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Chapter 1

General introduction
## Contents

1. Introduction
   1.1 The Complement system
   1.2 Complement system and autoimmunity
      1.2.1 Systemic lupus erythematosus
      1.2.2 Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides
      1.2.3 Anti-glomerular basement membrane (GBM) disease
   1.3 Alternative pathway initiation and activation
      1.3.1 Alternative pathway regulation
   1.4 Properdin
      1.4.1 Genetics
      1.4.2 Structure
      1.4.3 Biosynthesis
      1.4.4 Emerging roles
   1.5 Scope of the thesis
   1.6 References
1. Introduction

The complement system has been shown to have a role in various systemic autoimmune (AI) diseases which have a renal component. This includes systemic lupus erythematosus (SLE), goodpastures syndrome and anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides. In particular the classical pathway (CP) of complement is involved in SLE, with the exact mechanism of how these SLE autoantigens along with their autoantibodies interact with complement in the glomerulus remaining ambiguous. Furthermore, recent studies have demonstrated the expanding role of the alternative pathway (AP) of complement activation in steady state or in aspects of ANCA associated vasculitides or anti-GBM disease. The precise means of activation of the AP in these immune complex mediated disease settings remains unclear. Regulation is imperative in the AP due to its inherent ability to autoactivate. Factor H and properdin are both key opposing regulators in the AP with novel emerging roles in initiation and control of the AP. Deciphering the specific modes of AP activation and involvement is an ever expanding field requiring further elucidation, with previously unexpected roles for AP components revealing the diversity and complexity of this ancient pathway.

1.1 The Complement system

The complement system has long been viewed as a key member of innate immunity. Its biological activities range from defense against pathogens, regulation of self-tolerance and shaping of the adaptive immune response (1-4). The complement system consists of approximately thirty soluble and membrane proteins which are mainly produced in the liver along with white blood cells acting as an extrahepatic source. The system is divided into three pathways based on their mode of recognition, activation and regulation, the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP). The pathways all converge on the central complement component C3 which if activated and deposited can lead to assembly of the membrane attack complex (MAC) and resulting lysis of the target cell (5)(Figure 1).

The classical pathway is activated by binding of its recognition molecule C1q to various ligands including antigen bound IgM and IgG, acute phase proteins, apoptotic or necrotic material. The binding of C1q to its ligand induces a conformational change which allows the associated serine protease C1r and C1s located on the collagen-like tail region to become activated. The activated C1s cleaves C4 and C2 assembling the classical pathway C3 convertase (C4bC2a complex). The C3 convertase cleaves C3 into C3b and C3a. The resulting
Cleavage exposes an internal highly reactive thioester bond which covalently links C3b to its target. The bound C3b acts as opsonin while the C3a functions as an anaphylotoxin (5-7).

The lectin pathway is activated in a similar manner to the CP. It can be initiated by the recognition of certain carbohydrate ligands by mannan binding lectin (MBL) as well as H-ficolin or L-ficolin. MBL can bind mannose residues on pathogens leading to activation of MBL associated serine protease (MASP), in a similar manner as C1r and C1s activation (8). The MASPs associated with MBL are MASP-1, MASP-2 and MASP-3 (9, 10). MASP-2 once activated cleaves C4 and C2 generating the C4bC2a complex which is identical to the CP C3 convertase (11).

The AP is the oldest phylogenetically of all the complement pathways. The AP is unique in that its activation can occur spontaneously or more recently it has been proposed that it can be directed in a pattern recognition manner via properdin. The pathway comprises of C3, factor B, factor D and properdin. With the pathway being capable of activating spontaneously, regulation is key to prevent uncontrolled activation on the surface of self. A series of cell surface and fluid phase negative regulators are instrumental in controlling the AP (7, 12-14). With the alternative pathway being one of the major topics in this thesis it will be discussed later in more detail.

