THE ROLE OF COMPLEMENT iC3b IN DENSE DEPOSIT DISEASE

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A thesis submitted to Imperial College for the degree of Doctor of Philosophy

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Declaration of Originality

I certify that the work in this thesis is my own and that all else is appropriately referenced.

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Acknowledgements

I would like to thank my supervisors, Professor Matthew Pickering and Professor Marina Botto, for their guidance and unstinting support during this project. I am also indebted to Professor Terry Cook for his frequent advice. I wish to thank my interim assessors Dr Fred Tam and Dr Juthathip Mongkolsapaya for their help. I am especially grateful to Dr Marieta Ruseva for her direction and encouragement of my research. Dr Talat Malik and Dr Liliane Fossati-Jimack also provided valuable assistance. I wish to thank Ms Lorraine Lawrence for the processing of histology. I also wish to acknowledge the Centre for Biomedical Sciences staff and the Hammersmith Campus Library staff and Document Delivery service. I am grateful to Ms Claudia Rocchi, Dr Sharron Stubbs and all my colleagues at the Centre for Complement and Inflammation Research for their help during my fellowship. This project was undertaken with the generous financial support of Kidney Research UK (KRUK Clinical Research Fellowship TF12/2011). Finally I wish to thank Teresa Pereira for her love and support during our London years. This thesis is dedicated, in loving memory, to my grandmother, Dorrie Downie.
Abstract

Dense deposit disease (DDD) is a rare, progressive and incurable kidney disease characterized by complement C3 accumulation along the glomerular basement membrane (GBM). It is the prototypical form of C3 glomerulopathy, which comprises renal disorders due to excessive C3 activation via the alternative pathway (AP). Human and murine studies indicate that renal inflammation in DDD is initiated by the specific C3 activation product iC3b. I have assessed the role of iC3b in DDD using the uniquely informative experimental model of C3 glomerulopathy in factor H (FH)-deficient mice. I demonstrate that coexisting deficiency of CD11b, the specific leukocyte receptor for iC3b, exacerbates the spontaneous renal phenotype in FH-deficient mice. This suggests that the iC3b-CD11b interaction may mediate partial renal protection in DDD. I also show that CD11b deficiency produces hypersensitivity to experimentally triggered glomerular injury in both FH-deficient and FH-sufficient mice. My second experimental approach in vivo and in vitro was to assess whether C3 activation in the circulation or on the GBM surface is the predominant cause of iC3b accumulation in DDD. This included experiments in which administration of a novel engineered human FH protein fragment reduced glomerular C3 staining in FH-deficient mice.
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<tbody>
<tr>
<td>AFU</td>
<td>arbitrary fluorescence units</td>
</tr>
<tr>
<td>aHUS</td>
<td>atypical haemolytic uraemic syndrome</td>
</tr>
<tr>
<td>ANTN</td>
<td>accelerated nephrotoxic nephritis</td>
</tr>
<tr>
<td>AP</td>
<td>alternative pathway</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>C3-/-</td>
<td>(murine) homozygous deficiency of C3</td>
</tr>
<tr>
<td>C3GN</td>
<td>C3 glomerulonephritis</td>
</tr>
<tr>
<td>C3NeF</td>
<td>C3 nephritic factor</td>
</tr>
<tr>
<td>C4BP</td>
<td>C4b-binding protein</td>
</tr>
<tr>
<td>CB</td>
<td>coating buffer</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD11b-/-</td>
<td>murine homozygous deficiency of CD11b</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>Cfb-/-</td>
<td>murine homozygous deficiency of factor B</td>
</tr>
<tr>
<td>CFHR5 nephropathy</td>
<td>complement factor H-related 5 nephropathy</td>
</tr>
<tr>
<td>Cfh-/-</td>
<td>murine homozygous deficiency of factor H</td>
</tr>
<tr>
<td>Cfi-/-</td>
<td>murine homozygous deficiency of factor I</td>
</tr>
<tr>
<td>CGG</td>
<td>chicken gammaglobulin</td>
</tr>
<tr>
<td>CP</td>
<td>classical pathway</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>Crry</td>
<td>complement receptor 1-related gene/protein-y</td>
</tr>
<tr>
<td>CSS</td>
<td>chronic serum sickness</td>
</tr>
<tr>
<td>DAF</td>
<td>decay accelerating factor</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DDD</td>
<td>dense deposit disease</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DGKe</td>
<td>diacylglycerol kinase epsilon</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>Er</td>
<td>rabbit erythrocytes</td>
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<tr>
<td>ESKD</td>
<td>end-stage kidney disease</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FB</td>
<td>factor B</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystalized portion of immunoglobulin</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-gamma receptor</td>
</tr>
<tr>
<td>FcRγ</td>
<td>Fc receptor gamma chain</td>
</tr>
<tr>
<td>FD</td>
<td>factor D</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FH</td>
<td>factor H</td>
</tr>
<tr>
<td>FHDplHS</td>
<td>factor H-depleted human serum</td>
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<tr>
<td>FHL-1</td>
<td>factor H-like protein 1</td>
</tr>
<tr>
<td>FHR</td>
<td>factor H-related</td>
</tr>
<tr>
<td>FI</td>
<td>factor I</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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1. CHAPTER ONE: INTRODUCTION
1.1 Overview of my thesis

Dense deposit disease (DDD), sometimes called membranoproliferative glomerulonephritis (MPGN) type 2, is a rare glomerular disorder that typically progresses to end-stage kidney disease (ESKD) and recurs after transplantation [Appel et al. 2005, Smith et al. 2007, Smith et al. 2011]. Its name derives from the original pathological description identifying electron-dense deposits in the glomerular basement membrane (GBM) and other renal structures [Berger & Galle 1962]. Several lines of evidence suggest causation of DDD due to abnormal regulation of the alternative pathway (AP) of complement. These include: (1) glomerular accumulation of complement C3 metabolites, with little or no accumulation of immunoglobulin (Ig) or early classical pathway (CP) complement components, in patients with DDD; (2) evidence of excessive AP activation in patients with DDD, including low plasma levels of C3 and AP components due to secondary depletion; (3) presence of circulating C3 nephritic factors (C3NeFs) or other autoantibodies against AP-associated proteins in many patients with DDD; (4) a small number of family studies showing cosegregation of DDD or similar renal phenotypes with homozygous deficiency of the AP regulatory protein factor H (FH), or with other rare gene variants in FH, C3 or other complement genes; (5) identification of additional, rare complement gene variants in sporadic cases of DDD; (6) genome wide association studies (GWAS) indicating that the population attributable risk of DDD is influenced by common complement gene variants; (7) structural and functional data supporting an effect on AP regulation of some of the disease-associated gene variants; and (8) informative animal models in which genetic deficiency of the orthologous murine or porcine FH proteins is associated with renal disease reminiscent of human DDD.

In this thesis, I present my experimental work examining the role of complement fragment iC3b in DDD. My two research hypotheses are:

Hypothesis 1

That deficiency of CD11b, the specific leukocyte receptor for iC3b, ameliorates experimental renal disease in FH-deficient mice.

Hypothesis 2

That FH protects the GBM from surface phase C3 activation via the AP.
In this Introduction, I provide an overview of complement biology, focusing on the AP and its regulation by FH. I summarize the immune role of CD11b, including data implicating the iC3b-CD11b interaction in human autoimmune diseases. I describe the key clinicopathological features of dense deposit disease, and present an integrated model of pathophysiology. I then outline the renal disease models in FH-deficient and CD11b-deficient animals, and conclude with a discussion of my research aims.

1.2 The complement system

The complement system is an immune defense mechanism that recognizes and eliminates microbial pathogens and damaged host cells [Walport 2001, Ricklin et al. 2010]. It comprises over 30 proteins found in plasma and other body fluids or localized to cell membranes. These include pattern recognition molecules, proteases, opsonins, anaphylatoxins, the membrane attack complex (MAC), complement regulatory proteins and cell surface receptors. Complement activation is initiated via three proteolytic pathways, the AP, CP and lectin pathway (LP), converging at activation of C3 (Fig. 1). Downstream effects of C3 activation include: chemotaxis due to the anaphylatoxins C3a and C5a; opsonization, whereby cells are tagged with C3 fragments for complement C3 receptor (CR)-dependent removal by the mononuclear phagocyte system and stimulation of adaptive immune responses; and formation of the MAC (C5b-9) with the capacity for lysing cells. Tight control by regulatory proteins protects against indiscriminate complement activation and consequent host tissue injury.
Figure 1 The pathways of complement activation

Complement activation via the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP) leads to formation of C3 convertases for the activation of intact C3. C3 activation generates the anaphylatoxin C3a and opsonin C3b. Amplification of C3 activation via the C3 convertase of the AP (C3bBb) is inhibited by factor H (FH). Downstream formation of C5 convertases leads to C5 activation, releasing the anaphylatoxin C5a together with C5b, which initiates membrane attack complex (MAC) formation. Adapted from [Barbour et al. 2014] with permission of Elsevier.
1.2.1 C3 activation

C3 (185kDa) is the most abundant of the complement proteins [Müller-Eberhard et al. 1960]. It is an α₂-macroglobulin glycoprotein encoded on chromosome 19p13.3-13.2 and produced mainly by the liver [Alper et al. 1969]. Plasma concentrations (0.7-1.7 mg/mL) rise ~50% in acute phase response [Gabay & Kushner 1999]. Extrahepatic C3 synthesis occurs in numerous tissues including the kidney [Morgan & Gasque 1997], which contributes 5% to the circulating C3 pool under basal conditions [Tang et al. 1999]. Intact C3 consists of two disulfide-linked polypeptide chains, the alpha (α)-chain (110kDa) and beta (β)-chain (75kDa) [Janssen et al. 2005], and is biologically inactive. Sequential α-chain cleavage steps generate C3 fragments [West et al. 1966]. This is accompanied by major conformational rearrangements that determine interaction of the C3 fragments with biological surfaces and other components of the complement system including regulatory proteins and receptors [Nishida et al. 2006]. The first step (C3 activation) involves cleavage of a single peptide bond on the α-chain, releasing a small fragment, the anaphylatoxin C3a (8kDa, Fig. 2). This leaves C3b (177kDa), which consists of the cleaved α-chain, now termed α-prime (α’), joined to the β-chain. C3 activation occurs at a low rate due to the C3 tickover mechanism or the effect of plasma proteases, and is catalyzed by C3-cleaving enzyme complexes (C3 convertases) [Ruseva & Heurich 2014].

C3 activation disrupts the internal thioester bond of intact C3, resulting in a conformational change that exposes the reactive thioester-containing domain (TED) on the α-chain [Janssen et al. 2006]. The highly electrophilic exposed thioester confers on nascent or 'metastable' C3b (designated as C3b*) a capacity for covalent binding to the nucleophilic free hydroxyl (-OH) or amine (-NH₂) groups found on all biological surfaces [Müller-Eberhard et al. 1966, Law & Levine 1977, Tack et al. 1980]. However, in the circulation (fluid phase) C3b* is rapidly hydrolysed, with C3b(H₂O) no longer able to form covalent bonds on surfaces. This allows a window period of only 60μs for C3b* surface-binding [Sim et al. 1981], which is thus restricted to local sites of activation and is only ~10% efficient [Müller-Eberhard et al. 1967, Law & Dodds 1997]. C3 activation also exposes overlapping N-terminal sites on the α'-chain of C3b for non-covalent binding to proteins involved in formation and dissociation of the AP C3 convertase [Taniguchi-Sidle & Isenman 1994, Janssen et al. 2006, Ajees et al. 2006].
Figure 2 C3 activation products
Cleavage of intact C3 (C3 activation) is followed by sequential FI-mediated cleavage of C3b and iC3b utilizing both plasma and membrane cofactors (upper panel). C3 activation is associated with a conformational change that exposes the α-chain thioester-containing domain (TED), enabling covalent binding of C3b to biological surfaces. Complement receptors are shown below their respective C3 ligands.
1.2.2 The classical and lectin pathways

Activation of the CP is typically initiated when the globular heads of C1q, circulating in a complex with two C1r and two C1s serine proteases as the C1 proenzyme, recognise and bind the fragment crystallized (Fc) portion of IgM or IgG in immune complexes. This leads to autoactivation of C1r and its transactivation of C1s, which then cleaves C4 (isotypes A and B) generating the proteolytic fragments C4a and C4b. As described above for its homologue C3b, C4b presents an electrophilic carbonyl group within its newly exposed thioester domain for covalent binding to hydroxyl groups (C4B predominant binding) or amine groups (C4A predominant binding) on the CP-activating immune complex or cell surface, followed by attachment to C4b of C2. Where the resulting C4bC2 complex adopts a specific spatial orientation in relation to the bound C1s, C1s also cleaves C2 yielding C2a (large fragment [Kemper et al. 2014]) and C2b. This generates the C3 convertase of the CP, C4b2a, with the C2a site acting as a serine protease for C3 activation. The LP becomes activated through mannose-binding lectin (MBL) or ficolins (ficolin-1, -2, -3), which circulate in complexes with three MBL-associated serine proteases (MASP-1, -2, -3, acting as homologues for C1r and C1s) and other non-enzymatic MBL-associated proteins. On binding of MBL or ficolins to cell-surface carbohydrates or acetylated molecular patterns, autoactivation of MASP-2 (and possibly the other MASP proteins) leads to cleavage of C4 and C2, and formation of the identical C3 convertase (C4b2a) as for the CP. Physiological dissociation of C4b2a is enhanced by the soluble CP/LP regulator, C4b-binding protein (C4BP).

1.2.3 The alternative pathway

In evolutionary terms, the AP is the most ancient of the complement activation pathways, and probably predates the development of the adaptive immune system [Farries & Atkinson 1991]. It was discovered through the demonstration of Ig- and CP-independent C3 activation in vitro [Pillemer et al. 1953, Götze & Müller-Eberhard 1971]. Three aspects of C3 activation are unique to this pathway: (1) C3 activation via the AP occurs both on surfaces and in aqueous solution (‘fluid phase’) due to the generation of a freely diffusing C3 convertase of the AP; (2) fluid-phase C3 activation via the AP occurs spontaneously at a low level (so-called C3 ‘tick-over’ [Nicol & Lachmann 1973]); and (3) the AP constitutes a common ‘amplification loop’ for C3 activation [Lachmann 2009], irrespective of the initiating trigger of C3 activation.
1.2.3.1 C3 tickover

C3 tickover entails low-level, spontaneous activation of C3 in the fluid phase due to hydrolysis of C3. It was originally proposed as a solution to the paradox wherein ‘the alternate pathway for C3 fixation seems to possess an absolute requirement for fixed C3, the product of its reaction’ [Nicol & Lachmann 1973]. Incubation of intact C3 in water at 37°C was shown to be associated with slow decay (0.5% per h) of C3 haemolytic activity [Pangburn & Müller-Eberhard 1980]. More rapid C3 haemolytic decay during incubation with the nucleophile methylamine (CH₃NH₂) was proportional to the rate at which methylamine bound intact C3 (via covalent interaction with the TED [Nishida et al. 2006]). This interaction resulted in the acquisition of certain ‘C3b-like’ functional properties, including the ability to initiate C3 activation via the AP in normal human serum. As this property had also been observed following repeated freeze-thawing of intact C3 in solution, it was postulated that ‘under physiological conditions…C3b-like C3 arises by nonenzymatic spontaneous thioester-bond hydrolysis of C3 and thereby effects formation of a steady-state concentration of the initial C3 convertase of the alternative pathway of complement activation’ [Pangburn & Müller-Eberhard 1980]. Among its C3b-like properties, C3(H₂O) is able to form a C3 convertase of the AP [Pangburn et al. 1981].

