells could indeed be significant in pathogenesis of SLE (Scheller et al. 2011). Although we believe that decreased SMA-α mRNA expression was associated with properdin deficiency, but no study has been reported the direct role of properdin on SMA-α expression, thus, the mechanism for the decrease SMA-α expression in MRL/lpr properdin-deficient mice is unclear.

Nephrin could be upregulated in the inflamed kidney (Manson et al. 2014). Thus, the next step was to include a marker that could indicate renal inflammation in the filtration barrier, so the main purpose of this study was to identify the association between nephrin expression and properdin deficiency in MRL/lpr mice, therefore, we applied qPCR to examine the properdin deficiency on the renal nephrin expression. Our novel result showed for the first time that there is significantly decreased nephrin in MRL/lpr properdin-deficient mice compared to MRL/lpr properdin-wildtype mice (Figure 3-29 and Figure 3-30). However, nephrin is normally measured as a slit diaphragm marker, within the glomerular filtration barrier, which is closely correlated with inflammation (Li et al. 2017). This result was consistent with a study conducted by Manson et al. (2014) who demonstrated that in the patients with SLE, expression of renal nephrin was significantly upregulated compared to the controls, and that might be due to nephrin loss in the urine, although increased gene expression does not mean increased protein presence. Furthermore, the most convincing data for the
complement components role in the glomerular diseases come from the rat model studies, where it has been demonstrated that that complement depletion had a role in preserving slit diaphragm morphology and prevented the loss of nephrin and that was usually associated with passive nephritis (Turnberg and Cook 2005). Up to now, there is no published information on the underlying mechanism of properdin in SLE progression, thus, although properdin deficiency seems to affect nephrin expression in MRL/lpr mice, still we did not find a study report the direct role of properdin on nephrin. We assume that it may attribute to the role of the complement system, in particular C3b, in renal disease-mediated an enhanced renal inflammation (Novakovic et al. 2012).

Since we found that there is increased level of pro-inflammatory cytokines in this study, and according to a recent data for Muhl and Pfeilschifter (2003) who reported that caspase-3 might be involved in inflammatory response by cytokines cleaving; thus, in this study, in an attempt to understand the underlying mechanism behind the harmful effect of properdin on renal inflammation in MRL/lpr mice, western blot was conducted to measure the expression of Caspase-3 in the kidney lysates. We found for the first time that there is an association between properdin deficiency and reduced caspase-3 expression in MRL/lpr properdin-deficient mice compared to the MRL/lpr properdin wildtype mice (Figure 3-31 and Figure 3-32). However, caspase-3 is considered to be a downstream effector, interacts with caspase-8 and caspase-9 to mediate apoptosis (Krishnan et al. 2005). As in most previous studies, activation of the apoptosis results in increased caspase-3, careful consideration of Fas pathway blocking, caspase-3 must be produced via other pathways, in particular, TNF pathway and intrinsic pathway, this is consistent with the previous studies support that apoptosis is activated in MRL/lpr mice (Dieker et al. 2015). This result was consistent with an in vitro study by Nauta et al. (2002) who demonstrated for the first time that the complement components of the terminal complement pathway can induce caspase-3 dependent apoptosis leading to increased inflammation. However, although no previous study reported a direct link between properdin and caspase-3, but it is likely that decreased complement activation in properdin-deficient mice is linked reduction of inflammation and apoptosis.
4.1.3 Splenic response is attenuated in the absence of properdin.