Figure 1: Complement system overview

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1.2 Complement system and autoimmunity

The complement system is involved in a variety of biological activities. This includes the recognition and clearance of foreign pathogen via direct lysis, opsonisation and phagocytosis (5, 15, 16). Complement is also involved in the removal of self-antigens including immune complexes and apoptotic cells (17). In recent times, complement studies have focused on its ability to bridge innate and adaptive immunity in either lowering the threshold for B cell activation or activating T cells (3, 18). It comes as no surprise that both complement and autoimmunity are intimately interweaved which is clearly evident in conditions such as ANCA associated vasculitides and SLE. The targeting of the kidney in such autoimmune disease may be in part due to its ability to filter 120ml of plasma per minute. The complement arm in such autoimmune renal disease could be accounted for by complement in circulation along with the production of complement components locally in the kidney. Classical pathway (CP) activation has always been recognised as a feature of immune complex mediated diseases, however there has been an increasing array of data demonstrating a role for the alternative pathway (AP) in such systemic disease states (19, 20).

1.2.1 Systemic lupus erythematosus

SLE is an incurable systemic autoimmune disease characterised by loss of self-tolerance. This dysregulation of self-tolerance is exhibited by B cell hyperactivity, autoantibody production and immune complex formation (21, 22). The aetiology remains largely unknown with genetic, environmental and hormonal factors involved (23). SLE can target various organs including skin, central nervous system, serosa, joints and kidney (24, 25).

A central characteristic of SLE is the breakdown in self-tolerance resulting in autoantibody production which targets antigens that are widely expressed. These antigens include nuclear components such as DNA, histones and ribonucleoproteins with the believed source of autoantigens due to failure in the clearance of apoptotic cells, with the nucleosome being the driving autoantigen (26-30). Anti-nuclear antibodies are found in the majority of SLE patients with anti-dsDNA regarded as being highly specific for the disease (31, 32).

Lupus nephritis (LN) (renal disease found in up to 50% of SLE patients) is a major cause of morbidity and mortality for these SLE patients (33, 34). Patients with LN experience a rise in anti-dsDNA antibodies and a reduction in serum complement levels. Immune complexes become deposited in the glomeruli and induce complement activation both of which are involved in the pathogenesis of LN (35, 36). Complement is involved in the effector phase of LN in SLE however, the complement system can also be the target of autoantibody response (31, 37).
Anti-C1q autoantibodies may be predictive of LN flares in SLE patients however they can also be found in healthy individuals with no renal pathology (37-40). Studies by Trouw et al found that anti-C1q antibodies alone were not sufficient to induce full blown renal injury (i.e proteinuria) \textit{in-vivo}. The work indicated that additional triggers were required, with the addition of a sub threshold amount of anti-glomerular basement membrane which binds C1q, followed by anti-C1q, induced renal injury (41). Intriguingly, further links with complement exist as deficiency in C1q and C1r/C1s are associated with increased susceptibility to develop SLE (42). Deficiencies in complement are believed to be a prominent cause of defective clearance of apoptotic cells leading to the “waste disposal” hypothesis becoming a proposed mechanism of disease induction. However, the presence of the complement system can lead to activation of the system during active SLE which may lead to tissue injury (43). These findings demonstrate the paradoxical role of the complement system in this disease setting.

1.2.2 Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides

ANCA associated vasculitides (AAV) consist of a group of systemic AI diseases including granulomatosis with polyangiitis, microscopic polyangiitis, Churg-Strauss syndrome, “renal-limited” vasculitis and certain drug-induced vasculitis syndromes (44). They are characterised by necrotising inflammation of small vessel walls frequently targeting the kidneys. ANCA are predominantly used as serological markers of small vessel vasculitides, with proteinase 3 (PR3) targeted in granulomatosis with polyangiitis or mainly myeloperoxidase (MPO) in the remaining AAV (19, 45). In AAV, primed neutrophils express ANCA antigens on their cell surface followed by adherence of the neutrophil to susceptible endothelium. Following interaction of ANCA antibodies with the exposed antigens the neutrophil becomes activated. The activated neutrophil releases soluble factors to activate the complement system and damage the endothelium resulting in necrotising vasculitis (46). The hypothesised source of these autoantigens include TNF primed neutrophils and neutrophil extracellular traps (47, 48).