1.2.3.2 C3b amplification

The C3b amplification loop of the AP was originally demonstrated in vitro as a ‘positive feedback mechanism’ wherein C3b participates in formation of the AP C3 convertase (C3bBb) for activation of intact C3 to C3b [Müller-Eberhard & Götze 1972]. Mg²⁺-dependent binding of C3b to the inactive plasma serine protease factor B (FB, 93kDa) is followed by cleavage of FB to Ba and Bb by the plasma serine protease factor D (FD, 25kDa) [Forneris et al. 2010]. This yields C3bBb, the AP C3 convertase, with the Bb component responsible for C3 activation [Müller-Eberhard & Götze 1972, Götze & Müller-Eberhard 1976]. C3b amplification occurs via the AP in the fluid phase and on surfaces, regardless of the initiating pathway of C3 activation [Fearon et al. 1973, Harboe et al. 2004]. However, C3bBb is short-lived (t½ of ~90s) owing to spontaneous dissociation of Bb from C3b [Fishelson et al. 1984, Pangburn & Müller-Eberhard 1986]. Properdin (53kDa) is a glycoprotein that stabilizes C3bBb against spontaneous dissociation and prevents interaction of the C3b component with complement regulatory proteins [Pillemer et al. 1954, Fearon & Austen 1975, Alcorlo et al. 2013]. Properdin is thus the only physiological positive regulator of complement activation, and in addition may initiate surface assembly of C3bBb [Kemper et al. 2010].
1.2.3.3 Artificial surfaces

Artificial surfaces used in vitro (e.g. polystyrene-coated receptacles) or in vivo (e.g. vascular access and cardiopulmonary devices) may induce C3 activation via the AP. Firstly, hydrophobic artificial surfaces with increased density of free –OH or –NH_2 groups facilitate covalent surface-binding of C3b* from the circulation, anchoring formation of the AP C3 convertase [Chenoweth 1987]. Secondly, abundant plasma proteins including albumin, IgG and fibrinogen are adsorbed onto artificial surfaces, forming a layer of plasma proteins capable of binding C3b* covalently [Andersson et al. 2005, Nilsson et al. 2007]. Thirdly, adsorption of intact C3 to artificial surfaces may activate it non-proteolytically [Andersson et al. 2002]. Based on this observation, it has been argued that C3 tickover represents ‘contact activation’ due to surface interfaces including vanishing gas bubbles (formed in vivo by cardiopulmonary bypass or in decompression sickness), activated platelets, lipoproteins and foreign biomaterials [Nilsson & Nilsson Ekdahl 2012].

1.2.4 Factor I

Factor I (FI, 88kDa) is a plasma serine protease encoded on chromosome 4q25 and produced mainly by the liver (plasma concentration 35-45μg/mL) [Reynolds et al. 2009, Lay et al. 2014]). It was originally identified based on functional ‘inactivation’ of surface-bound C3b in vitro [Tamura & Nelson 1967, Lachmann & Müller-Eberhard 1968]. The ability of FI to cleave C3b [Abramson et al. 1971, Ruddy & Austen 1971] and inhibit the AP C3 convertase [Alper et al. 1972] was subsequently demonstrated in fluid phase assays. In human serum depleted of FI, uncontrolled C3 activation via the AP indicated that C3 tickover is ‘normally efficiently damped’ by this regulatory protein [Nicol & Lachmann 1973]. In individuals with rare homozygous deficiency of FI, secondary depletion of C3, FB and properdin due to uncontrolled AP activation results in low plasma levels of these proteins [Alper et al. 1970, Vyse et al. 1994]. Circulating C3 is detected almost exclusively in the form of C3b, consistent with a physiological requirement for FI in the generation of C3b metabolites.

FI cleaves the α’-chain of C3b, releasing the α’-chain C3f fragment (2kDa) to leave iC3b (176kDa), which consists of the residual α’-chain joined to the β-chain. FI-mediated C3b cleavage is accompanied by a conformational change that sequesters the TED and disrupts the FB binding site [Nishida et al. 2006, Alcorlo et al. 2011]. This conformational change renders iC3b unable to form the AP C3 convertase [Law et al. 1979]. FI-mediated C3b cleavage requires a C3b-binding cofactor, prototypically FH [Pangburn et al. 1977, Harrison & Lachmann 1980]. The membrane-expressed regulators CR1 (CD35) and membrane cofactor protein (MCP, CD46) are additional FI cofactors expressed on cellular surfaces. A
second FI-mediated cleavage step degrades iC3b, generating an $\alpha'$-chain fragment, C3dg (40kDa), and C3c (135kDa), which consists of the residual $\alpha'$-chain joined to the $\beta$-chain. FI-mediated iC3b cleavage utilizes CR1 as the principal cofactor [Medicus et al. 1983].

On surfaces, efficient FI-mediated cleavage of covalently bound C3b prevents C3b amplification via the AP and its downstream effects including MAC formation. It also results in a relatively short $t_{1/2}$ for surface-attached C3b (~90s) compared to its cleavage product iC3b (~35 min) [Ross et al. 1985]. Subsequent FI-mediated cleavage of iC3b (in the presence for CR1) leaves surface-bound C3dg, which is further trimmed by proteases to C3d (corresponding to the TED) [Lachmann et al. 1982]. Far from being immunologically inert, surface-bound iC3b and C3d(g) are important ligands for CRs expressed on leukocytes [Ueda et al. 1994]. A critical role for these opsonic C3 fragments in immune clearance is underscored by the predisposition of individuals with severe FI deficiency to recurrent bacterial infection and immune complex-mediated disease [Nilsson et al. 2011].

1.2.5 Factor H

FH (155kDa) is a plasma glycoprotein that regulates the AP in the fluid phase and on surfaces [Nilsson & Müller-Eberhard 1965, Makou et al. 2013]. It is encoded in the regulators of complement activation (RCA) cluster of chromosome 1q32 that also contains the genes for C4BP, CR1, CR2, MCP, decay accelerating factor (DAF, CD55) and the five FH-related (FHR) proteins [Rodriguez de Cordoba et al. 1985]. FH consists of a single chain of 20 homologous modules each containing ~61 amino acids, termed short consensus repeat (SCR) domains, linked by 40 disulfide bridges (Fig. 3) [Rodriguez de Cordoba et al. 2004]. Extensive post-translational modification of FH, including N-glycosylation at 8 sites, is essential to protein conformation [Fenaille et al. 2007]. FH is constitutively produced by the liver, but also extrahepatically [Ferreira et al. 2010]. In a recent, large population study, the median plasma FH level in healthy individuals was 226.6μg/mL (range 63.5-847.6μg/mL) [Sofat et al. 2013].

The mechanisms by which FH regulates the AP were originally demonstrated using surface (haemolysis) assays with FH purified from normal human plasma [Weller et al. 1976, Whaley & Ruddy 1976]. In the fluid phase, uncontrolled C3 activation via the AP was demonstrated in normal human serum depleted of FH [Whaley & Thompson 1978]. Similar to FI deficiency, individuals with rare homozygous FH deficiency show secondary depletion of plasma C3, FB and properdin due to uncontrolled C3 activation via the AP [Thompson & Winterborn 1981]. FH competes with FB for C3b binding and also displaces the Bb fragment from any preformed AP C3 convertase, thereby both impeding formation and promoting dissociation.
of the AP C3 convertase (decay accelerating activity). FH is also a cofactor for FI-mediated cleavage of C3b to iC3b (FI cofactor activity). The two major C3b-binding sites on FH are located within the amino (N)-terminal SCR domains 1-4 and carboxy (C)-terminal SCR domains 19-20, respectively. N-terminal SCR domains 1-4 are necessary and sufficient for fluid phase decay accelerating activity and FI cofactor activity [Schmidt et al. 2008b, Wu et al. 2009]. FH-like protein 1 (FHL-1, 43kDa), which results from alternative splicing of FH and comprises N-terminal SCR domains identical to FH SCR domains 1-7 and four additional C-terminal amino acids, is also capable of fluid phase AP regulation [Friese et al. 1999].

The C-terminal C3b-binding site of FH plays a critical role in FH-mediated regulation of the AP on surfaces [Ferreira et al. 2006]. Despite recognising the C3d moiety of C3b, the C-terminal binding site interacts only weakly, if at all, with the C3b cleavage products iC3b and C3d(g) [Alcorlo et al. 2011]. Glycosaminoglycans (GAGs) and sialylated carbohydrates on cellular surfaces enhance the interaction of FH with surface-bound C3b. This was originally demonstrated through desialylation of sheep erythrocytes, which abrogated their protection by FH against AP-mediated haemolysis in in vitro assays [Fearon 1978, Pangburn & Müller-Eberhard 1978]. Similarly, deficiency of GAGs on zymosan and rabbit erythrocytes was shown to mediate resistance of these AP-activating surfaces to FH regulatory activity [Kazatchkine et al. 1979, Meri & Pangburn 1990]. Subsequently, binding of FH to a wide array of cellular surfaces has been demonstrated in vitro, for example using cultured human vascular endothelial cells (HUVEC) [Jozsi et al. 2004, Jokiranta et al. 2005]. Several GAG-binding sites on the FH molecule include one on SCR 20 that overlaps with the C3b-binding site [Hellwage et al. 2002, Schmidt et al. 2008a]. Structural studies indicate that FH adopts a bent-back FH conformation in which N-terminal binding to C3b facilitates complement regulation, with simultaneous C-terminal binding to the same C3b molecule and to adjacent surface GAGs [Aslam & Perkins 2001, Morgan et al. 2011].

1.2.6 Factor H-related proteins

The five structurally related FHR proteins are encoded downstream of the FH gene in the RCA cluster. C-terminal SCR domains are highly conserved between FH and the FHR proteins. By contrast the FHR proteins lack the first four N-terminal SCR domains that mediate the complement regulatory activity of FH (and FHL-1). Instead, distinct N-terminal SCR domains 1 and 2 are highly conserved on FHR-1, -2 and -5 and these are important in FHR dimerization [Goicoechea de Jorge et al. 2013]. The FH-FHR locus is prone to large genomic deletions, insertions and rearrangements (detected as copy number variation) [Jozsi & Zipfel 2008].
Figure 3 The factor H protein family

FH consists of 20 short consensus repeat (SCR) domains. Those at the C-terminus (depicted in yellow) mediate surface recognition and are common to the five FHR proteins (FHR-4 has two isoforms, FHR-4A and FHR-4B). By contrast, N-terminal SCR domains 1-4 mediating FH complement regulatory function (red) are found only on FH and FHL-1. The N-terminal SCR domains 1 and 2 are highly conserved on FHR-1, FHR-2 and FHR-5 (blue) but not FHR-3 and FHR-4A/B. Reproduced from [Barbour et al. 2015] with permission from Oxford University Press (License N° 3510930508784).
1.2.7 C5 activation

Binding of additional C3b molecules to the C3 convertases yields the C5 convertases, C3bC4bC2a and C3bC3bBb, for activation of C5 [Pangburn & Rawal 2002]. C5 (190kDa) is structurally related to C3 and C4, and comprises an α-chain (115kDa) and β-chain (75kDa). C5 activation by cleavage of the α-chain releases the potent anaphylatoxin C5a and C5b, which initiates MAC formation. The MAC inserts pores into the lipid bilayer of cell surfaces, culminating in cellular activation or lysis [Tegla et al. 2011].

1.2.8 In vitro and ex vivo studies of surface phase alternative pathway activation

1.2.8.1 Rabbit haemolysis assay

The rabbit haemolysis assay utilizes the susceptibility of unsensitized rabbit erythrocytes (E_R) to AP-dependent lysis following incubation in normal human serum (NHS) [Platts-Mills & Ishizaka 1974]. This property has led to the routine use of E_R in the laboratory evaluation of human sera for AP defects. The key mechanisms by which AP-dependent E_R lysis occurs were elucidated in a series of experiments using purified human AP components [Fearon & Austen 1977b]. E_R or sheep erythrocytes (E_S) were coated with C3b by incubation in Mg^{2+}-containing buffer with C3, FB and FD. AP-dependent haemolysis was then triggered through a second incubation with FB, FD and properdin in buffer to which heterologous (rat) serum was added (as a source of C3-C9).

Previous studies had shown that haemolysis of C3b-coated E_S in rat serum could be reduced through prior incubation in human FH (especially in combination with human FI) [Weiler et al. 1976, Whaley & Ruddy 1976]. It was now demonstrated that: (1) the relative reduction in haemolysis achieved through prior incubation with FI with or without FH was much greater for E_S than E_R [Fearon & Austen 1977b]; (2) following an initial incubation with FB, FD and properdin, a subsequent incubation with FH led to dissociation of the AP C3 convertase on C3b-coated E_S but not C3b-coated E_R (based on the extent of haemolysis during a final incubation with rat serum); and (3) whereas E_R lysis following incubation in buffer containing C3, FB, FD, properdin, FI and FH was associated with C3 activation (based on haemolytic decay of the supernatant), neither lysis nor C3 activation occurred using E_S. Based on these experiments, and earlier results using zymosan [Fearon & Austen 1977a], the authors concluded that ‘activators of the alternative pathway, such as zymosan and E’T [E_R], provide privileged sites that protect C3b deposited by the low grade fluid phase reaction and the P,C3b,Bb [properdin-stabilized AP C3 convertase] formed at that site from the action of regulatory proteins, resulting in membrane-associated amplified C3 cleavage’ [Fearon & Austen 1977b].
Next, the authors assessed whether ‘increased C3b fixation by E’ resulted from deposition by particle-bound amplification C3 convertase rather than by fluid phase convertase’ [Fearon & Austen 1977b]. Incubation of E\textsuperscript{S} in solution containing purified AP components and FH led to 10% C3 activation at 60 minutes, with negligible cell surface uptake of (radiolabelled) C3. By contrast, incubation of E\textsuperscript{R} in the presence of FH led to 64% C3 activation after only 15 minutes, with surface uptake of 12.4% of total cleaved C3 at 60 minutes. In the absence of FH, activation of total C3 occurred within 15 minutes, and was associated with surface uptake of only 0.14% and 0.16% C3 on E\textsuperscript{R} and E\textsuperscript{S}, respectively. The authors concluded that ‘deregulation of endogenous controls at the surface of E\textsuperscript{R} but not E\textsuperscript{S} results in local deposition of C3b by formation and function of surface-bound amplification convertase. In the absence of the regulatory protein, β1H [FH], the fluid phase reaction spontaneously advances to amplification, consuming C3 and [F]B, so that specific deregulation at the surface of E’ with concomitant accumulation of C3b cannot occur’ [Fearon & Austen 1977b].

1.2.8.2 Renal proximal tubular C3 staining

Renal proximal tubular cells may provide an AP-activating surface. Incubation of normal rat or human cryosections ex vivo with NHS led to preferential C3 deposition on the apical (luminal) side of proximal tubules that was AP-dependent [Camussi et al. 1982, Camussi et al. 1983]. This was also demonstrated using human cultured proximal tubular epithelial cells (PTEC), but not HUVEC [Biancone et al. 1994, Gaarkeuken et al. 2008]. AP-dependent C3 deposition on cultured human PTEC following incubation with urinary proteins from proteinuric patients was attributed to the ability of tubular epithelium to provide a ‘protected site’ from AP regulation [Peake et al. 2002]. However, human FH has been shown to bind to human cultured PTEC [Buelli et al. 2009, Zaferani et al. 2012, Nagamachi et al. 2014]. Tubular staining for FH has also been reported on human renal sections [Licht et al. 2007]. The degree to which FH binds to the apical surface of PTEC may be influenced by surface expression and/or sulfation of GAGs [Buelli et al. 2009, Zaferani et al. 2012].

C3 deposition on the apical surface of cultured murine TEC following incubation of WT serum was enhanced in the presence of a recombinant mouse FH fragment comprising C-terminal SCR domains 19-20 [Renner et al. 2010]. This was attributed to competitive inhibition of full-length FH on the TEC surface by the recombinant FH fragment. The authors concluded that ‘regulation of complement by factor H is inadequate to prevent spontaneous complement activation on the apical surface of the cells’ [Renner et al. 2010]. However, by addition to WT mouse serum of supraphysiological purified FH from WT mice, C3 deposition on the TEC surface was reduced, indicating that ‘at a high enough concentration factor H is capable of suppressing CAP activation on the TEC surface’ [Renner et al. 2011].
1.2.9 Membrane-expressed regulators

CR1 is a multifunctional type 1 transmembrane glycoprotein comprising up to 44 SCR domains and expressed on most peripheral blood cells [Krych-Goldberg & Atkinson 2001]. Its complement regulatory functions include decay accelerating activity for the C3 and C5 convertases and cofactor activity for FI-mediated cleavage of C3b and iC3b [Fearon 1979, Ross et al. 1982, Medicus et al. 1983]. Soluble CR1 (sCR1) is present at low physiological concentrations in plasma as a result of surface cleavage of membrane-bound CR1 on leukocytes [Yoon & Fearon 1985, Danielsson et al. 1994].

MCP is a type 1 transmembrane protein comprising 4 SCR domains that is expressed on all nucleated cells and has cofactor activity for FI-mediated C3b cleavage [Seya et al. 1986]. Soluble MCP has also been detected in small amounts, typically in association with cancer or immune diseases [Seya et al. 1995].

DAF is a glycoporphosphatidylinositol (GPI)-anchored protein comprising four SCR domains, and is expressed on peripheral blood cells, with decay accelerating activity for C3 and C5 convertases [Medof et al. 1984]. CD59 is a GPI-linked inhibitor of MAC formation expressed on erythrocytes and leukocytes [Meri et al. 1990].