We hypothesised further that complement system activation would enhance CR2 expression on B cell resulting in physically increased B lymphocytes; thus, the fourth step was to analyse CD21/CD35 surface expression in splenic B220 positive cell population. Our results showed for the first time that CD21/CD35 surface expression in splenic B220 positive cell population was significantly decreased in MRL/lpr properdin-deficient- mice compared to MRL/lpr properdin wildtype mice (Figure 3-36). Chen et al. (2000) showed that CR2-deficient mice had B cell function defects including decreased switching of isotype and blunted responses to T-lymphocytes resulting in lower anti-DNA Abs production, suggesting that binding of CR2 on B-cell to C3 could play a significant role in complement activation process causing B-cell stimulation. Lisnevskaya et al. (2014) demonstrated that MRL/lpr mice develop activated complement system associated with increased hyper-reactive B cells and associated with high serum levels of auto-antibodies resulting in progressive renal disease. Thus, it seems that properdin deficiency could have an effect on the B cell activation and proliferation. The increase in CD21/CR2 expression in human SLE is likely to be the effect of increased complement activation in the patients with SLE (Rickert 2005; Wu et al. 2007). Therefore, these results showed that there is a possibility of affecting CR2 expression on B lymphocytes by properdin deficiency, and that could reduce B cell activation and proliferation resulting in less anti-DNA Abs production.

Venkatesh et al. (2009) demonstrated that FcγRIIB deficient mice showed more activation of high affinity autoreactive B lymphocytes resulted in increased antibody production, suggesting that can influence immune response. It seems that decreased production of anti-DNA Abs and less disease severity noticed in this study could be due to effect of properdin deficiency on FcγRIIB receptor expression. Probably upregulation FcγRIIB as a counter measure to greater IC become of higher B cell activation and their binding to activation FcRs. FcγRIIB has a role in human SLE and we found a difference between MRL/lpr PWT/PKO. Thus, we measured the splenic mRNA expression of FcγRIIB. There is a higher mRNA expression of FcγRIIB in MRL/lpr PKO mice comparing with MRL/lpr PWT.
Jhou et al. 2017 reported that MRL/lpr mice develop activated complement system associated with hyper-reactive B-cells and with down-regulated FcγRIIB in the spleen by IHC and PCR; that down-regulates the autoreactive B-cells leading to reduced auto-antibodies production.

MRL/lpr P<sup>WT</sup> mice require more C5aR to react to properdin amplified C5a generation. In addition, it has been reported that FcγRIIB deficient mice show higher C5aR expression (Karsten et al. 2012), suggesting that FcγRIIB suppresses the expression of C5aR. Thus, we hypothesised that increased C5aR expression leads to less B cell activation; thus, we measured the mRNA expression of C5aR in the spleen. Our finding showed for the first time that properdin deficiency was associated with increased splenic C5aR mRNA expression in MRL/lpr properdin-deficient mice compared to MRL/lpr properdin wildtype mice (Figure 3-41 and Figure 3-42). It has been reported that activated complement system is associated with increased C5aR expression (Neumann et al. 2002). In addition, complement component C5a binding to C5aR induced T and B cells activation resulting in elevated anti-DNA Abs production (Gerard and Gerard 1994). Wenderfer et al. (2005) showed that reduction of anti-dsDNA IgG level was associated with reduced responses of CD4+ Th-1 in the MRL/lpr C5aR deficient mice, suggesting that deficiency of C5aR attenuates functions of T/B cells and the renal disease in MRL/lpr mice. Bao et al. (2005) found that renal C5aR mRNA and protein expression was significantly increased in MRL/lpr mice compared to the controls, supporting the importance of C5aR in lupus nephritis, and that C5aR blockade could represent a potentially viable treatment. Bao et al. (2015) found that C3aR and C5aR expression was significantly upregulated in MRL/lpr mouse kidneys. Furthermore, another study demonstrated that of C5a receptor inhibition alleviates lupus in MRL/lpr mice (Jacob et al. 2010). It seems that the lack of properdin in the MRL/lpr properdin-deficient mice may have an effect on C5aR expression resulting in diminished anti-DNA Abs production and disease severity.
5 Conclusion.

Since properdin is the only positive regulator for the complement system; thus, its gene absence was examined in this IC-disease model. It was proposed that absence of properdin could determine SLE disease activity; thus, this study was conducted to assess the direct or indirect effect of properdin deficiency on SLE in MRL/lpr mice. Our work showed that the lack of properdin deficiency has a consistent and significant effect on the renal disease development in MRL/lpr mice.