Recent studies have demonstrated that ANCA activated neutrophils can activate the complement system. Neutrophils act as potent source of various complement components such as C3, factor B and properdin (49-51). The proteases stored within the neutrophil granule have been shown to activate complement C5 with oxidants demonstrating a similar capability (52-54). Studies by Xiao et al illustrated that activation of human neutrophils with ANCA antibodies releases factors which can activate complement leading to the generation of C3a (55). Further work by Schrieber et al revealed that supernatants from ANCA activated
neutrophils activated the complement system in normal serum generating C5a. The presence of C5a can prime the neutrophil allowing for ANCA induced respiratory burst in the neutrophil, releasing free oxygen radicals and proteases (56).

With various *in-vitro* data demonstrating the role of complement in the induction or effector phase of the disease, the *in-vivo* studies focused mainly on elucidating which complement pathway was involved. A mouse model of the disease using anti-MPO IgG demonstrated that C4 deficient mice developed comparable disease as to wild type, while factor B or C5 deficient mice were protected from damage, indicating a role for the AP (55). Recent studies in the ANCA field demonstrated the presence of AP components at the site of injury as well as activation of the AP in the circulation of patients with active ANCA vasculitis (57, 58). Cumulatively the data illustrates the role of the AP in ANCA vasculitis as well as highlighting the varied interactions between various neutrophil components and the AP, which will be discussed further in this thesis.

### 1.2.3 Anti-glomerular basement membrane (GBM) disease

Anti-GBM disease refers to what was originally called goodpastures syndrome or goodpastures disease. Recently, anti-GBM disease has been classified in the new nomenclature of immune complex small vessel vasculitides (59). Anti-GBM disease is an autoimmune disease characterised by the role of B cell and T cells in the pathogenesis. HLA-DRB1*1501 and DRB1*1502 are associated with the disease however it is also believed to have environmental triggers. It is characterised by the presence of autoantibodies targeting typically the non-collagenous domain of the α3-chain of type IV collagen. The α3-chain of type IV collagen is a component of alveolar basement membrane and GBM, which are the principle targets in the condition. The autoantigen is a cryptic epitope which can be exposed upon oxidant damage. Patients suffering from anti-GBM disease would present with anti-GBM autoantibodies with glomerulonephritis and/or alveolar bleeding. The anti-GBM autoantibody is integral to the disease based on elution studies and transfer to primates (60). With the anti-GBM antibody a central characteristic and driving force in the disease it is the focus of many murine models of anti-GBM disease. Histopathologically, the glomeruli of patients with anti-GBM present with a linear deposition of IgG on the GBM generally accompanied by C3 deposition, with recent studies demonstrating the extent of complement activation in circulation and urine of patients with anti-GBM disease, including AP activation (61, 62). In particular a murine model of anti-GBM disease, demonstrated a role for the AP, through the ability to induce significant disease in C1q and C4 knockout mice (63).

Anti-GBM antibodies are crucial for diagnosis, however approximately 20-35%
of patients with anti-GBM antibodies also have ANCA, generally targeting MPO (61). This demonstrates the close overlap between both ANCA and anti-GBM, both autoantibody mediated diseases with complement and renal components. The exact role of the AP in anti-GBM disease remains unclear and continues to be an area requiring further elucidation.

1.3 Alternative pathway initiation and activation

Activation of the AP can occur to the best of our knowledge by two described mechanisms. The traditional model of AP activation involves a process known as “tickover”. Tickover describes the ability of fluid phase C3 to undergo spontaneous conformational changes to generate C3(H₂O) or C3i. This C3(H₂O) or “C3b like molecule” is formed by interaction of the internal thioester bond with water. This “C3b like molecule” has the C3a fragment still attached, however it can associate with the inactive serine protease factor B in the presence of Mg²⁺. The factor B attached to C3(H₂O) is cleaved by factor D (serine protease) into a Bb fragment and a Ba segment. The Ba fragment is released while the Bb fragment remains attached to the C3(H₂O). The active C3(H₂O)Bb complex is then stabilised by properdin forming the “initiation C3 convertase”. The stabilised “initiation C3 convertase” can cleave additional fluid phase C3 into metastable C3b (lacks C3a fragment) which can covalently attach to its target surface using its thioester bond. The newly formed metastable C3b can in the presence of factor B, Mg²⁺ and factor D assemble the AP C3 convertase (C3bBb) which can be stabilised by properdin (Figure 2). This stabilised multimeric complex can generate more metastable C3b feeding back into the AP activation (7, 12-14).