In the kidney, DAF, MCP and CD59 are ubiquitously expressed in all three glomerular cell types (mesangial cells, endothelial cells and podocytes) whereas CR1 is expressed exclusively on podocytes [Ichida et al. 1994, Nangaku 1998, Naik et al. 2013]. MCP is the only membrane-expressed regulatory protein detected in renal tubules (on the basolateral aspect of cortical collecting ducts and proximal tubules) [Ichida et al. 1994]. Cytokine-enhanced FH production has been demonstrated using cultured PTEC and mesangial cells [Zhou et al. 2001].

1.2.10 Complement receptors

Of the five recognised CRs, the SCR modular receptors CR1 and CR2 (CD21) and the beta-2 (β2)-integrins CR3 (CD11b/CD18, αmβ2, macrophage-1 antigen [Mac-1]) and CR4 (CD11c/CD18, αxβ2, p150,95) recognise distinct C3 α-chain sites [van Lookeren Campagne et al. 2007]. CR1g is a member of the Ig superfamily expressed on tissue resident macrophages in the liver, where it binds the β-chain of C3b and iC3b and is important in phagocytosis [Helmy et al. 2006]. CR1g may also function as an inhibitor of the C3/C5 convertases [Wiesmann et al. 2006]. The anaphylatoxin chemotactic receptors C3aR (55kDa) and C5aR (CD88) are G-coupled receptors present on peripheral blood leukocytes and tissue-derived cells, and mediate important proinflammatory responses [Wetsel 1995].
CR1 was originally identified through its role as the C3b/C4b receptor on erythrocytes in immune adherence [Nelson 1953, Rothman et al. 1975]. It binds the C3c moiety of C3b and has reduced affinity for iC3b [Kalli et al. 1991]. In addition to erythrocytes, it is expressed on myeloid leukocytes, follicular dendritic cells (FDCs) and B and T cells [Khera & Das 2009]. Its expression on myeloid leukocytes facilitates cooperation with Fc-gamma receptor (FcyR) and CR3 in phagocytosis [Sutterwala et al. 1996].

CR2 is expressed mainly on B cells and FDCs, and binds the C3d moiety of iC3b and C3d(g) [Weis et al. 1984, van den Elsen & Isenman 2011]. Under conditions of limiting antigen, co-ligation of CR2 and the B cell antigen receptor (BCR) by C3d lowers the threshold dose of antigen required for B cell signalling leading to clonal expansion [Carter et al. 1988, Matsumoto et al. 1991]. Trapping of C3d-opsonized antigen by CR2-expressing FDCs in germinal centres facilitates antigen presentation and induction of B cell immunity [Carroll & Isenman 2012]. The molecular adjuvant effects of C3d mediated via CR2 have been shown as more potent than those of complete Freund’s adjuvant (CFA) [Dempsey et al. 1996].

1.2.10.1 β2-integrins

CR3/Mac-1 and CR4 were originally identified based on their specificity for the iC3b fragment and their expression on a distinct cell population from that expressing CR1 and CR2 [Ross & Lambris 1982]. They are members of the leukocyte-restricted β2-integrin subfamily, also comprising lymphocyte function-associated antigen 1 (LFA-1, or CD11a/CD18, αLβ2) and CD11d/CD18 (αDβ2) [Tan 2012]. The broader integrin family of 24 heterodimers constitutes the major adhesion receptors in metazoa [Hynes 2002]. Each β2-integrin consists of a common, smaller β2-subunit and a variable, larger α-subunit that are non-covalently associated. Both the α-subunit and β2-subunit comprise a large extracellular domain, a single pass transmembrane domain and a cytosolic tail [Arnaout 1990]. The presence of CR3/Mac-1 on human leukocytes was first shown using phagocyte-specific monoclonal antibodies [Springer et al. 1979]. CR3/Mac-1 was subsequently recognised as the phagocyte receptor for iC3b [Beller et al. 1982, Wright et al. 1983]. It is the predominant β2-integrin on neutrophils and is also expressed on monocytes/macrophages and myeloid dendritic cells (DCs) as well as lymphoid natural killer (NK) cells, γδ-T cells and a minor subset of B cells [Arnaout 1990, Ross 2000, Graff & Jutila 2007, Griffin & Rothstein 2011]. CR4 is expressed on a similar cell population and has many ligands in common with CR3/Mac-1, including iC3b [Chen et al. 2012].
Integrins are constitutively inactive, with activation mediated via bidirectional signalling between the extracellular and cytosolic integrin domains [Hynes et al. 2002]. This paradigm of integrin activation is commonly referred to as ‘outside-in’ and ‘inside-out’ signalling [Tan 2012]. Binding of extracellular ligands to integrins leads to transduction of outside-in signals through the cell membrane, culminating in cellular processes including adhesion, phagocytosis and cytokine production. Inside-out signaling involves transmission of intracellular signals, including those produced through stimulation of adjacent cell surface receptors, to the cytosolic tail. This induces conformational changes in the extracellular domains that are associated with a high-affinity ligand-binding state [Diamond & Springer 1994, Luo et al. 2007]. Thus an inactive integrin is converted from its bent extracellular conformation to an extended conformation, and its ligand-binding domain from a closed conformation to an open one [Springer & Dustin 2012]. Inside-out signalling is less clearly established for Mac-1/CR3 than other members of the β2-integrin subfamily (notably LFA-1) [Fagerholm et al. 2006]. Clustering of integrins on cellular surfaces is an additional determinant of avidity for ligand-dependent cellular processes [Ley et al. 2007].

1.2.10.2 The iC3b-CD11b interaction

The CD11b subunit of CR3/Mac-1 is a 165kDa glycoprotein encoded by the integrin alpha M (ITGAM) gene on chromosome 16p11.2. It confers specificity of CR3/Mac-1 for a wide range of structurally unrelated endogenous ligands and pathogen molecules [Yakubenko et al. 2002]. Many of these, including iC3b, intracellular adhesion molecule (ICAM)-1 (CD54), fibrinogen and heparin, bind overlapping binding sites within the CD11b inserted (I)-domain [Diamond et al. 1993, Diamond et al. 1995]. The I-domain contains a metal ion-dependent adhesion site (MIDAS) in which Mg2+ coordinates ligand binding [Stewart & Hogg 1996]. iC3b ligation of CD11b-expressing leukocytes is critical to phagocytosis of complement-opsonized particles, immune complexes and apoptotic cells [Arnaout et al. 1983, Takizawa et al. 1996, Dupuy & Caron 2008] and to antigen presentation during induction of peripheral immune tolerance [Hammerberg et al. 1998, Sohn et al. 2003]. Both CD11b and CD11a (LFA-1) bind ICAM-1, leading to leukocyte adhesion and transendothelial migration [Diamond et al. 1990, Ley et al. 2007]. Cytotoxicity and adhesion are thus viewed as the classic CD11b-mediated leukocyte functions [Ross 2000], whereas a wide array of other CD11b ligands contribute to novel roles including oxidative burst and degranulation, and apoptosis [Tan 2012]. The I-domains of CD11b [Ueda et al. 1994, Ustinov & Plow 2005] and CD11c (CR4) [Malhotra et al. 1986] recognise distinct iC3b moieties. CD11b binds the C3d moiety of iC3b with high affinity, but interacts only weakly with the iC3b cleavage product C3d(g) [Bajic et al. 2013]. CD11c interacts with the C3c moiety [Chen et al. 2012].
1.2.10.3 CD11b in human disease

The importance of β₂-integrins in innate immunity was elucidated in patients with leukocyte adhesion deficiency type 1 (LAD-1), an autosomal recessive disorder characterized by recurrent bacterial infection [Arnaout et al. 1982, Fischer et al. 1983]. LAD-1 is associated with variants in the \( ITGB2 \) gene encoding CD18 that result in reduced expression or dysfunction of Mac-1/CR3, LFA-1 and CR4 [Kishimoto et al. 1987, Hogg et al. 1999]. A case is reported of CD11b deficiency with normal antigenic levels of CD18, in a patient with non-healing lower limb ulcers [Darveaux et al. 2014].

Single nucleotide polymorphisms (SNPs) in \( ITGAM \) are associated with increased risk of developing systemic lupus erythematosus (SLE) based on GWAS. Most strongly associated is a missense variant at exon 3 of \( ITGAM \) (230G>A, rs1143679, Arg77His). Arg77His occurs with 9-11% frequency in populations of European and African descent, with an odds ratio of 1.4-2.2 for development of SLE [Harley et al. 2008, Nath et al. 2008, Hom et al. 2008]. Additional GWAS have shown association of Arg77His specifically with renal manifestations of SLE [Yang et al. 2009, Kim-Howard et al. 2010, Warchol et al. 2011, Sanchez et al. 2011, Toller-Kawahisa et al. 2014].

The functional effect most strongly associated with Arg77His is impaired phagocytosis of iC3b-coated targets by CD11b-expressing monocytes/macrophages or neutrophils [MacPherson et al. 2011, Rhodes et al. 2012, Fossati-Jimack et al. 2013]. This polymorphism has also been identified as the Mart alloantigen in neonatal autoimmune neutropenia [Simsek et al. 1996, Sachs et al. 2004]. Rare SLE-associated \( ITGAM \) variants have also been identified in individuals with impaired CD11b-dependent phagocytosis [Roberts et al. 2014]. An earlier study noted impaired phagocytosis of iC3b-coated cells in a patient with SLE in whom an abnormal iC3b-binding epitope on CD11b appeared to have a genetic basis [Witte et al. 1993].

1.2.11 Complement in T cell responses

An emerging concept in complement biology is that the generation of complement activation products by immune cells plays a critical role in adaptive immunity [Kolev et al. 2014]. For example, during the interaction of cognate APCs and T cells, the production and activation of C3 and C5 mediates T cell co-stimulation [Strainic et al. 2008]. It appears that intracellular complement activation and receptor systems may also be important in induction of T cell – mediated responses [Liszewski et al. 2013]. Conversely, immune cell-derived complement activation products may contribute to negative regulation of T cells (for example, via the interaction of C3b with MCP) [Le Friec et al. 2012].
1.3 The glomerular basement membrane

The glomerular capillary wall (GCW) comprises three layers: (1) the GBM, an acellular, extracellular matrix (ECM); (2) on its vascular aspect, a layer of fenestrated endothelium with an overlying glycocalyx (composed of glycoproteins, glycolipids and proteoglycans); and (3) on its urinary aspect, the podocyte layer (visceral epithelial cells) [Slater et al. 2011, Suh & Miner 2013]. The adult GBM is composed of three layers in turn: (1) the lamina densa; (2) on its endothelial/vascular side, the lamina rara interna; and (3) on its epithelial/urinary side, the lamina rara externa. The lamina rara interna and externa are composed largely of GAGs, especially heparan sulfate and hyaluronic acid. The lamina densa is composed of a fine (~3nm), filamentous meshwork in which several glycoproteins are present in addition to GAGs. Although these glycoproteins are common to all basement membranes, specific isoforms are found in the lamina densa of the GBM. Thus polymerised laminin-521 (α5β2γ1) provides the basic scaffold for incorporation of collagen type IV (α3α4α5), nidogen/entactin-1 and -2 and the heparan sulfate proteoglycans agrin and perlecan in the GBM [Miner 2011].

Some of the matrix components are secreted by podocytes to maintain GBM structure and function [Pavenstadt et al. 2003]. Others, including fibronectin, a plasma glycoprotein also found in other ECM and on cell surfaces, are probably derived from the circulation. GBM protein composition may be altered in association with pathological GBM thickening, for example in diabetic nephropathy [Mason & Wahab 2003]. Atypical proteoglycans are also be identified within the GBM in certain disease states (e.g. podocan secreted by podocytes in HIV-associated nephropathy) [Ross et al. 2003].

The GBM is generally believed to be devoid of membrane-expressed complement regulatory proteins [Zipfel 2006]. A single study reported that CR1 is present in human foetal GBM [Appay et al. 1988]. Binding of plasma FH to the GBM has been reported based on indirect immunofluorescence (IF) staining and immuno-EM of normal human kidney sections [Licht et al. 2007]. The primary antibody used was said to be specific for N-terminal FH domains, although cross-reactivity with common epitopes on FHR proteins and/or FHL-1 was not strictly excluded. Elsewhere a linear IF staining pattern for FH in normal kidney specimens or C3 glomerulopathy biopsy samples has been attributed to GBM-binding of FH [Meri et al. 1992, Clark et al. 2013]. Aside from the question of cross-reactivity, this pattern could equally be consistent with FH binding/expression in other layers of the GCW including the endothelium [Vaziri-Sani et al. 2006, Bridoux et al. 2011].
1.4 Dense deposit disease

1.4.1 Histological features

DDD is defined by the appearance on electron microscopy (EM) of extremely dark (osmiophilic), ribbon-like, intramembranous dense deposits transforming the GBM [Cook H. T. & Pickering M.C. 2015] (Fig. 4a,b). Electron-dense deposits are also sometimes seen in the basal lamina of Bowman’s capsule, tubular basement membrane (TBM) and mesangium [Berger & Galle 1962, Joh et al. 1993, Nasr et al. 2009]. Even at high magnification, these deposits lack substructure. On light microscopy (LM), reduplication of the GBM and thickening of the GCW may be visualized by double contours or ‘tram tracks’ on silver stain (Fig. 4c). Together with mesangial expansion and hypercellularity, this constitutes a membranoproliferative pattern [Cook H. T. & Pickering M. C. 2015]. However, in one series in 69 patients with DDD, a mesangioproliferative pattern (43% of biopsies) was more common than a membranoproliferative one (25% of biopsies) [Walker et al. 2007]. Crescentic GN (17%) and an acute proliferative and exudative pattern of ‘diffuse endocapillary proliferation with numerous macrophages and neutrophils’ (12%) were also observed. Recurrence of DDD may even produce crescentic GN following renal transplantation [Hoschek et al. 2002, Braun et al. 2005]. For these reasons, the term MPGN type 2 [Mathew & Kincaid-Smith 1971, Habib et al. 1975] is no longer used for DDD.

IF or immunohistochemistry (IHC) using immunoperoxidase shows intense glomerular staining for C3, corresponding to the dense deposits seen on EM [Cook H. T. & Pickering M.C. 2015]. Thus C3 is detected in the mesangium and in a variably linear or granular pattern in the GCW (Fig. 4d,e), whereas little or no glomerular Ig or early CP components (C1q and C4) are detected [Berger et al. 1969]. Isolated or predominant C3 is indicative of C3 activation via the AP. C3-dominant staining is seen not only in DDD, but also in cases where the GCW deposits are subendothelial rather than intramembranous, or otherwise lack the classic appearance of DDD [Bariéty et al. 1971]. In one series of 19 such cases, the term glomerulonephritis C3 (or C3 glomerulonephritis, C3GN) was proposed [Servais et al. 2007]. The term C3 glomerulopathy has since been introduced for all forms of GN involving ‘a disease process due to abnormal control of complement activation, deposition, or degradation and characterized by predominant glomerular C3 fragment deposition with electron-dense deposits on electron microscopy’ [Pickering et al. 2013]. Recognizable entities within C3 glomerulopathy include those with distinct morphology (DDD and electron-dense intramembranous GBM transformation) or aetiology (CFHR5 nephropathy and the presence of an abnormal FHR-5 protein [Gale et al. 2010]). The more common histological lesion of MPGN type 1 is characterized by glomerular deposition of C3 and IgG, consistent
Figure 4 Histological features of dense deposit disease

(a) Electron microscopy (EM) showing typical osmiophilic, ribbon-like electron-dense deposits (indicated by arrow) within the lamina densa (middle layer) of the glomerular basement membrane (GBM). Reproduced from [Barbour et al. 2013a] with permission of Oxford University Press (License N° 3510930958829). (b) EM showing characteristic osmiophilic deposits (arrow). (c) Light microscopy with silver stain showing a membranoproliferative pattern with double contours of the GBM (arrow). (d) Immunofluorescent and (e) immunoperoxidase staining of glomerular capillary wall C3 and some granular mesangial C3. Reproduced from [Barbour et al. 2013b] with permission of Elsevier.
with immune complex formation. MPGN type 1 is therefore not included in the classification of C3 glomerulopathy, although some overlap probably exists, in light of reports of MPGN type 1 in association with genetic complement abnormalities (discussed below).