This study presents novel findings in this field. Thus, we propose that our results summarise practical messages for the patients with SLE. As we noticed that properdin blocking leads to less lupus severity, thus, it could be a novel therapy strategy, and suggesting that the patients with SLE who have higher properdin levels could develop progressive end stage renal disease quicker.

Since we found that the severe lupus is consistent with increased C3c, C3b and iC3b presence, thus, we propose that in the patients with SLE, levels of C3 can indicate the activity of the disease, and it is likely that when C3 is normal, the disease is more likely calm. We hypothesise that these activated fragments could activate the inflammatory cells and B lymphocytes leading to increased anti-DNA Ab production. Therefore, blocking of activated C3 fragments could be a new therapy strategy.

Since anti-DNA Abs are the hallmark of SLE, and their binding to complement components develop circulating-ICs which deposit in the kidney resulting in renal damage. Thus, reducing auto-antibodies production could be a beneficial strategy to treat the patients with SLE. Therefore, blocking of CD21 and C5a receptors, and BAFF could be a novel therapy.
6 Future plan.

To test whether treating MRL/\textit{lpr} P^{WT} mice with anti-properdin antibodies (Gullipalli \textit{et al.} 2018) recapitulates the improvement seen in MRL/\textit{lpr} P^{KO} mice as preclinical study.
7 Appendices

British Society for Immunology (BSI) Congress, 4-7th December 2017, Brighton.

Characterisation of Murine IgAN. Local Inflammatory Changes and Systemic Complement Activation

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Introduction
IgA nephropathy (IgAN) was first described by Berger and Hinglais in 1968, and it is now known as the most common form of primary glomerulonephritis in the world. The causes of IgAN are not fully understood (Donadio and Grande 2003). Pathogenically, the complement system could be activated on IgA immune complexes in plasma and/or mesangial deposits via the alternative pathway, involving iP, a molecule that stabilises complement convertase complexes (Zhou et al. 2012). Therefore, we investigated the evidence that complement activation is a feature of the immunopathology of our recently established mouse model of IgA nephropathy.

Methods

Figure 1: Developing of IgA nephropathy mouse model

Results
Cytoplasmic vacuoles in tubules were observed in IgAN WT mice (Figure 2). CP and AP were activated in sera of IgAN WT mice (Figure 3). Intense staining for iP was observed in the kidneys from IgAN WT mice, together with activated C3 and immunoglobulins, indicative of IC deposition. iP reactivity was present only in IgAN WT mice and colocalised with F4/80+ macrophages (Figure 4).

Figure 3: Binding of IgM to C1q and LPS to iP to activate the CP and AP to C9 formation by ELISA in 20% serum samples from IgAN and Sham control WT mice (n=16), heat inactivated NMS (56℃, 30 minutes). *** p < 0.0001 (each done in triplicate).

Figure 4: Representative images of 5 µm- kidney sections from IgAN and Sham control WT mice (n=16). Yellow spectrum indicates merging signals as indicated. Comparisons of intensities Image J was used to measure the intensity of stained glomeruli, interstitium and tubules, p < 0.0001.

Conclusion:
Cytoplasmic vacuoles in the epithelium of tubules. Systemic complement activity (CP and AP) in IgAN. Glomerular IC deposition (colocalisation of Ig with activated C3 / iP). Tubulointerstitial infiltration of F4/80+ iP+ macrophages.

Acknowledgements:
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References:
Dear Hasanain Alaridhee

On behalf of the British Society for Immunology, we want to thank you for attending the BSI Congress 2017 held in Brighton from December 4-7, 2017. Your participation helped to make this year’s congress a great success and we hope you found it a valuable learning experience.