![Figure 2: C3 initiated alternative pathway activation](image-url)
Recently, another method of AP activation has been described termed the properdin-directed model. This mechanism is dependent on the pattern recognition ability of properdin to initially recognise and bind its target surface. Properdin can recognise ligands such as zymosan, bacteria and glycosaminoglycans acting as a focal point for complement activation (64, 65). Once properdin has bound the target surface it can bind C3b followed by factor B and Mg^{2+}, with the inactive factor B cleaved by factor D to form the fully stabilised and active AP C3 convertase (C3bBb) as described earlier. Both mechanisms lead to the generation of the same AP C3 convertase with the initiation molecules being the only variation (64)(Figure 3).

One of the unique features of the AP is the “amplification loop C3 convertase”. This loop is initiated when C3b generated from any of the three pathways then binds factor B and assembles the AP C3 convertase leading to augmentation of C3 activation. The “amplification loop” can play a major role in AP activation in particular in settings of local injury where infiltrating cells can act as source of C3 and properdin or where regulatory mechanisms are impaired or due to a reduction in the expression of complement regulators (66-68). The loop can deposit $10^7$ C3b molecules on a target in less than five minutes (69). Furthermore, autoantibodies termed C3 nephritic factors can stabilise the C3 convertase inhibiting them from degradation allowing for propagation of AP activation (70). The ability of the AP to become initiated spontaneously allowing for continuous C3 activation demonstrates the importance the cell surface and fluid phase regulators play in the AP, preventing complement depletion and uncontrolled deposition on host cells.
1.3.1 Alternative pathway regulation

The regulators involved in limiting AP activation can be sub-divided into cell surface or fluid phase regulators. The plasma regulators of the AP include factor H, factor H-like protein 1 (FHL-1), factor H-related proteins (FHRs) and factor I. Factor H is a major fluid phase regulator of the AP including the “amplification loop”. Factor H acts as a co-factor for factor I mediated cleavage of C3b and has decay accelerating activity for the AP C3 convertase. Furthermore factor H can exhibit surface inhibitory effects based on its ability to interact with C3b or sialic acid on target surfaces (71). Also FHL-1 can act as a co-factor for factor I while FHR members can bind C3 controlling its fate. Factor I is a serine protease with proteolytic degrading activity which requires co-factors for cleavage of C3b and C4b, which is imperative to controlling the classical and alternative pathway (71, 72).

Cell surface regulators: membrane co-factor protein (MCP, CD46) and decay-accelerating factor (DAF, CD55) are both involved in AP regulation. MCP acts as a co-factor for factor I mediated cleavage of C3b and C4b while DAF accelerates the decay of the AP and CP C3 and C5 convertase (7). CR1 (CD35) expressed on red blood cells functions as a co-factor for factor I mediated cleavage of C3b while CD59 inhibits C9 polymerisation and assembly of the membrane attack complex (MAC) (73). A novel aspect of the AP is that it contains the only positive regulator of the complement system, properdin which promotes complement activation. The delicate balance between properdin and the various negative regulators of the AP in particular factor H is an integral axis for determining the fate of the AP activation.

1.4 Properdin

Properdin was discovered by Louis Pillemer in 1954 and was the first AP component to be found (74). The name properdin originates from the latin word perdere “to destroy” which fittingly describes many aspects of this serum protein. Properdin is known as the only positive regulator in the complement system. It stabilises the short lived AP C3 convertase (C3bBb) by approximately five to ten fold (75). Properdin bound more readily C3bB and C3bBb as compared to C3b (76).