1.4.2 Clinical features

Estimated prevalence per million population ranges from 1-2 C3 glomerulopathy cases (inclusive of DDD) [Medjeral-Thomas et al. 2014] to 2-3 DDD cases [Smith et al. 2011]. DDD is traditionally viewed as a diagnosis of childhood and young adulthood, with recent series showing a younger age at diagnosis for DDD than C3GN [Servais et al. 2012, Medjeral-Thomas et al. 2014]. However, one recent DDD series included numerous patients aged over 60 years [Nasr et al. 2009]. Some studies indicate that DDD is more common in females than males (~2:1 ratio) [Nasr et al. 2009, Wang et al. 2012], although most show no sex differences in DDD (or C3GN) [Walker et al. 2007, Servais et al. 2012, Lu et al. 2012, Medjeral-Thomas et al. 2014]. DDD presents clinically as nonspecific intrinsic renal disease, comprising any or all features of haematuria, proteinuria, peripheral oedema, hypertension and renal failure [Appel et al. 2005]. It is associated with extrarenal conditions of acquired partial lipodystrophy [Misra et al. 2004] and ocular drusen [Duvall-Young et al. 1989]. These conditions may also be associated with other types of C3 glomerulopathy and MPGN [Barbour et al. 2013b]. Similarly, an association with monoclonal gammopathy of undetermined significance (MGUS) has been reported in adults with DDD [Sepandj & Trillo 1996, Hill & Desmond 2007, Nasr et al. 2009, Sethi et al. 2010] and C3GN [Bridoux et al. 2011, Zand et al. 2013]. No renal clinical or pathological features appear to distinguish the MGUS-associated forms of C3 glomerulopathy from non-MGUS-associated forms. Increased prevalence of type I diabetes mellitus was also reported in families with a DDD-affected member in a self-reporting study [Lu et al. 2012].

Prognosis of DDD is poor, with 10-year progression to ESKD of 40-50% [Smith et al. 2007, Servais et al. 2012, Lu et al. 2012]. Renal survival may be reduced in adults compared to children [Nasr et al. 2009, Servais et al. 2012]. Reduced GFR at diagnosis appears to predict progression to ESKD [Nasr et al. 2009, Servais et al. 2012, Medjeral-Thomas et al. 2014]. Crescentic GN may predict poor renal outcome, although this is inconsistent between studies, as is the case with numerous other pathologic variables [Barbour et al. 2013b]. Prognosis may be slightly better for C3GN than DDD [Medjeral-Thomas et al. 2014]. In CFHR5 nephropathy, progression to ESKD is more common in males than in females for reasons that are not understood [Athanasiou et al. 2011]. Following renal transplantation, histological recurrence of DDD and C3GN is common, although the impact of this on graft survival is controversial [Braun et al. 2005, Little et al. 2006, Zand et al. 2014].
In DDD, dense deposits containing C3 fragments accumulate within the lamina densa, leading to disruption of the GBM and consequent renal functional injury (e.g. proteinuria). In addition to causing dense transformation of the GBM, C3 activation leads to downstream proinflammatory pathways mediating progressive renal failure. For example, acute glomerular inflammation may be due to MAC activation, with a linear staining pattern for C5b-9 sometimes observed in patients with DDD [Cook H. T. & Pickering M.C. 2015]. Immune cells recruited to the glomerulus in response to anaphylatoxins mediate chronic inflammation with ongoing release of proinflammatory cytokines.

Limited evidence from human biopsy studies implicates iC3b as the specific C3 activation product present within the GBM deposits, with additional support from a model of C3 glomerulopathy in FH-deficient mice (discussed below in section 1.5.1). The presence within the glomerulus of immune cells expressing CD11b, the specific leukocyte receptor for iC3b, has not been directly assessed in DDD, although the findings of a biopsy study in patients with MPGN type 1 may be relevant [Soma et al. 1998]. This showed that CD11b+ cells were prominent in the glomerulus, especially in peripheral capillary loops (similar to the C3 staining pattern). The glomerular CD11b+ cell count also correlated with both the intensity of C3 staining and the degree of proteinuria. It was therefore suggested that the interaction of CD11b on myeloid cells with subendothelial deposits of C3 was important in disease progression. Although expression of CD11b+ on glomerular cells mainly correlated with that of CD15, a neutrophil/monocyte marker, glomerular cells expressing the monocyte/macrophage marker, CD68+, are also frequently noted in patients with MPGN [Soma et al. 1994, Yang et al. 1998, Wu et al. 2012].

Clinicopathological overlap between C3 glomerulopathy and post-infectious GN (PIGN) is exemplified by: (1) DDD case series in which renal symptoms are preceded by respiratory tract infection [Habib et al. 1975, Nasr et al. 2009, Wang et al. 2012]; (2) C3 abnormalities in many cases of PIGN, including a transient fall in plasma C3 and the presence of glomerular C3 without Ig [Cook H. T. & Pickering M. C. 2015]; (3) EM showing the presence in many DDD/C3GN cases of subepithelial, hump-like dense deposits classically associated with post-streptococcal GN [Walker et al. 2007, Nasr et al. 2009]; and (4) case reports showing persistent renal disease following a diagnosis of PIGN, in which biopsies and/or genetic association data were later consistent with C3 glomerulopathy [Sandhu et al. 2012, Vernon et al. 2012, Meleg-Smith 2012, Sethi et al. 2013]. One possible interpretation is that infection may initiate or exacerbate the disease process that leads to C3 glomerulopathy (Fig. 5), for example via exogenous (or cryptogenic endogenous) triggers of complement activation.
Figure 5 Pathophysiology of DDD and other forms of C3 glomerulopathy

Triggers of C3 activation (depicted at centre) may be exogenous (e.g. infection) or endogenous (i.e. C3 tickover). C3 activation generates C3b molecules for attachment to surfaces including the GBM. In health (upper panel), C3b amplification via the AP is tightly controlled by FH in the circulation and on surfaces. In theory, any surface-attached C3b is then metabolized, leaving C3 fragments (iC3b, C3d) attached to the GBM, and releasing FH back into the circulation. In the setting of abnormal FH (bottom left panel), uncontrolled C3 activation generates excessive C3 fragments in the circulation, which may accumulate along the GBM. Alternatively, enhanced FH de-regulation due to abnormal FHR proteins (bottom right panel) may be one mechanism by which C3 accumulates along the GBM despite intact FH. Reproduced from [Barbour et al. 2015] with permission from Oxford University Press (License No 3511371147517).
1.4.4 Associated complement abnormalities

Low plasma C3 levels are identified in ~80% of DDD cases, compared to only ~40% of C3GN cases [Nasr et al. 2009, Servais et al. 2012, Medjeral-Thomas et al. 2014]. AP functional studies typically show impaired AP function in patients with DDD, including both those with severe FH deficiency [Levy et al. 1986, Schejbel et al. 2011, Rusai et al. 2013] and those with detectable plasma FH levels [Sethi et al. 2010, Hawfield et al. 2013]. This has generally been attributed to chronic secondary depletion of C3 and/or other AP substrates [Seelen et al. 2005], as a result of acquired or inherited factors. One study reported that a diagnostic panel comprising assays for C3NeFs, anti-FB and anti-FH autoantibodies, and FH genetic sequence variants yields one or more positive findings in almost 90% of DDD cases [Zhang et al. 2012]. Taken together, these data suggest that uncontrolled C3 activation occurs in the circulation in DDD, whereas normal plasma C3 levels in many cases of C3GN may indicate that abnormal AP control is restricted to surfaces, specifically within the glomerulus. For example, in familial C3GN (including CFHR5 nephropathy) associated with genetically abnormal FHR proteins, enhanced ‘FH deregulation’ on the GBM surface has been proposed as one disease mechanism.

1.4.4.1 Autoantibodies

C3NeF, first described in a patient with ‘persistent hypocomplementemic glomerulonephritis’ [Spitzer et al. 1969], is an autoantibody that binds a neo-epitope on C3bBb, stabilizing it against spontaneous and FH-mediated decay and prolonging its C3-cleaving action [Daha et al. 1976]. A second type of C3NeF was found to display slower C3 and C5 activation [Mollnes et al. 1986] and properdin dependence [Clardy et al. 1989]. C3NeFs are more common in DDD than C3GN or MPGN type 1 [Schwertz et al. 2001, Servais et al. 2012], especially properdin-independent C3NeFs [Tanuma et al. 1990]. However, plasma levels of C3NeF often do not correlate with clinical parameters of disease [Appel et al. 2005, Zhang et al. 2012]. In addition, C3NeFs are occasionally seen in post-streptococcal GN [Fremeaux-Bacchi et al. 1994], lupus nephritis [Walport et al. 1994] and membranous nephropathy [Niel et al. 2015]. Hence a role of C3NeFs in specific causation of C3 glomerulopathy is unlikely.

Enhanced AP C3 convertase activity has also been demonstrated in association with autoantibodies that bind to native FB [Strobel et al. 2010, Zhang et al. 2012] or both FB and C3b [Chen et al. 2011] in patients with DDD. An early report concerned a patient with C3 glomerulopathy and circulating monoclonal \( \lambda \) light chain dimers targeted to SCR 3 of FH [Meri et al. 1992, Jokiranta et al. 1999]. The light chain dimers blocked N-terminal-dependent binding of FH to C3b, producing increased C3 activation via the AP in vitro. A
patient with a possible diagnosis of DDD was later reported with monoclonal IgG-λ anti-FH autoantibodies against N-terminal SCR domains impairing FI cofactor activity [Nozal et al. 2012]. High-titre anti-FH autoantibodies targeted against N-terminal SCR domains and associated with AP effects were reported in two further patients with DDD and MPGN [Goodship et al. 2012]. However, they were also detected in healthy individuals.

1.4.4.2 Family studies

Family studies have shown a genetic basis for a small number of cases of C3 glomerulopathy (Table 1). The first report in which the genetic variant was characterized concerned two brothers with autosomal recessive ‘atypical dense intramembranous deposit disease’ in association with very low plasma C3, C5, FB and FH levels [Levy et al. 1986]. Renal immunostaining showed C3 without Ig in the glomerulus (granular mesangial and linear pattern) but not the TBM or renal arteries, with discontinuous intramembranous and subendothelial dense deposits on EM. Genetic assessment in the elder brother revealed a homozygous missense FH variant mapping to SCR 7 [Dragon-Durey et al. 2004]. Both consanguineous parents and two other children had half-normal FH levels (without renal disease), consistent with heterozygosity for the FH variant causing FH deficiency (so-called type 1 variant). A case of endocapillary GN with predominant glomerular C3 was later described in an infant with a homozygous type 1 FH variant mapping to SCR 2 (the result of paternal isodisomy) [Schejbel et al. 2011]. A young girl with C3GN was also described with a homozygous type 1 FH variant mapping to SCR 16 [Rusai et al. 2013]. A different amino acid substitution at the identical FH protein domain, resulting in non-secretion of FH from cells both in vivo and in vitro, had earlier been reported in a family with autosomal recessive type 3-collagen glomerulopathy [Vogt et al. 1995, Ault et al. 1997, Schmidt et al. 1999].

Before these reports of familial C3 glomerulopathy, severe FH deficiency had been found to co-segregate with the alternative renal lesion of thrombotic microangiopathy (TMA) in one family [Thompson & Winterborn 1981]. Renal TMA is characterized by microvascular endothelial injury and associated thrombosis in renal arterioles and glomerular capillaries. Aetiology of renal TMA due to genetic or acquired abnormalities in complement regulation is commonly referred to as atypical haemolytic uraemic syndrome (aHUS) [Barbour et al. 2012]. Renal TMA lacks well-defined electron dense deposits and C3 accumulation, and hence is pathologically distinct from C3 glomerulopathy. A number of family studies have since shown renal TMA rather than C3 glomerulopathy in association with homozygous FH deficiency [Pickering & Cook 2008, Sethi et al. 2009, Habibi et al. 2010, Wilson et al. 2013, Michaux et al. 2014]. In one aHUS pedigree a family member developed C3 glomerulopathy following renal transplantation (later complicated by allograft TMA) [Boyer et al. 2008].
<table>
<thead>
<tr>
<th>Original report</th>
<th>Diagnosis</th>
<th>Affected family member (case N°)</th>
<th>Glomerular IF/IHC</th>
<th>EM deposits</th>
<th>C3 levels</th>
<th>C3NeF</th>
<th>FH levels</th>
<th>Abnormal protein</th>
<th>Rare genetic variant</th>
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</table>

Subend subendothelial; Subepi subepithelial; IM intramembranous; Mesang mesangial; Undetect undetectable. Adapted from [Barbour et al. 2013b] with permission of Elsevier
The reasons for this phenotypic variance are unclear. However, mechanistic insights into C3 glomerulopathy and aHUS have derived from functional characterization of the genetic defects in some cases together with the study of animal models (discussed below). Notably, the familial aHUS-associated type 1 FH variants have invariably mapped to the C-terminal SCR domains 15-20 responsible for FH surface recognition. C-terminal clustering of mainly heterozygous FH variants has been noted in familial and (more common) sporadic cases of aHUS [Warwicker et al. 1998, Rodriguez et al. 2014]. In most of these cases, plasma FH levels are normal (type 2 mutations). These clinical data implicate impaired AP control on endothelial surfaces in aHUS pathogenesis [Richards et al. 2001, Caprioli et al. 2001].

Both autosomal recessive and autosomal dominant C3 glomerulopathy are described in families with type 2 FH variants mapping to N-terminal SCR domains. Two sisters developed autosomal recessive C3GN in association with a homozygous FH variant in SCR 4 [Licht et al. 2006, Habbig et al. 2009]. Circulating FH displayed impaired C3b-binding, decay accelerating activity and FI cofactor activity in vitro. By contrast, binding to heparin, C3d and HUVEC was normal. A mother and son developed autosomal dominant MPGN with predominant glomerular C3 in association with a heterozygous FH variant in SCR 2 [Power et al. 1990, Wong et al. 2014]. A recombinant FH fragment (SCR domains 1-4) comprising this genetic variant showed reduced C3b-binding and complement regulatory activity in vitro compared to a non-variant SCR1-4 fragment. The reasons for a dominant negative effect of the abnormal FH protein on AP regulation remain unclear.

Autosomal dominant DDD was also reported in a mother and twin sons with a heterozygous C3 variant producing a gain of function in vitro [Martinez-Barricarte et al. 2010]. C3 encoded by the variant C3 allele was identified as the dominant circulating form of C3 in these patients. Although this C3 molecule could not be cleaved by the AP C3 convertase, it was activated by proteases and also formed C3(H2O). It then formed an AP C3 convertase that was resistant to FH-mediated decay accelerating and FI cofactor activity. On surfaces, DAF-mediated decay accelerating activity and MCP-mediated FI cofactor activity were intact. Thus uncontrolled activation of the C3 encoded by the normal C3 allele was restricted to the fluid phase. In two earlier family studies, C3 glomerulopathy was associated with hyperfunctional C3 molecules [Marder et al. 1983, Linshaw et al. 1987]. Autosomal dominant C3GN was also reported in association with a heterozygous FB variant producing a putative gain of function [Imamura et al. 2015].
Autosomal dominant C3GN associated with a heterozygous copy number variation in FHR-5 in two Greek Cypriot families led to the designation ‘CFHR5 nephropathy’ [Gale et al. 2010]. The subsequent identification of numerous affected individuals across Cyprus with an identical gene variant is consistent with a founder effect. The clinical phenotype is of microscopic and episodic macroscopic haematuria, and progression to ESKD, mostly in males (for unknown reasons). Autosomal dominant C3GN has since been reported in association with other FHR copy number variations. Strikingly, all these cases involve internal duplication of exons or formation of hybrid genes, resulting in abnormal circulating FHR proteins with duplicated N-terminal SCR domains 1 and 2. These SCR domains on FHR-1, -2 and -5 contain a dimerization motif that facilitates formation of FHR homodimers, heterodimers [Goicoechea de Jorge et al. 2013] and even larger complexes [Tortajada et al. 2013]. Compared to dimeric recombinant FHR proteins, monomeric recombinant FHR proteins lacking the dimerization motif show less avidity for ligands including C3b both in vivo [Goicoechea de Jorge et al. 2013] and in vitro [Tortajada et al. 2013, Goicoechea de Jorge et al. 2013]. As FHR-1, -2 and -5 lack the complement regulatory domains of FH, competition for surface C3b appears to influence the degree to which C3b amplification is inhibited (predominant FH binding) or allowed to proceed (predominant FHR binding) on surfaces. This effect of abnormal FHR proteins, referred to as FH deregulation, has been shown in vitro through enhanced lysis of guinea pig erythrocytes in the presence of FH.