In order to ensure future congresses are even more successful, we kindly ask you to complete the congress evaluation form which can be accessed by clicking here. We thank you in advance for your comments and suggestions. Each will be given thoughtful consideration so that future congresses and BSI events will continue to provide value. Upon completion of the evaluation form you will be able to download your certificate of attendance.

Thank you and we hope to welcome you at the next BSI Congress in Liverpool, 2 – 5 December 2019. Save the date!

Best regards,

British Society for Immunology

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Deficiency of Complement Properdin Improves phenotype of MRL/lpr mice

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Introduction:
In systemic lupus Erythematosus (SLE), immune complexes (ICs) and activated complement deposit in the kidney, leading to initiate an inflammation resulting in glomerulonephritis. Properdin is one of the alternative pathway components and stabilizes C3 convertases generated by classical, lectin or alternative pathways. It was found that inhibition of the alternative pathway reduce 80% of the classical pathway activity in terms of formation TCC. It was demonstrated that absence of DI and DII could improve the phenotype of MRL/lpr mice (Watanabe et al., 2000; Elliott et al. (2004)). To determine the significance of the positive regulator properdin in SLE, IP deficient mice were crossed with MRL/lpr mice. MRL/lpr mice have a mutation in their Fas gene and develop a spontaneous lupus-like disease that is characterized glomerulonephritis.

Hypothesis:
Properdin aggravates renal disease in MRL/lpr mice.

Methodology:
1. Study mice: MRL/lpr mice were crossed with C57LJ/6J mice to develop MRL/lpr C57LJ/6J mice.
2. Histopathological assessment: glomerular and tubular changes (mesangial proliferation and loss of brush border) in PAS-stained kidney sections. The values were expressed as means±SD for 10 fields.
3. Immunofluorescence staining: activated C3 fragments (C3c, C3b and C3b), immunoglobulins (IgA, IgG and IgM) and immune complexes (overlapped C3 and Ig).
4. ELISA: TNF-alpha and IL-6.
5. Measuring of serum creatinine.

Results:
Absence of IP reduces renal disease

Figure 1: Histopathological scoring of glomerular and tubular changes, per
1mm2/10 different fields. (HG) healthy glomerulus. (MP) mesangial
proliferation. (LBB) loss of brush border. The data are represented as
means of 10 fields ± SD. Dunnet’s multiple comparisons test/One-way
ANOVA (*p = 0.0137, ** and **** p < 0.0001).

Figure 2: Representative images of 5 μm kidney sections. Yellow spectrum
indicates merging signals as indicated. Comparisons of intensities of
image J used to measure the intensity of stained glomeruli, interstitium and
tubules. Dunnet’s multiple comparisons test/One-way ANOVA, p < 0.0001.

Conclusion:
Our results suggest that complement factor D play a significant role in the
immunopathogenesis of renal disease in MRL/lpr mice suggesting the
similar effect of DI and DII deficiency in MRL/lpr mice was also due to
the lack of AP activation.

Future plan:

- FACS: to analyse the splenocytes (CD21 and B220).
- qPCR: FcyR2b and C5aR.

References:
MRL/lpr mice'. Kidney Int. 65(1), pp. 126-38.
renal disease in MRL/lpr mice genetically deficient in the alternative
Certificate of Attendance

This certificate confirms that:

Hasanain Alaridhee

Attended the

Complement UK Symposium & Training Course

held on

Monday 26th & Tuesday 27th March 2018

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COMPLEMENT UK
Collaborative UK Research Network
Complement Properdin Deficiency Reduces Kidney Damage In the phenotype of MRL/lpr mice

Hasanain Alarichee

Supervisors: Dr Stover and Prof Barnett
8 Bibliography


Isfort, K., Ebert, F., Bornhorst, J., Sargin, S., Kardakaris, R., Pasparakis, M., Bahler, M., Schwerdtle, T., Schwab, A. and Hanley, P.J. (2011) 'Real-time imaging reveals that P2Y2 and P2Y12 receptor agonists are not chemoattractants and macrophage chemotaxis to complement C5a is phosphatidylinositol 3-kinase (PI3K)- and p38 mitogen-activated protein kinase (MAPK)-independent', The Journal of biological chemistry, 286(52), pp. 44776-44787.


activation reduces B-cell numbers and ameliorates lupus', *Exp Mol Med*, 49(9), e381.