1.4.1 Genetics

The human properdin gene is located on the short arm of the X chromosome at Xp11.23 - Xp11.3 encoding for the thrombospondin repeats (TSR), the basic subunit of properdin (77, 78). The properdin gene is composed of ten exons encompassing 6kb of the genome. Exon 1 is transcribed however it is not
translated with the translation start site located in the second exon. The 49 amino acid N-terminal domain is encoded for by exon 3. Exon 4-8 codes for TSR 1-5 with exon 9 encoding for the initial 38 amino acid of TSR 6 with the remainder of TSR 6 encoded for by exon 10, which also encodes for the C-terminal domain (79).

A deficient state does exist in humans, with properdin deficient individuals at 3000 fold greater risk of meningococcal disease caused by *Neisseria meningitidis*. The properdin deficient individuals are generally male as the properdin gene is encoded on the X chromosome (77). Properdin deficiency is categorised into three subtypes: Type 1 deficiency is the complete absence of properdin in the plasma. Type 2 deficiency is categorised as plasma properdin of less than 10% of the normal range. Type 3 deficiency presents with normal plasma levels of properdin however the protein is aberrant (80).

1.4.2 Structure
Properdin consists of rod like subunits of 53kDa which form head to tail associations, forming dimers, trimers, tetramers and the recently described pentamers respectively (Figure 4) (81-83). In plasma the approximate ratio of dimers: trimers: tetramers is 26:54:20 (82). Properdin function increases with size as tetramers are ten times more active compared to dimers (84). Each 53kDa subunit consists of seven type 1 thrombospondin repeats (TSR), numbered from TSR 0-6 in order from the N-terminus (85). TSR 4 is involved in stabilisation of the C3 convertase with TSR 5 involved in binding C3b while TSR 6 is involved in the oligomerisation of properdin subunits (86). Properdin can interact with the C345C domain of C3b and the von Willebrand factor type-A (vWA) domain in Bb allowing it to stabilise the AP C3 convertase by holding the two components

![Figure 4: Properdin Isoforms](image)

...
of the complex together. Furthermore, properdin promotes stability of the C3 convertase by inhibiting the ability of C3b to interact with DAF or factor I mediated co-factors thus prolonging activation (83).

1.4.3 Biosynthesis
In contrast to the majority of complement factors which are produced in the liver, properdin is synthesised mainly by white blood cells. Properdin is synthesised by monocytes, T lymphocytes, neutrophils and is stored in dendritic cells and the granules of mast cells (51, 87-90). Limited data is available on the regulation of production or secretion by white blood cells, with data from neutrophils indicating that TNF, C5a and IL-8 can induce properdin secretion (51). Intriguingly, activated neutrophils can release properdin from its granules which then becomes bound to the neutrophil cell surface independent of any C3, allowing for C3 convertase assembly (91). Normal properdin levels in plasma (approx. 25 μg/ml) are relatively low compared to various other AP components such as C3 (1000-1500 μg/ml) and factor B (74-286 μg/ml), demonstrating its potent effects on the AP even as a minority (92). This is of particular interest when you focus on the local complement production at a site of injury or the immunological synapse. Previous studies have demonstrated a key role for the alternative pathway in the APC T cell synapse, whereby the AP is produced locally and enhances T cell proliferation and APC cytokine release (93). Infiltrating cells such as dendritic cells acting as a source of properdin at the site of injury, can dramatically alter the ability to induce AP activation in the local environment. The exact source of plasma derived properdin still remains unclear, with murine studies offering us some insight into its origin. Studies by Miwa et al, illustrated that tissue specific deletion of properdin in myeloid cells of mice resulted in a 95% reduction in plasma properdin levels (94), indicating that myeloid cells are the majority properdin “shareholder”.