Autosomal recessive MPGN/glomerular TMA with positive glomerular C3 staining was associated with a homozygous variant in the gene encoding diacylglycerol kinase epsilon (DGKε) in a large family (denoted as UT-062) [Ozaltin et al. 2013]. The mechanism by which DGKε, an intracellular lipid kinase expressed in podocytes, influences glomerular disease has not been determined. Additional studies have reported homozygous or compound heterozygous DGKE variants in patients with aHUS, some of whom also have complement-associated gene variants [Lemaire et al. 2013, Sanchez Chinchilla et al. 2014].

1.4.4.3 Other genetic associations

Additional rare or novel genetic variants in complement-related genes have been reported in individuals with C3 glomerulopathy (albeit without family data supporting disease causation). For example, one series identified rare FH, F1 and MCP variants in 18% of patients with DDD, C3GN or MPGN type 1 [Servais et al. 2012]. GWAS have also identified common allelic variants in FH and C3 associated with increased or reduced risk of DDD [Abrera-Abeleda et al. 2011]. In some cases, in vitro AP effects have been attributed to the at-risk variant, supporting the disease association [Heurich et al. 2011]. The term ‘complotype’ has been coined for the pattern of complement gene variants that appears to influence an
individual's risk of C3 glomerulopathy and/or other complement-related diseases [Harris et al. 2012]. For example, FH risk haplotypes comprising the single nucleotide polymorphism (SNP) Tyr402His on SCR 7 or Ile62Val on SCR 1 confer common susceptibility to DDD and age-related macular degeneration (AMD) [Hageman et al. 2005, Pickering et al. 2007]. SCR 7 contains one of the GAG-binding sites, with some functional data indicating that the 402H variant of FH and FHL-1 displays reduced binding to GAGs and Bruch's membrane (the macular ECM) [Clark et al. 2013, Clark et al. 2014].

1.4.5 Treatment

No treatment is proven as beneficial in DDD. A 1992 randomized controlled trial demonstrated benefit with alternate day prednisolone in 70 children with MPGN, of whom 14 had DDD [Tarshish et al. 1992]. Long-term plasma infusion was effective and well tolerated in the report of familial C3GN related to a circulating abnormal FH protein [Licht et al. 2006]. Elsewhere plasma infusion/exchange is reported with only mixed success in DDD [Appel et al. 2005]. Case reports attributing a disease-modifying effect in patients with C3 glomerulopathy to the anti-CD20 monoclonal antibody rituximab are fewer than those showing no effect at all. Eculizumab (Soliris®, Alexion Pharmaceuticals, USA) is a humanized monoclonal IgG1-κ anti-human C5 antibody approved for use in patients with paroxysmal nocturnal haemoglobinuria and atypical HUS. Off-label use of eculizumab was assessed in a prospective trial of 6 patients with native or post-transplant recurrent DDD or C3GN, 3 of whom showed a clinical response over a median 1-year treatment period [Bomback et al. 2012]. Glomerular accumulation of eculizumab was noted in post-treatment biopsy specimens [Herlitz et al. 2012]. Published case reports in over a dozen patients with C3 glomerulopathy show an 86% rate of clinical response to eculizumab, raising the possibility of publication bias (Table 2). On the basis of these limited data, patients in whom eculizumab appears most likely to be effective include those with biopsies showing active renal inflammation and/or glomerular C5b-9 deposition. Patients require vaccination against meningococcal infection prior to beginning eculizumab treatment, although this does not eliminate risk especially in the setting of concurrent immunosuppressive therapy (e.g. post-transplantation) [Struijk et al. 2013]. A Phase 1 clinical trial in children and adults with DDD (NCT01791686) is evaluating recombinant human sCR1 therapy following promising preclinical data in vitro [Chen et al. 2013] and in vivo [Couser et al. 1995, Zhang et al. 2013].
<table>
<thead>
<tr>
<th>Report</th>
<th>Study design</th>
<th>Reported Diagnosis (treatment age/sex)</th>
<th>Treatment duration (months)</th>
<th>Clinical response</th>
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<td>Case report</td>
<td>DDD (22/F)</td>
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<tr>
<td>[Vivarelli et al. 2012]</td>
<td>Case report</td>
<td>DDD (17/M)</td>
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<td>[McCaughan et al. 2012]</td>
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<td>DDD (22/M)</td>
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<td>DDD (42/M)</td>
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<td></td>
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<td>Yes 50%</td>
</tr>
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<td></td>
<td></td>
<td>C3GN (25/M)</td>
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</tr>
<tr>
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<td></td>
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<td>Allograft recurrent C3GN (20/M)</td>
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<td>[Gurkan et al. 2013]</td>
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<td>[Besbas et al. 2014]</td>
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<td>[Kerns et al. 2013]</td>
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<td>[Roussset-Rouviere et al. 2014]</td>
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<td>[Ozkaya et al. 2014]</td>
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<td>[Berthe-Aucejo et al. 2014]</td>
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<td>Allograft recurrent C3 glomerulopathy (63/F)</td>
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<tr>
<td></td>
<td></td>
<td>C3 glomerulopathy (45/M)</td>
<td>6</td>
<td>Yes</td>
</tr>
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</table>
1.5 Animal models

1.5.1 Experimental C3 glomerulopathy

Murine FH is structurally and functionally very similar to human FH [Kristensen & Tack 1986, Cheng et al. 2006]. Mice with homozygous FH deficiency (Cfh/-) were engineered through targeted deletion of exon 3 (encoding SCR 1) in embryonic stem cells [Pickering et al. 2002]. Cfh/- mice have proved to be an informative experimental model of C3 glomerulopathy. An earlier model was described in FH-deficient Norwegian Yorkshire piglets (now extinct) [Høgåsen et al. 1995]. Porcine dense deposit disease/MPGN type 2 was due to a homozygous mutation that affected SCR 20, preventing FH secretion [Hegasy et al. 2002].

Cfh/- mice on a 129/SvxC57BL/6 mixed genetic background develop albuminuria and MPGN with ~25% mortality at 8 months. Plasma levels of C3 and C5 are extremely low, and C3 is detected within the glomerulus in a florid linear pattern [Pickering et al. 2002, Goicoechea de Jorge et al. 2011]. By contrast, mesangial IgG is seen only after MPGN develops. Electron-dense deposits are seen within the GCW, initially in the subendothelial layer, and stain positively for C3 on immuno-EM [Pickering et al. 2002]. C3 accumulation along the GBM is the earliest pathological renal abnormality detected in Cfh/- mice, as also reported in FH-deficient piglets [Jansen et al. 1998]. Cfh/- mice crossed with FB-deficient mice (Cfb/-), which are unable to form the AP C3 convertase, do not show glomerular C3 accumulation or spontaneous renal disease. This indicates that pathogenesis is dependent on uncontrolled C3 activation via the AP [Pickering et al. 2002]. Transplantation of kidneys from mice with combined homozygous deficiency of FH and C3 (Cfh/-C3/-) into Cfh/- recipients results in the appearance of linear C3, which therefore derives from the circulating C3 pool rather local renal C3 synthesis [Rose et al. 2008]. Laser dissection microscopy followed by western blot identified C3 β-chain and α'-chain fragments in glomerular tissue from Cfh/- mice, consistent with the presence of iC3b (with or without C3d[g]) along the GBM. Accordingly, linear C3 was also detected using specific antibodies that recognise a C3d epitope on iC3b and C3d(g) [Paixão-Cavalcante et al. 2009, Ruseva et al. 2013].

Injection of purified murine FH [Paixão-Cavalcante et al. 2009] or human FH [Fakhouri et al. 2010] restores plasma C3 levels in Cfh/- mice to normal at 24h. This is accompanied by a significant reduction in linear C3 staining, and the appearance of mesangial and tubulointerstitial C3. Daily administration of human FH for 5 days results in negative linear and mesangial C3 staining and significantly reduces linear C3d staining. Following transplantation of kidneys from Cfh/- mice into WT recipients, linear C3 staining is absent at five weeks [Alexander et al. 2007]. In FH-deficient piglets, a single infusion of WT porcine plasma or purified porcine FH led to a sustained rise in plasma C3 levels [Høgåsen et al.
Weekly WT plasma infusions increased median survival and reduced histological severity of MPGN in comparison to untreated animals [Høgåsen et al. 1995]. The critical role of FH deficiency in pathogenesis is underscored by absence of glomerular C3 or progressive renal disease in mice deficient in CR1-related gene/protein-y (Crry) [Ruseva et al. 2009]. This is despite uncontrolled C3 activation via the AP due to deficiency of this rodent-specific, membrane-expressed regulator [Li et al. 1993].

FI-deficient mice (Cfi-/-) have very low plasma levels of C3 (in the form of C3b) [Rose et al. 2008]. This is similar to severe FI deficiency in humans, which has never been reported in association with C3 glomerulopathy (notwithstanding several reports of immune complex GN [Nilsson et al. 2011]). The renal phenotype in Cfi-/ mice consists of mesangial C3 accumulation and nodular mesangial expansion without MPGN [Rose et al. 2008]. Remarkably, mice with combined homozygous deficiency of FH and FI (Cfh-/-,Cfi-) were shown to have an identical plasma C3 profile and renal phenotype to Cfi-/ mice. Injections of serum from Cfh-/-,C3-/ mice (as a source of murine FI) into Cfh-/-,Cfi-/ mice resulted in cleavage of circulating plasma C3b and the appearance of linear C3 staining, similar to that observed in Cfh-/ mice [Rose et al. 2008]. These data showed that cleavage of C3b to iC3b by circulating FI was critically required for C3 accumulation along the GBM and consequent glomerular inflammation and injury in FH-deficient mice [Rose et al. 2008].

1.5.2 Experimental aHUS

Cfh-/- mice with transgenic expression of a murine FH protein comprising SCR domains 1-15 (Cfh-/-,FHΔ16-20) do not develop DDD [Pickering et al. 2007]. The FHΔ16-20 protein in plasma displayed impaired binding to heparin and HUVEC in vitro. In Cfh-/-,FHΔ16-20 mice, plasma C3 levels are significantly higher than in Cfh-/ mice, indicating that FHΔ16-20 is able to regulate C3 activation via the AP in vivo. As the transgenic mice generally show only mild mesangial and endothelial C3 accumulation, it appears that the C-terminal SCR domains responsible for FH surface recognition are not required to prevent C3 accumulation along the GBM. Also unlike Cfh-/ mice, Cfh-/-,FHΔ16-20 mouse frequently develop spontaneous renal TMA and mesangiolysis, supporting the role of impaired AP surface regulation (on the endothelium) in aHUS pathogenesis. Accompanying deficiency of C5 in Cfh-/-,FHΔ16-20 mice abrogates renal TMA without altering plasma C3 levels or the glomerular C3 staining pattern [Goicoechea de Jorge et al. 2011]. In Cfh-/ mice (on a DBA/2 background), accompanying C5 deficiency ameliorated spontaneous MPGN at 1 year and protected against experimentally induced acute glomerular inflammation using the heterologous NTN model [Pickering et al. 2006]. These studies led to the clinical evaluation of eculizumab in patients with aHUS or C3 glomerulopathy.
1.6 Studies in CD11b-deficient mice

CD11b has an identical cellular distribution in mice and humans [Ault & Springer 1981]. At baseline, CD18-deficient mice (lacking all four β2-integrins) have increased circulating leukocyte and neutrophil counts and increased susceptibility to bacterial infections, similar to human LAD-1 [Wilson et al. 1993, Scharffetter-Kochanek et al. 1998, Etzioni et al. 1999]. By contrast, mice with homozygous CD11b deficiency (CD11b-/-) display WT levels of circulating leukocytes and neutrophils, with no increase in rates of spontaneous infection [Coxon et al. 1996, Ding et al. 1999] (except for one study [Lu et al. 1997]). Consistent with human data, neutrophils from CD11b-/- mice show adhesion and complement-dependent phagocytosis in vitro [Rothlein & Springer 1985, Coxon et al. 1996, Lu et al. 1997]. However in vivo studies in CD11b-/- mice have produced conflicting results in a variety of inducible disease models including peritonitis, GN and sepsis (Table 3). Reasons for this include: (1) the differing anatomic sites and inflammatory stimuli examined; (2) the opposing roles of CD11b-expressing immune effector cell types under the different experimental conditions; (3) the variable cellular processes mediated via CD11b ligation, culminating in both protective and deleterious functions; and (4) redundancy of Mac-1/CR3 and LFA-1 in mediating leukocyte adhesion. In one adroit summary, ‘complex biological circuits predict dual and sometimes opposing roles for the same receptor that is cell type and context dependent’ [Rosetti et al. 2012].