Mohamed, F. (2013) 'The Role of Complement Properdin in Murine Infection with *Listeria monocytogenes*', University of Leicester, Leicester, UK.


A., Melis, M., Gonzalez, A., Alarcon Riquelme, M. E., da Silva, B. M.,
Marchini, M., Danieli, M. G., Del Giacco, S., Mathieu, A., Pani, A.,
Montgomery, S. B., Rosati, G., Hillert, J., Sawcer, S., D’Alfonso, S., Todd,
J. A., Novembre, J., Abecasis, G. R., Whalen, M. B., Marrosu, M. G.,
Meloni, A., Sanna, S., Gorospe, M., Schlessinger, D., Fiorillo, E.,


Stover, C. M., Luckett, J. C., Echtenacher, B., Dupont, A., Figgitt, S. E., Brown,
J., Mannel, D. N. and Schwaeble, W. J. (2008) 'Properdin plays a
protective role in polymicrobial septic peritonitis', *J Immunol*, 180(5),
3313-8.


Takahashi, K., Kozono, Y., Waldschmidt, T. J., Berthiaume, D., Quigg, R. J.,
Baron, A. and Holers, V. M. (1997) 'Mouse complement receptors type 1
(CR1;CD35) and type 2 (CR2;CD21): expression on normal B cell
subpopulations and decreased levels during the development of autoimmunity in MRL/lpr mice', *J Immunol*, 159(3), 1557-69.

antibody complexes in the process of solubilization of immune

management and understanding of autoimmune lymphoproliferative

functional recovery in MRL/MpJ mice after spinal cord dorsal

Tian, C., Gagnon, L.H., Longo-Guess, C., Korstanje, R., Sheehan, S.M.,
'Hearing loss without overt metabolic acidosis in ATP6V1B1 deficient
MRL mice, a new genetic model for non-syndromic deafness with


renal disease in MRL/lpr mice genetically deficient in the alternative

analysis of MRL-lpr mice: relationship of the Fas apoptosis gene to
disease manifestations and renal disease-modifying loci', *J Exp Med*,
176(6), 1645-56.

Watson, S., Cailhier, J. F., Hughes, J. and Savill, J. (2006) 'Apoptosis and
glomerulonephritis', *Curr Dir Autoimmun*, 9, 188-204.

Wenderfer, S., Stepkowski, S and Braun, M. (2008) 'Increased survival and
reduced renal injury in MRL/lpr mice treated with a novel sphingosine-1-
phosphate receptor agonist', *Kidney International*, 74, 1319-1326

(2005) 'C5a receptor deficiency attenuates T cell function and renal
disease in MRL/lpr mice.', *J Am Soc Nephrol*, 16(12):3572-82.

B. H., Birmingham, D. J., Chang, D. M., Chen, C. J., McCurdy, D.,
Badsha, H. M., Thong, B. Y., Chng, H. H., Arnett, F. C., Wallace, D. J.,
of a common complement receptor 2 haplotype with increased risk of
systemic lupus erythematosus', *Proc Natl Acad Sci U S A*, 104(10), 3961-
6.

Xu, W., Berger, S.P., Trouw, L.A., de Boer, H.C., Schlagwein, N., Mutsaers, C.,
and necrotic cells independently of C3b and regulates alternative

Zhang, Y., Garcia-Ibanez, L. and Toellner, K. M. (2016) 'Regulation of germinal

Zhou, H. F., Yan, H., Stover, C. M., Fernandez, T. M., Rodriguez de Cordoba,
S., Song, W. C., Wu, X., Thompson, R. W., Schwaeble, W. J., Atkinson,