1.4.4 Emerging roles
Next to the ability of properdin to act as stabiliser it can act as a pattern recognition molecule. Initially it was described to recognise microbial stimuli including Neisseria gonorrhoeae and zymosan, inducing complement activation (64). In-vitro, properdin can bind LPS coated wells and native forms of properdin can bind to Chlamydia pneumonia resulting in C3b deposition (95, 96). Properdins pattern recognition capabilities stretch further than its ability to bind exogenous ligands. Properdin can bind mammalian cells via glycosaminoglycans (Figure 3) as was observed with proximal tubular epithelial cells (PTEC). Properdin bound viable PTEC by attaching to tubular heparin sulphates (65). Gaarkeuken et al demonstrated properdin deposition along the brush border of patients with
proteinuric kidneys which was absent in healthy controls. *In-vitro* purified properdin could bind specifically PTEC, which when exposed to serum could support complement activation. Indicating that properdin can act as an anchor on PTEC and induce AP activation on its surface (97). Properdin can bind viable cells however it seems just as capable as a pattern recognition molecule binding altered self. Properdin can bind necrotic mouse splenocytes, either in the absence or presence of C3, illustrating the properdin directed model of AP activation (98). Properdin binding altered self is not limited to just necrotic splenocytes as it can also bind apoptotic T cells. The properdin bound apoptotic T cell can direct deposition of C3b or induce phagocytic uptake of the apoptotic T cell independent of complement activation (99).

The generation of a properdin knockout mouse has expanded the field of investigation and our understanding of properdin *in-vivo* in AP mediated tissue injury. Murine models of arthritis have demonstrated a beneficial effect in deficiency of properdin, with neutralisation of properdin also improving arthritis development (100, 101). In a model of abdominal aortic aneurysm, properdin deficient mice had a better outcome as well as wild type mice treated with an anti-properdin antibody (102). Also, a mouse model of renal ischemia reperfusion injury generating AP mediated tubular injury, demonstrated amelioration of the condition in a properdin depleted setting (94). Properdin has exhibited a fatal role in various AP mediated tissue injury settings as above, surprisingly in C3 glomerulopathy which is characterised by AP activation, the presence of properdin can be protective (103-105). The ever diverse roles of properdin including its “traditional” and emerging roles are the focus of studies in both healthy and disease states, to elucidate the depth of capabilities of this previously disregarded protein.

1.5 Scope of the thesis

The scope of this thesis surrounds the role of the complement system in various aspects of autoimmunity. Our experiments were targeted towards investigating the possible roles of the classical pathway and alternative pathway in various AI settings. Properdin is the only positive regulator of the complement system by stabilizing the alternative pathway convertase, and both in human and mouse it’s precise role in steady state or disease conditions remains to be elucidated. In chapter 2 we investigated the causal relationship between autoantibodies and the development of lupus nephritis. Our studies focused on endothelial cells which are a major target in LN and their interaction with autoantigens C1q and nucleosomes. The aforementioned *in-vitro* studies were performed in the presence or absence of autoantibodies against the respective autoantigens, followed by addressing
its ability to induce classical pathway activation. In chapter 3 we describe the generation of a novel mouse properdin ELISA and histological stain to elucidate the contribution of properdin in murine disease models. In chapter 4 we focused on generating a method for the purification and functional testing of human properdin for in-vitro studies. As the AP has been implicated in many neutrophil-mediated diseases, in chapter 5 we investigated the interaction of properdin with neutrophil components and their role in alternative pathway activation. Our studies focused on the interactions between the neutrophil enzymes including those which are autoantigens in ANCA vasculitis. Our findings demonstrated a novel method of AP activation by human neutrophil enzymes. Recent studies have indicated the ability of properdin to act as stabiliser and initiator of the AP, however there has never been a physiological inhibitor for properdin described. In chapter 6 we describe our findings on a novel inhibitor that can directly interact with properdin, inhibiting properdin directed complement activation on human cells. With regulation of the AP being particularly important due to the amplification loop, we focused in chapter 7 on the role in adaptive immunity of the two opposing AP regulators properdin and factor H. Our findings demonstrated novel diverging roles of DC derived properdin and factor H production and roles in T cell immunity. In chapter 8 we discuss the findings and future lines of investigation encompassing the ever expanding role of properdin activation, regulation and downstream effects on the alternative pathway.
References