The first renal studies in CD11b-/- mice showed protection in the heterologous NTN model (discussed in Chapter 4). However, recent studies have instead shown increased severity of experimentally induced immune complex GN in CD11b-/- mice. These include a lupus passive transfer model using mice with selective neutrophil transgenic expression of human FcγRIIA (Fcγ+/-,hFcγRIIA or Fcγ-/-,hFcγRIIA) or FcγRIIA and FcγRIIIB (Fcγ-/-,hFcγRIIA/IIIB). Mice were sensitized with subcutaneous (s.c.) human IgG/CFA injection followed by immunization via two i.v. injections of serum from patients with SLE. This led to increased day 10 glomerular neutrophil influx, day 14 albuminuria and day 21 mesangial expansion, hypercellularity and crescents (~5% of glomeruli affected) in transgenic mice with accompanying CD11b deficiency [Rosetti et al. 2012]. Glomerular macrophage influx was minor, and monocyte/macrophage depletion using clodronate prior to lupus serum transfer had no effect on susceptibility to renal injury. A pristane-induced model of immune-complex-mediated multiorgan injury was associated with increased proteinuria and glomerular hypercellularity in CD11b-/- mice [Shi et al. 2013]. Induction of complement-mediated tubulointerstitial nephritis by transplantation of kidneys from Crry-/-,C3-/- donors into C3-sufficient mice was also associated with more severe disease in recipients with accompanying CD11b deficiency [Chaves et al. 2014].
<table>
<thead>
<tr>
<th>Disease/study</th>
<th>Induction method</th>
<th>Cellular response: CD11b/− versus WT</th>
<th>Disease outcome: CD11b/− versus WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peritonitis/colitis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Coxon et al. 1996]</td>
<td>i.p. thioglycollate</td>
<td>↑ nφ, Mφ (peritoneum)</td>
<td></td>
</tr>
<tr>
<td>[Lu et al. 1997]</td>
<td>i.p. thioglycollate</td>
<td>⇔ nφ (peritoneum)</td>
<td></td>
</tr>
<tr>
<td>[Cao et al. 2005]</td>
<td>i.p. thioglycollate</td>
<td>↑ Mφ (peritoneum)</td>
<td></td>
</tr>
<tr>
<td>[Abdelbaqi et al. 2006]</td>
<td>oral dextran sodium sulfate (DSS)</td>
<td>↓ nφ, Mφ, ↑ plasma cells (colon)</td>
<td>n/s ↑ colitis</td>
</tr>
<tr>
<td><strong>Nephritis</strong></td>
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<td></td>
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<tr>
<td>[Tang et al. 1997]</td>
<td>i.v. rabbit NTS</td>
<td>↓ early (2-4h) nφ (glomerulus)</td>
<td>↓ 4-8h albuminuria</td>
</tr>
<tr>
<td>[Hirahashi et al. 2009]</td>
<td>i.v. rabbit NTS×2 then LPS</td>
<td>↓ 4-24h nφ (glomerulus); ↓ NE</td>
<td>↓ glomerular thrombosis (day 4)</td>
</tr>
<tr>
<td>[Rosetti et al. 2012]</td>
<td>s.c. human IgG/CFA then i.v. human lupus serum ×2 (Fcγ+/−.hFcγRIIA, Fcγ−/−.hFcγRIIA or Fcγ−/−.hFcγRIIA/IIIB mice)</td>
<td>↑ nφ (glomerulus, synovium)</td>
<td>↑ proteinuria, nephritis, arthritis</td>
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<tr>
<td>[Ding et al. 2013]</td>
<td>i.v. apoptotic thymocytes (anti-snRNP Ig transgenic mice)</td>
<td>↑ anti-snRNP Ig, ANA IgG (plasma); ↑ IgG (glomerulus)</td>
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<tr>
<td>[Shi et al. 2014]</td>
<td>i.p. pristane</td>
<td>early (16h) ↑ nφ, later ↓ nφ, M1 Mφ (peritoneum, lungs); ↑ eosinophils, M2 Mφ (peritoneum)</td>
<td>↓ DPH, mortality</td>
</tr>
<tr>
<td>[Shi et al. 2013]</td>
<td></td>
<td>↑ nφ (glomerulus)</td>
<td>↑ proteinuria, glomerular hypercellularity</td>
</tr>
<tr>
<td>Disease/study</td>
<td>Induction method</td>
<td>Cellular response: CD11b−/− versus WT</td>
<td>Disease outcome: CD11b−/−</td>
</tr>
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<td>-------------------------------</td>
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<tr>
<td><strong>Sepsis</strong></td>
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<tr>
<td>[Rosenkranz et al. 1998]</td>
<td>caecal ligation/puncture</td>
<td>↓ nφ; ↓ mast cells at baseline (peritoneum)</td>
<td>↑ mortality</td>
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<tr>
<td>[Rijneveld et al. 2005]</td>
<td>intranasal <em>Streptococcus pneumoniae</em></td>
<td>↑ nφ (pulmonary airspace)</td>
<td>↑ pneumonia, bacteraemia</td>
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<tr>
<td>[Morrison et al. 2008]</td>
<td>footpad inoculation with Ross River virus</td>
<td>transient ↑ Mφ, NK cells, T cells (synovium, muscle)</td>
<td>↓ arthritis/myositis</td>
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<tr>
<td>[Zhang et al. 2009]</td>
<td>i.p. poly I:C</td>
<td>↑ inflammation (liver)</td>
<td>↑ hepatocellular injury, mortality</td>
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<tr>
<td>[Han et al. 2010]</td>
<td>i.p LPS, poly I:C, CpG oligodeoxynucleotides</td>
<td>↑ inflammation (lung); ↑ proinflammatory cytokines (serum)</td>
<td>↑ mortality (versus CD11b+/−)</td>
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<td></td>
<td>i.p. <em>Escherichia coli</em></td>
<td>↑ proinflammatory cytokines (serum)</td>
<td>↑ mortality</td>
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<tr>
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<td>i.v. <em>Listeria monocytogenes</em></td>
<td>↑ proinflammatory cytokines (serum)</td>
<td>↓ bacteraemia</td>
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<td>[Zhou et al. 2013]</td>
<td>i.p. poly I:C</td>
<td>↓ proinflammatory cytokines (serum, liver)</td>
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<td>[Liu et al. 2014]</td>
<td>caecal ligation/puncture</td>
<td>↔ nφ (lung)</td>
<td>↑ mortality</td>
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<td><strong>Brain ischaemia-reperfusion injury</strong></td>
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<td>[Soriano et al. 1999]</td>
<td>Transient MCA occlusion</td>
<td>n/s ↓ nφ (brain)</td>
<td>↓ cerebral infarct volume</td>
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<td>[Arumugam et al. 2004]</td>
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<td>↓ late (24h) nφ (brain)</td>
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<td>[Kanwar et al. 2001]</td>
<td>i.p. ova with alum, then intranasal ova</td>
<td>↑ eosinophils (pulmonary airways)</td>
<td>↑ bronchial hyperreactivity</td>
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<td>Disease/study</td>
<td>Induction method</td>
<td>Cellular response: CD11b−/− versus WT</td>
<td>Disease outcome: CD11b−/− versus WT</td>
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<td><strong>Arthritis</strong></td>
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<td>[Ji et al. 2002]</td>
<td>i.p. serum transfer from K/B×N mice</td>
<td>↔ leukocytes (synovium)</td>
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<td>[Watts et al. 2005]</td>
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<td>n/s ↑ leukocytes (synovium)</td>
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<td>[Bullard et al. 2005]</td>
<td>s.c. MOG + CFA</td>
<td>↓ leukocytes in late disease (spinal cord)</td>
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<td>[Hirahashi et al. 2006]</td>
<td>intradermal LPS + TNF</td>
<td>n/s ↓ nφ (skin); ↓ NE</td>
<td>↓ thrombosis and (absent) haemorrhage</td>
</tr>
<tr>
<td><strong>Bullous pemphigoid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Liu et al. 2006]</td>
<td>intradermal rabbit IgG anti-mouse BP180</td>
<td>early (4h) ↑ nφ, later (12-24h) ↓ nφ (skin); late ↓ NE</td>
<td>↓ (absent) skin blisters</td>
</tr>
<tr>
<td><strong>Immune tolerance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Ehirchiou et al. 2007]</td>
<td>oral low-dose ova, then i.v. ova/CFA</td>
<td>↑ T&lt;sub&gt;H&lt;/sub&gt;-17 cells (draining lymph nodes)</td>
<td>↑ delayed-type hypersensitivity</td>
</tr>
<tr>
<td>[Ling et al. 2014]</td>
<td>intranasal HY peptide + LPS in female mice then syngeneic male skin graft</td>
<td>↓ primed T cells (spleen)</td>
<td>↓ skin graft rejection</td>
</tr>
<tr>
<td><strong>Allogeneic transplantation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Shimizu et al. 2008]</td>
<td>allo-mismatched cardiac transplantation</td>
<td>↓ nφ, Mφ, CD8+ T cells (heart)</td>
<td>↓ allograft rejection</td>
</tr>
<tr>
<td><strong>Tumour angiogenesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Soloviev et al. 2014]</td>
<td>s.c. cancer cells or Matrigel + growth factor</td>
<td>↓ nφ, Mφ (tumours, Matrigel implant)</td>
<td>↓ tumour angiogenesis</td>
</tr>
</tbody>
</table>

LPS lipopolysaccharide; MCA middle cerebral artery; MOG myelin oligodendrocyte glycoprotein; Mφ macrophages; NE neutrophil elastase; NK natural killer; nφ neutrophils; poly I:C polyinosinic:polycytidylic acid; n/s non-significant; TNF tumour necrosis factor
1.7 Aims of my project

The overarching aim of my doctoral thesis is to examine the role of iC3b in DDD. My specific aims are:

1.7.1 To define the contribution of CD11b to spontaneous renal disease associated with FH deficiency.

I approached this experimentally by examining the spontaneous renal phenotype in Cfh−/− mice with accompanying deficiency of CD11b. I present these experiments in Chapter 3.

1.7.2 To define the contribution of CD11b to experimentally induced renal injury.

I assessed the response of Cfh−/− mice with accompanying CD11b deficiency to accelerated NTN (ANTN). I further assessed the role of CD11b in ANTN independent of FH deficiency. These experiments are discussed in Chapter 4.

1.7.3 To assess the functional domains of FH necessary for prevention or treatment of DDD.

I examined the therapeutic effect of a novel recombinant human ‘mini-FH’ protein comprising SCR domains 1-5 and 18-20 in Cfh−/− mice. These experiments, undertaken as part of a collaborative project, are detailed in Chapter 5.

1.7.4 To assess C3 activation on the GBM in the setting of FH deficiency.

I utilized in vitro assays to assess the ability of GBM components to activate C3 in the presence or absence of FH. Comparison was with AP-activating surfaces including zymosan and rabbit erythrocytes. These experiments are presented in Chapter 6.
2. CHAPTER TWO: MATERIALS AND METHODS
2.1 Antibodies

Antibodies are summarized in Table 4.

2.2 Experimental mice

C57BL/6 mice were purchased from Harlan Ltd (Bicester, UK). Cfh-/- mice were generated as previously described [Pickering et al. 2002]. Cfh-/-, C3-/- [Wessels et al. 1995] and CD11b-/- mice [Coxon et al. 1996] were back-crossed for 10 generations onto a C57BL/6 background. C3-/-,Cfh-/- and Cfh-/-,Cfi-/- mice were generated by intercrossing the respective strains as previously described [Rose et al. 2008]. Cfh-/-,CD11b-/- mice, developed by intercrossing the Cfh-/- and CD11b-/- strains, were born at the expected Mendelian ratios and were viable and fertile under specific pathogen-free conditions. Animals were housed in the animal facility at Imperial College London and all experiments were conducted in accordance with institutional guidelines under my supervisor’s UK Home Office project licences (PPL 70/6877 and 70/7850) and my personal licence (PIL 70/23892).

Experiments were performed in age- and weight-matched mice (unless specified otherwise in the Results). Prior to each experiment, all mice tested negative for dipstick haematuria (0) and proteinuria (≤ 1+) using Hema-Combistix® urine reagent strips (Siemens, Germany, Product N° 2877). In some experiments, blood was collected at serial time-points via tail venesecion onto ethylene diamine tetraacetic acid (EDTA) dipotassium salt Microvette® tubes (Sarstedt, Germany, Product N° 16.444), with prompt chilling on ice. At the conclusion of each experiment, terminal anaesthesia was induced by intraperitoneal (i.p.) injection of 300µL of 25% v/v Hypnorm® fentanyl citrate/fluanisone (VetaPharma, UK) and 25% v/v Hypnovel® midazolam (Roche, Switzerland) in double-distilled water (ddH₂O). Blood was collected via cardiac puncture onto EDTA-coated Microvette® tubes (Sarstedt, Product N° 16.444), with prompt chilling on ice. Plasma separation by centrifugation of whole blood for 10 minutes at 16,060×g was followed by pooling and storage in 0.6mL microtubes at -80°C. All mice were killed by Schedule 1. Kidneys were collected onto phosphate buffered saline (PBS) for IF microscopy or fixative for LM (either Bouin’s solution [Sigma-Aldrich, USA, Product N° HT101128] or formalin for mice found dead).
Table 4 Antibodies

<table>
<thead>
<tr>
<th>Primary conjugated antibodies</th>
<th>Conjugate</th>
<th>Assay</th>
<th>Concentration</th>
<th>Manufacturer</th>
<th>Product N°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat polyclonal IgG anti-human C3</td>
<td>HRP</td>
<td>ELISA/western blot</td>
<td>1 in 10,000/1 in 3,000</td>
<td>Cappel™, USA</td>
<td>0855237</td>
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<td>HRP</td>
<td>ELISA</td>
<td>1 in 25,000</td>
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<td>0855557</td>
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<td>Sheep polyclonal IgG anti-mouse IgG</td>
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<td>ELISA</td>
<td>1 in 1000</td>
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<td>515-035-062</td>
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<td>ELISA</td>
<td>1 in 3000</td>
<td>Sigma-Aldrich, USA</td>
<td>A3438</td>
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<td>Mouse monoclonal IgG₁ anti-goat/sheep IgG</td>
<td>FITC</td>
<td>Immunostaining</td>
<td>1 in 100</td>
<td>Sigma-Aldrich, USA</td>
<td>F4891</td>
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<td>Goat polyclonal IgG anti-mouse C3</td>
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<td>1 in 200</td>
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<td>Rabbit polyclonal anti-human fibrin(ogen)</td>
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<td>Immunostaining</td>
<td>1 in 800</td>
<td>Dako, Denmark</td>
<td>F0111</td>
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<td>Immunostaining/FACS</td>
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<td>0855167</td>
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<td>Immunostaining/FACS</td>
<td>1 in 200/1 in 50</td>
<td>Sigma-Aldrich, USA</td>
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<td>Mouse monoclonal IgG₁ anti-human FH (OX24)</td>
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<td>Immunostaining</td>
<td>1 in 25</td>
<td>See Methods and Materials</td>
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<td>Goat polyclonal IgG anti-mouse C3d</td>
<td>Biotin</td>
<td>Immunostaining</td>
<td>1 in 10</td>
<td>R&amp;D Systems, USA</td>
<td>BAF2655</td>
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<td>Goat polyclonal anti-PNA</td>
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<td>Immunostaining</td>
<td>1 in 400</td>
<td>Vector Labs, USA</td>
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<td>Primary unconjugated antibodies</td>
<td>Conjugate</td>
<td>Assay</td>
<td>Concentration</td>
<td>Manufacturer</td>
<td>Product N°</td>
</tr>
<tr>
<td>------------------------------------------------------</td>
<td>-------------------</td>
<td>----------------</td>
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<td>--------------</td>
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<tr>
<td>Rabbit polyclonal IgG anti-mouse albumin</td>
<td>Radial</td>
<td>immunodiffusion</td>
<td>1 in 150</td>
<td>AbD Serotec, UK</td>
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<tr>
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<td></td>
<td>1 in 1,000</td>
<td>Sigma-Aldrich, USA</td>
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<td>Sigma-Aldrich, USA</td>
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<td>Rabbit polyclonal IgG anti-collagen type IV</td>
<td>ELISA</td>
<td></td>
<td>1 in 4,000</td>
<td>Abcam, UK</td>
<td>ab6586</td>
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<tr>
<td>Goat polyclonal IgG anti-mouse C3</td>
<td>ELISA (capture)</td>
<td></td>
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<td>Goat anti-human C5 antisera</td>
<td>Western blot</td>
<td></td>
<td>1 in 2,500</td>
<td>Quidel, USA</td>
<td>A306</td>
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<td>Goat anti-human FH antisera</td>
<td>Western blot</td>
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<td>1 in 20,000</td>
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<td>A312</td>
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<td>Rabbit polyclonal anti-human FHR-5</td>
<td>Western blot</td>
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<td>1 in 1,000</td>
<td>Abnova, Taiwan</td>
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<tr>
<td>Goat anti-mouse C3 antisera</td>
<td>Western blot</td>
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<td>1 in 4,000</td>
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<tr>
<td>Goat anti-human FI antisera</td>
<td>Western blot</td>
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<td>1 in 20,000</td>
<td>Quidel, USA</td>
<td>A313</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary conjugated antibodies</th>
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<td>ELISA</td>
<td>1 in 30,000</td>
<td>Sigma-Aldrich, USA</td>
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<td>Monoclonal mouse IgG1 anti-goat/sheep IgG</td>
<td>HRP</td>
<td>Western blot</td>
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<td>Sigma-Aldrich, USA</td>
<td>A9452</td>
</tr>
<tr>
<td>Polyclonal swine anti-rabbit Ig</td>
<td>HRP</td>
<td>Western blot</td>
<td>1 in 2,000</td>
<td>Dako, Denmark</td>
<td>P021702-2</td>
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<tr>
<td>Streptavidin</td>
<td>PE</td>
<td>Immunostaining</td>
<td>1 in 400</td>
<td>BD Pharmingen™, USA</td>
<td>S54061</td>
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<tr>
<td>Streptavidin</td>
<td>Alexa Fluor 555</td>
<td>Immunostaining</td>
<td>1 in 150-200</td>
<td>Invitrogen, USA</td>
<td>S-21381</td>
</tr>
<tr>
<td>Avidin</td>
<td>Alexa Fluor 488</td>
<td>Immunostaining</td>
<td>1 in 200-400</td>
<td>Invitrogen, USA</td>
<td>A-21370</td>
</tr>
</tbody>
</table>
2.3 Urine analysis

Dipstick haematuria and proteinuria were assessed throughout the experiments using Hema-Combistix®. At the end of some experiments, mice were placed into individual metabolic cages for 16h collection of urine. Albuminuria was then assessed by radial immunodiffusion [Ruseva et al. 2013]. 1.2% agarose deoxyribonucleic acid (DNA) pure grade Electran® (VWR, USA, Product N° 443666A) was dissolved in PBS using a microwave oven, followed by temperature equilibration at 55°C in a water bath. 150µL rabbit polyclonal IgG anti-mouse albumin antibody (AbD Serotec, UK, Product N° 0220-1829) was added per 10mL of the agarose solution, and gels poured onto 3 inch glass plates and allowed to set for 30 minutes at RT. 6µL equidistant wells were punched in the gels using a hollow metal tube, with the glass plates as the base. 5µL mouse urine samples were added to the wells, with 5µL purified mouse albumin (Sigma-Aldrich, USA, Product N° A3139) added separately as a positive control in doubling dilutions starting at 2mg/mL in PBS. The gels/glass plates were placed in a wet box overnight at 4°C. After washing three times for 30 minutes in PBS using a large reservoir, with separation of the gels from the glass plates, each gel was placed onto the hydrophobic side of an equivalent-sized piece of GelBond® film (Cambrex, USA, Product N° 53750) for transfer. Wet Whatman® 3mm cellulose chromatography paper (Sigma-Aldrich, USA, Product N° 3030917) was placed on top, followed by a stack of paper towels, a flat tile, and finally a weight. The transfer apparatus was left for 1h at RT, after which the gelbond was removed and dried with a hairdryer until transparent. It was then immersed in 0.2% w/v Coomassie Brilliant Blue R250 (Sigma-Aldrich, UK, Product N° 27815) in buffer (45% v/v methanol, 10% v/v acetic acid in ddH2O) for 30 minutes, followed by intermediate destaining buffer for 30 minutes and pure destaining buffer (20% methanol, 10% acetic acid in ddH2O) for 30 minutes. After drying overnight at RT, the gelbond was examined under a measuring eyepiece. The square of the diameter of precipitin circles for the albumin standard was plotted against concentration on a logarithmic scale, and a sigmoid curve obtained. The albumin concentration of mouse urine samples was calculated from this standard curve using linear regression.

2.4 Assessment of plasma for renal and haematological function

Plasma urea was measured using an enzymatic ultraviolet method as part of a kit (Boehringer Mannheim/R-Biopharm, Germany, Product N° 10542946035). This assay measures urea concentration based on the oxidization of nicotinamide adenine dinucleotide (NADH) that occurs (in the presence of glutamate dehydrogenase) due to ammonia, which is generated by hydrolyzation of urea only in the presence of urease. Mouse plasma samples were diluted 1 in 10 in PBS. 1 tablet of NADH was dissolved per 1mL of triethanolamine
buffer/2-oxoglutarate, with 100µL/well of this solution added to 96-well flat-bottom Nunc Maxisorp<sup>®</sup> plates (Thermo Scientific, USA, Product N° 442404) together with 200µL/well ddH<sub>2</sub>O and 10µL/well duplicates of the diluted plasma samples. Purified urea 140mg/L (5mmol/L) and PBS were used as positive and negative controls, respectively. 2µL of urease was then added to one of the duplicate wells for each sample. After mixing, wells were left at room temperature (RT) for 5 minutes before absorbance was read at 340nm. 2µL/well of glutamate dehydrogenase was then added to all wells. After mixing, wells were left for 20 minutes at RT before absorbance was read at 340nm. For all mouse plasma specimens, the difference between the optical density (OD) before and after the NADH oxidation reaction was between 0.1 and 1.0, with plasma urea concentration calculated as specified by the kit manufacturer.

Haematocrit was calculated using EDTA-treated blood in ammonium-heparin capillary tubes (Hirschmann<sup>®</sup>-Laborgeräte, Germany, Product N° 9100418) following centrifugation at 13,000rpm in a Mikro 20 centrifuge (Hettich, Germany) for 5 minutes. The packed cell height is expressed as a percentage of total height. Peripheral blood smears were prepared on SuperFrost<sup>®</sup> glass slides (VWR, USA, Product N° 631-0117) and stained using the Diff Quik staining kit (Polysciences, USA, Product N° 24606-500). Red cell fragments were quantified as the number per 200 red blood cells counted using a BX40 microscope (Olympus, Japan).

2.5 Measurement of mouse plasma C3 by sandwich ELISA

Nunc-Immuno™ MicroWell™ Maxisorp™ 96-well, flat-bottom, polystyrene enzyme-linked immunosorbert assay (ELISA) plates (Sigma Aldrich, USA, Product N° 442404) were coated with 50µL/well of 1 in 8,000 goat polyclonal IgG anti-mouse C3 antibody (Cappel<sup>TM</sup>, USA, Product N° 0855463; 4mg/mL) in coating buffer (0.1 M NaHCO<sub>3</sub> pH 9.5) and left overnight at 4°C. After washing with 200µL/well of 0.1% v/v TWEEN<sup>®</sup> 20 Detergent (Calbiochem, Germany, Product N° 655204) in PBS (PBST), plates were incubated with 100µL/well of 2% w/v bovine serum albumin (BSA, Sigma-Aldrich, USA, Product N° A7030) in 0.1% PBST for 1h at RT. After washing twice, plates were incubated with 50µL/well of each mouse plasma specimen diluted 1 in 12,000 in 2% BSA/0.1% PBST for 1h at RT. For the standard curve [Rose et al. 2008], murine serum amyloid P component (Calbiochem, Germany, Product N° 565193), which contains mouse C3 protein at a concentration of 263mg/mL, was used in doubling dilutions commencing at 1 in 500. Control sera from an unmanipulated Cfh<sup>−/−</sup> mouse and a WT mouse were also tested for each assay. After washing five times, plates were incubated with 50µL/well of 1 in 25,000 horseradish peroxidase (HRP)-conjugated goat polyclonal IgG anti-mouse C3 antibody (Cappel<sup>TM</sup>, USA, Product N° 0855557) in 0.1% PBST for 1h at RT. After washing five times, the assay was developed using 50µL/well of
tetramethylbenzidine (TMB) substrate reagents and 20µL/well of 2N sulfuric acid, and the absorbance read at 450nm. The OD for the standard was plotted against known C3 concentration on a logarithmic scale, and a sigmoid curve obtained. C3 concentration of mouse plasma samples was calibrated against the standard curve using linear regression.

2.6 Detection of mouse proteins in plasma by western blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-PROTEAN® Tetra Cell (Bio-Rad, USA, Product N° 165-8003). For separation of mouse C3 under reducing questions, a 1.0mm-thick 10% polyacrylamide resolving gel was poured containing 33% v/v ProtoGel® (30% polyacrylamide, National Diagnostics, USA, Product N° EC890), 26% v/v ProtoGel® resolving buffer (National Diagnostics, USA, Product N° EC892) 0.15% w/v ammonium persulfate (APS) and 0.15% v/v N,N,N′,N′-tetramethylethylenediamine (TEMED) in ddH₂O. For separation of mouse C5 under non-reducing conditions, a 7.5% polyacrylamide resolving gel was poured using 25% v/v ProtoGel® 30% solution and the other reagents. After the resolving gel had set, a 4% polyacrylamide stacking gel containing 13% v/v ProtoGel® 30% solution, 25% ProtoGel® stacking buffer (National Diagnostics, USA, Product N° EC893), 1% w/v APS and 0.3% v/v TEMED in ddH₂O was then poured on top and a 10-tooth comb inserted. Once set, the gel was placed into electrophoresis buffer (25mM Tris hydroxymethylaminomethane, 192mM glycine, 0.1% SDS [Sigma Aldrich, USA, Product N° 05030]) and the comb removed.

Stored mouse plasma samples were thawed and diluted in NuPAGE® lithium dodecyl sulfate (LDS) buffer 4× (Life Technologies, USA, Product N° NP007) with (reducing) or without (non-reducing) 5% v/v 2-mercaptoethanol (MCE, Sigma-Aldrich, USA, Product N° 63689). Specimens containing 1µL of plasma were loaded into wells in the stacking gel. As a control for C5 western blot, 0.1µg purified human C5 protein (hC5, Quidel, USA, Product N° A403) was used. ColorPlus™ pre-stained protein ladder with a range of 10-230kDa (New England BioLabs, USA, Product N° P7711S) was loaded into a separate well for each gel.

Proteins were separated by SDS-PAGE at 170mV constant voltage supplied by a PowerPac™ Basic Power Supply (Bio-Rad, USA, Product N° 164-5050). The gel was then separated from the glass plates in a reservoir containing transfer buffer (25mM Tris hydroxymethylaminomethane, 192mM glycine, 20% v/v methanol in ddH₂O). A transfer cassette was then assembled in transfer buffer using the Mini Trans-Blot Module (Bio-Rad, USA, Product N° 170-3935). On the cathode side, pieces of Whatman® 3mm cellulose chromatography paper (Sigma-Aldrich, 3030917) were loaded first, followed by the gel, followed by a similar-sized 0.45µm pore-size polyvinylidene fluoride (PVDF) Immobilon-P
membrane (Merck-Millipore, Germany, Product N° IPVH00010) that had been activated for 15 seconds in methanol, followed by pieces of chromatography paper on the anode side. Electrophoretic protein transfer was performed in transfer buffer at 100mV for 1h.

Following transfer, nonspecific binding was blocked using 20mL of 5% w/v non-fat dry milk (NFDM, Marvel, UK) in PBS for 1h at RT. The membrane was then incubated overnight at 4°C in detecting antibodies diluted in 5% NFDM/PBS as follows: 1 in 4,000 goat anti-mouse C3 antisera (Cappel™, USA, Product N° 0855444), 1 in 20,000 goat anti-human F1 antisera (Quidel, USA, Product N° A313) or 1 in 2,500 goat anti-human C5 antisera (Quidel, USA, Product N° A306). After washing three times in 0.1% PBST and reblocking with 5% NFDM/PBS for 1h at RT, secondary detection was with 1 in 2,000 (for mouse C3), 1 in 5,000 (for mouse C5) or 1 in 20,000 (for mouse F1) HRP-conjugated mouse monoclonal IgG, anti-sheep/goat IgG (Sigma-Aldrich A9452) in 5% NFDM/PBS for 1h (for mouse C3 and F1) or 30 minutes (for mouse C5) at RT. After washing three times, the membrane was covered in Pierce enhanced chemiluminescence (ECL) western blot substrate A+B (Thermo Scientific, Belgium, Product N° 32106) followed by dark room exposure using Amersham Hyperfilm ECL films (GE Healthcare, UK, Product N° 28-9068-37).

2.7 Plasma IgG quantification by ELISA

For measurement of murine IgG against sheep IgG, 96-well flat-bottom Nunc-Immuno™ MicroWell™ plates (Thermo Scientific, USA, Product N° 167008) were coated with 100µL/well of 3.5µg/mL sheep serum IgG (Sigma-Aldrich I5131) in coating buffer (0.1M NaHCO₃ pH 9.6) overnight at 4°C. After washing three times with 0.05% PBST, nonspecific binding was blocked using 100µL/well 1% BSA/PBS for 1h at 37°C. After washing three times, 100µL/well mouse plasma samples were added in doubling dilutions beginning at 1 in 100 in 1% BSA/PBS, with incubation at 37°C for 1h. As a positive control, pooled sera for mice immunized with sheep IgG in CFA was used. After washing three times, captured mouse IgG was detected using 100µL/well of 1 in 1000 HRP-conjugated sheep polyclonal IgG anti-mouse IgG antibody (Jackson ImmunoResearch, USA, Product N° 515-035-062, 0.8mg/mL) in 0.05% PBST for 1h at RT. After washing three times, the assay was developed using 50µL/well TMB substrate reagents (BD Biosciences 555214) and 25µL/well of 2N sulfuric acid (BD Biosciences DY994) at RT and absorbance was read at 450nm and 540nm. The OD at 540nm was subtracted from that at 450nm for each well, with results expressed as the minimal IgG titre (i.e. maximal mouse plasma dilution) at which the OD₄50nm-540nm was at least double that for blocking buffer alone.
2.8 Light microscopy

Mouse kidneys collected onto Bouin’s solution were removed after 3h and transferred to 70% v/v ethanol in ddH₂O for storage. All kidneys in fixative were processed for LM by Ms Lorraine Lawrence. Briefly, the kidneys were embedded in paraffin wax, and 3µm sections were mounted on glass slides and dried overnight at 37°C. Sections were then stained with periodic acid-Schiff (PAS) and covered with glass coverslips. Sections were visualized using an Olympus BX40 microscope and photographs taken using a Retiga 2000R digital camera (QImaging, Canada) and Image-Pro Plus® 7.0 software (Media Cybernetics, USA). Histology was assessed for 50 glomeruli per section in a blinded manner. Glomerular neutrophils were counted based on the appearance of typical basophilic, multi-lobed nuclei within capillary lumens. PAS-positive (PAS+) material was assigned a score between 0 and 4 for the number of glomerular quadrants affected. Crescents were assessed based on characteristic morphology of circumferential cellular lesions inside Bowman’s capsule compressing the glomerular tuft. MPGN was assessed by glomerular cell counts and by ranking of specimens in terms of mesangial expansion from 1 (least prominent) to 21 (most prominent). Original magnification is provided for each image.

2.9 Renal immunostaining for mouse proteins

Unfixed kidneys were embedded in OCT embedding matrix (CellPath Ltd, UK) and snap frozen in isopentane for storage at -80°C. 5µm coronal cryosections were obtained using a Bright OTF5000 cryostat (A-M Systems, USA) and mounted on SuperFrost® glass slides (VWR, USA, Product N° 631-0910) coated with 0.1% poly-L-lysine (Sigma-Aldrich, USA), before fixing in acetone and storage at -80°C. Thawed sections were encircled with a Pap pen (The Binding Site, UK, Product N° AD100.1) and the slides then placed into a closed chamber to prevent evaporation of applied solutions.

Immunostaining was performed by pipetting 60µL of each solution onto sections and incubating at RT. Detection of glomerular sheep IgG in NTS-injected mice was with 1 in 100 fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal IgG₁ anti-goat/sheep IgG antibody (Sigma-Aldrich, USA, Product N° F4891) in PBS for 1h. For detection of mouse C3, IgG or fibrin/fibrinogen, sections were blocked using 20% v/v goat serum (Sigma-Aldrich, USA, Product N° G9023) in PBS for 30 minutes. After tapping off, detection was with 1 in 200 FITC-conjugated goat polyclonal IgG anti-mouse C3 antibody (Cappel™, USA, Product N° 0855500, 4mg/mL), 1 in 200 FITC-conjugated goat polyclonal anti-mouse IgG-Fc portion antibody (Sigma-Aldrich, USA, Product N° F5387) or 1 in 800 FITC-conjugated rabbit polyclonal anti-human fibrin(ogen) antibody (Dako, Denmark, Product N° F0111, 1.6mg/mL) in PBS for 1h.
For detection of mouse C3d, sections were blocked using 2% BSA/PBS for 30 minutes. After rinsing, the Biotin Blocking System (Dako, Denmark, Product No X-0590) was applied. C3d was detected using 1 in 10 biotinylated goat polyclonal IgG anti-mouse C3d antibody (R&D Systems, USA, Product N° BAF2655) in 2% BSA/PBS for 1h, followed by rinsing three times. Secondary detection was with 1 in 400 Alexa Fluor® 488-conjugated avidin (Invitrogen A-21370) or 1 in 400 phycoerythrin (PE)-conjugated streptavidin (BD Pharmingen™, USA, Product N° 554061, 0.5 mg/mL) for 1h.

Co-staining was performed for peanut (Arachis hypogaea) agglutinin (PNA) and either CD68 or CD11b. The Biotin Blocking System was applied, followed by blocking in 1 in 10 Carbo-Free™ blocking solution (Vector Laboratories, USA, Product No SP-5040) in ddH2O. After rinsing, PNA staining was performed using 1 in 400 biotinylated goat polyclonal anti-PNA antibody (Vector Laboratories, USA, Product No BA-0074, 1mg/mL) for 30 minutes. This antibody binds to glycan moieties with a terminal β-galactose residue at the core-1 branch of O-linked glycans. This was followed by rinsing in 0.1% PBST and then PBS. Visualization was with 1 in 200 Alexa Fluor® 555-conjugated streptavidin (Invitrogen S-21381) mixed with either 1 in 100 Alexa Fluor® 488-conjugated rat monoclonal IgG2a anti-mouse CD68 antibody (BioLegend, USA, Product N° 137012, 0.5mg/mL) or 1 in 100 Alexa Fluor® 488-conjugated rat IgG2a anti-mouse CD11b (BioLegend, USA, Product N° 101217, 0.5mg/mL), followed by rinsing in 0.1% PBST and then PBS.

After rinsing three times, all slides were mounted using VECTASHIELD® HardSet™ Mounting Medium with 4′,6′-diamidino-2-phenylindole (DAPI, Vector Laboratories, USA, Product N° H-1500) and glass cover slips (BDH, USA, Product N° 406/0188/52). Sections were visualized using a BX40 microscope with a BX-FLA vertical fluorescence illuminator (Olympus, Japan). IF photographs were taken using a Retiga 2000R digital camera (QImaging, Canada) and Image-Pro Plus® 7.0 software (Media Cybernetics, USA). Comparisons between sections used equivalent exposure times.

Quantitative IF for C3 and C3d was assessed for 10 glomeruli per section using Image-Pro Plus® 7.0 software (Media Cybernetics). Mean fluorescence intensity (MFI) is expressed in arbitrary fluorescence units (AFU). Representative images were obtained from the median section in each experimental group. Glomerular fibrin/fibrinogen positivity was assessed by counting fibrin-positive (fibrin+) glomerular quadrants for 50 glomeruli. For CD68-positive (CD68+) or CD11b-positive (CD11b+) cells, 20 glomeruli per section were identified on the basis of clustered DAPI-positive cell nuclei. Images obtained enabled bright signals for CD68 or CD11b to be counted as glomerular cells within the PNA-negative glomerular area.
2.10 Nephrotoxic nephritis

Sheep IgG against mouse GBM (NTS) was prepared as previously described [Pickering et al. 2002]. In accelerated NTN (ANTN), mice received i.p. injection of 200µL water-in-oil emulsion containing 200µg sheep serum IgG (Sigma-Aldrich, USA, Product N° I5131) and sterile Dulbecco’s PBS in 100µL vortexed CFA [Pickering et al. 2002]. The emulsion was prepared by vigorous mixing in a glass bottle using a glass syringe and fine-bore (21 gauge) needle, until a sample drop did not dissipate in cold water [Stills & Bailey 1991]. Five days later, mice received i.v. tail vein injection of 200µL 10% v/v NTS in sterile Dulbecco’s PBS. In heterologous NTN, mice received an identical i.v. NTS dose to that used for ANTN but without prior antigen sensitization using IgG/CFA.

2.11 Subcutaneous base-of-tail immunization

Mice received s.c. base-of-tail injection of 100µL emulsion containing 50µg 4-hydroxy-3-nitrophenylacetyl (NP₄)-haptenated chicken gammaglobulin (CGG, Biosearch Technologies, USA, Product N° N-5055A-5) and sterile Dulbecco’s PBS in 50µL CFA.

2.12 Measurement of murine IgG response to hapten by sandwich ELISA

96-well flat-bottom Nunc Maxisorp® plates (Thermo Scientific, USA, Product N° 442404) were coated with 50µL/well of 5µg/mL 4-hydroxy-3-nitrophenyl (NP₄)₁₀-conjugated BSA (Biosearch Technologies, USA, Product N° N-5050M-10) in coating buffer (100mM boric acid [H₃BO₃], 25mM sodium tetraborate, 75mM NaCl; pH 8.3) for 3h at 37°C. After washing three times with PBS, plates were blocked in 100µL/well blocking buffer (PBS 0.5% BSA, 0.02% w/v sodium azide [NaN₃]) for 1h at RT. After discarding the blocking buffer, 50µL/well duplicate 1 in 500 mouse plasma samples in sample buffer (PBS 2% BSA, 0.02% NaN₃, 0.05% TWEEN® 20) were added, with incubation overnight at 4°C. Single samples were also added to an uncoated well as a control for nonspecific binding. As a standard, duplicate pooled mouse plasma samples from primary NP₄-CGG-immunized WT mice were added in doubling dilutions beginning at 1 in 100 in sample buffer. After washing five times, captured mouse IgG was detected using 50µL/well of 1 in 3000 alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, USA, Product N° A3438) in sample buffer for 5h at 4°C. After washing five times, the assay was developed using 50µL/well of ALP substrate reagents (SigmaFAST p-nitrophenyl phosphate tablet set, Sigma-Aldrich, USA, Product N° N-2770) at RT and the absorbance read at 405nm. The OD for uncoated wells was subtracted from the OD for coated wells for each sample. The OD for the standard was plotted against percentage concentration on a logarithmic scale, and a
sigmoid curve obtained. The IgG titre of mouse plasma samples was calibrated against the standard curve using linear regression, and is expressed in arbitrary units.

2.13 Administration of human FH reagents in mice

*Cfh*⁻/⁻ mice were injected i.p. with recombinant human FH reagents or full-length human plasma-derived FH (hFH) in PBS, or PBS alone. hFH was either purified from human plasma by Dr Kevin Marchbank at Newcastle University, Institute of Cellular Medicine, Newcastle upon Tyne, or acquired commercially from Complement Technology, USA (Product N° A337). The reagents used, and doses and volumes injected, are presented in Chapter 5, Table 10. Recombinant human mini-FH and FH1-5 were produced by Dr Kevin Marchbank and Dr Eva-Maria Nichols at Newcastle University. Briefly, the FH1-5 expression vector was generated by PCR amplification of SCR domains 1-5 from a pBluescript vector containing the FH cDNA. Primers were designed to introduce a Sall restriction site at the 3’ end and a hexa histidine tag, stop codon and Nhel restriction site at the 5’ end. For mini-FH, SCR domains 1-5 were amplified with primers introducing Sall/KpnI restriction sites at 3’/5’ ends and SCR domains 18-20 were amplified with primers introducing a KpnI restriction site and hexa histidine tag at the 3’ end and stop codon and Nhel restriction site at the 5’ end. Both constructs were cloned into the pDR2EF1α mammalian expression vector using the Sall/Nhel restriction sites and transfected into Chinese Hamster Ovary (CHO) cells. The proteins were purified using nickel-affinity chromatography. Mini-FH was polished by affinity chromatography using OX24 antibody. Mini-FH and FH1-5 were functionally validated by a number of in vitro tests carried out at Newcastle University.

In a separate experiment, 12 week-old *Cfh*⁻/⁻.*C3*⁻/⁻ mice were exsanguinated via cardiac puncture under terminal anaesthesia. Blood was collected into TubeOne® 1.5mL microcentrifuge tubes (Starlab, Germany, Product N° S1615-5500) and allowed to clot for 10 minutes at RT and on ice for a further 1h. Pooled serum was separated by centrifugation and stored at -80°C. *Cfh*⁻/⁻.*Cfi*⁻/⁻ mice were injected i.p. with hFH (Comp Tech, A137) or via tail vein injection with 400μL of pooled *Cfh*⁻/⁻.*C3*⁻/⁻ serum or PBS. Tail vein injections were performed by my supervisor and by Dr Marieta Ruseva.

2.14 Detection of injected human FH reagents by western blot

SDS-PAGE was performed using 1μL mouse plasma in a 10% gel under reducing conditions. Detection of injected FH reagents was with 1 in 20,000 goat anti-human FH antisera (Quidel, USA, Product N° A312) in 5% NFDM/PBS overnight at 4°C. After washing three times in 0.1% PBST and reblocking with 5% NFDM/PBS for 1h at RT, secondary detection was with 1 in 20,000 HRP-conjugated mouse monoclonal IgG₁, anti-sheep/goat IgG
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(Sigma-Aldrich A9452) in 5% NFDM/PBS for 1h at RT. Blots were developed as described above.

2.15 Renal immunostaining for injected human FH reagents

Mouse monoclonal IgG₁ anti-human antibody (OX24, 1mg/mL) was prepared from hybridoma tissue culture and biotinylated by Dr Kevin Marchbank. Use of the Biotin Blocking System was followed by application of 1 in 25 biotinylated OX24 in PBS for 1h at RT. After rinsing, visualization was with 1 in 400 PE-conjugated streptavidin (BD Pharmingen™ 554061), 1 in 150 Alexa Fluor® 555-conjugated streptavidin (Invitrogen, USA, Product N° S-21381) or 1 in 200 Alexa Fluor® 488-conjugated avidin (Invitrogen, USA, Product N° A-21370) in PBS for 1h at RT. Representative images were obtained from the median section in each experimental group.

2.16 Human sera and purified proteins

NHS was pooled from healthy donors following cubital fossa venesection into BD Vacutainer® glass serum tubes (BD, USA, Product N° 366430) with clotting for 45 minutes at RT and for 1h on ice [Lachmann 2010]. Serum was separated from whole blood by centrifugation at 16,060×g for 10 minutes at 4°C. The supernatant was carefully removed and stored as single-use aliquots in Axygen® 0.6mL microtubes (Corning, USA, Product N° MCT-060-C) at -80°C. Human depleted sera and purified proteins were purchased from Complement Technology, USA (Table 5). The sera are depleted of FH, FI, C3 or C5 by immunoaffinity chromatography and stored in 0.1mM EDTA. On arrival (on dry ice), sera were thawed by hand and promptly transferred onto ice to minimise spontaneous complement activation. They were then pipetted into microtubes on ice for storage as single-use aliquots at -80°C. For each assay, human sera were thawed by hand and kept on ice for short period prior to use. Human purified proteins were also stored at -80°C.
Table 5 Human complement-depleted sera and purified complement proteins

<table>
<thead>
<tr>
<th>Human reagent</th>
<th>Manufacturer</th>
<th>Product N°</th>
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<tr>
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</tr>
<tr>
<td>Human purified factor H protein (hFH)</td>
<td>Complement Technology, USA</td>
<td>A137</td>
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2.17 Water bath incubation of human sera/proteins

Human sera (NHS or FH\textsuperscript{dpl}HS) were prepared in microtubes on ice at a 50% v/v concentration in incubation buffer (PBS with a final concentration of 10mM EGTA and 5mM MgCl\textsubscript{2}; pH 7.4) [Des Prez et al. 1975]. FH\textsuperscript{dpl}HS was also prepared to which hC3 was added at 1.0mg/ml serum (FH\textsuperscript{dpl}HS+hC3\textsuperscript{1.0mg/mL}), or hFH protein at 0.05mg/mL serum (FH\textsuperscript{dpl}HS+hFH\textsuperscript{0.05mg/mL}). Identical amounts of hC3 or hFH were also prepared in buffer without serum. Microtubes containing 10µL of each serum/protein preparation were then placed into a shaking water bath (Medline, UK, Product N° BS-06) at 37°C. An additional 4µL aliquot of each preparation was diluted for western blot in microtubes containing LDS buffer with MCE. At 10 minutes and 60 minutes, 4µL aliquots of each of the incubating specimens was also set aside for western blot in MCE-containing LDS buffer. western blot samples were heat-inactivated at 99°C for 3 minutes in a Thriller thermoshaker incubator (Peqlab, Germany, Product N° 91-7010) before storage at -80°C.

A standardized protocol for water bath incubation was subsequently developed using triplicate 5.4µL specimens of 50% human sera in incubation buffer with or without 10mM EDTA (final concentration) [North & Whaley 2005]. FH\textsuperscript{dpl}HS was also prepared in incubation buffer containing hFH 0.5mg/ml serum (FH\textsuperscript{dpl}HS+hFH\textsuperscript{0.5mg/mL}). After two samples had been placed into the water bath, which was then diluted in LDS buffer to which 10mM EDTA had been added with or without MCE. One of the incubating samples was removed and set aside for western blot at 10 minutes and the other at 60 minutes.
2.18 Measurement of C3 activation in human sera using ELISA

Collagen type IV from human placenta (Sigma-Aldrich, USA, Product N° C7521), laminin from human placental liquid (Sigma-Aldrich, USA, Product N° L6274), fibronectin from human plasma (BD, USA, Product N° 354008) and zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich, USA, Product N° Z4250) were stored in sterile Dulbecco’s PBS (Sigma-Aldrich, USA, Product N° D8537) at -80°C. 96-well flat-bottom polystyrene microtitre plates (Greiner Bio-One, Germany, Product N° 655001) were coated with 50µL/well of the purified proteins in doubling dilutions beginning at 25µg/mL in coating buffer (0.1M NaHCO₃ pH 9.5), or coating buffer alone. Plates were kept overnight at 4°C. The coating solution was then discarded, and plates were washed three times in 0.1% PBST. Nonspecific binding was blocked using 100µL/well of blocking buffer (2% BSA/0.1% PBST) for 3h at RT. Blocking buffer was discarded and the plates washed three times in PBS.

Human sera/protein were prepared at a 5% v/v concentration in incubation buffer (PBS; 10mM EGTA; 5mM MgCl₂; pH 7.4) with or without 10mM EDTA. 50µL of duplicate (NHS) or triplicate (FH₃plHS, FH₃plHS+hFH₀.5mg/mL or FI₃plHS) sera/protein preparations was added to wells. Single samples of human sera/proteins in incubation buffer containing 10mM EDTA, and of incubation buffer without serum, were also added to wells. Plates were placed in an incubator (Gallenkamp, UK) at 37°C, after which additional samples of each serum/protein preparation were added to EDTA-containing LDS buffer with MCE for storage at -80°C. After incubation for 45 minutes, plates were removed from the incubator and washed three times with 0.1% PBST with or without 1M NaCl. Captured human C3 was detected using 50 µL/well of 1 in 10,000 HRP-conjugated goat polyclonal IgG anti-human C3 antibody (Cappel™, USA, Product N° 0855237) in blocking buffer for 1h at RT. After washing three times with 0.1% PBST, plates were incubated in 50µL/well of TMB substrate reagents (BD, USA, Product N° 555214), after which the reaction was stopped using 20µL/well of 2N sulfuric acid (BD, USA, Product N° DY994). The absorbance was read at 450nm using a Multiskan Ascent® spectrophotometer (Thermo Scientific, USA) and Ascent Software version 2.6. Results are expressed as the mean OD.

Separately, plates were coated overnight with collagen type IV, laminin and fibronectin as described above before washing three times. The respective GBM components were detected using 50 µL/well of 1 in 1,000 rabbit polyclonal IgG anti-fibronectin antibody (Sigma-Aldrich, USA, Product N° F3648, 0.6mg/mL) or anti-laminin antibody (Sigma-Aldrich, USA, Product N° L9393, 0.7mg/mL) or 1 in 4,000 rabbit polyclonal IgG anti-collagen type IV antibody (Abcam, UK, Product N° ab6586, 1.0mg/mL) in PBS for 1h at RT. After washing
plates three times, secondary detection was with 50 µL/well of 1 in 30,000 ALP-conjugated goat polyclonal anti-rabbit IgG antibody (Sigma-Aldrich, USA, Product N° A3812) in PBS for 1h at RT. After washing three times, the assay was developed using 50µL/well of ALP substrate reagents (SigmaFAST p-nitrophenyl phosphate tablet set, Sigma-Aldrich, USA, Product N° N-2770) at RT, and absorbance was read at 405nm.

2.19 Measurement of rabbit haemolysis in human sera

1mL of rabbit blood in Alsever's solution (TCS Biosciences, UK, Product N° RB053, 25mL) stored at 4°C was gently pipetted into 10mL of lysis buffer (5mM Na barbitone, 150mM NaCl, 10mM EGTA, 7mM MgCl₂, 0.01% v/v gelatin from porcine skin [Sigma-Aldrich, USA, Product N° G-2500] in ddH₂O; pH7.4) in a Sterilin™ tube (Thermo Scientific, USA, Product N° Z10PS). Mixing by slow inversion of the tube was followed by centrifugation at 760×g for 4 minutes at 4°C. The supernatant was then discarded and the sediment gently resuspended in 10mL of lysis buffer. Centrifugation and resuspension steps were repeated several times until the supernatant was clear [Ruseva & Heurich 2014]. 2% v/v E₅₇ sediment was then gently resuspended in lysis buffer, with 20µL/well of the E₅₇ suspension, or lysis buffer alone, added to 96-well round-bottom Nunc™ MicroWell™ polystyrene microtitre plates (Thermo Scientific, USA, Product N° 163320). NHS, FHdplHS, FIdplHS, FHdplHS+hFH0.5mg/mL, C³dplHS and C₅dplHS underwent doubling dilutions in lysis buffer with or without 10mM EDTA on ice. C₃dplHS was also prepared in lysis buffer to which hC3 was added in doubling dilutions beginning at 1.0mg/mL serum. FHdplHS was also prepared in lysis buffer, to which hFH was added in doubling dilutions beginning at 0.5mg/mL serum.

50µL triplicate samples were added to wells containing E₅₇ beginning at a final 50% serum concentration, and a fourth sample added to wells containing lysis buffer alone. Four samples of water (positive control) or lysis buffer were also added to wells, and the plates were placed in an incubator at 37°C. A fifth sample of each serum/protein preparation was then added to EDTA-containing LDS buffer with MCE for storage at -80°C for western blot. After incubation for 1h, plates were centrifuged at 760×g for 4 minutes at 4°C. 50µL/well of the supernatant was transferred to 96-well flat-bottom Nunc™ MicroWell™ microtitre plates (Thermo Scientific, USA, Product N°, 167008) and spectrophotometry performed at an absorbance of 414nm for detection of haem. Samples of supernatant were then set aside for western blot.

For some assays, supernatant left over after incubation in non-lytic sera was discarded, and the E₅₇ pellet was gently resuspended on the plate in 50µL of lysis buffer followed by recentrifugation. These steps were repeated once. Fresh FHdplHS, NHS or C₃dplHS...
underwent doubling dilutions beginning at 20% v/v in lysis buffer, with triplicate 50µL/well serum samples used to resuspend the E⁰ pellets, and a fourth sample added to an empty well. Plates were reincubated for 1h at 37°C, before centrifugation and spectrophotometry as above. For all assays, the OD for sera/protein preparations incubated in wells not containing E⁰ was subtracted from the mean OD of the respective preparations in E⁰-containing wells. The value obtained for each preparation was divided by that for the positive control (water), with the results expressed as mean percent haemolysis due to H₂O.

2.20 FACS of rabbit erythrocytes following incubation in human sera

Following a single incubation of E⁰ suspension in non-lytic sera, the E⁰ pellet obtained by centrifugation was gently resuspended in 200µL/well of wash buffer (1% BSA in PBS) to which 10mM EDTA had been added, followed by centrifugation and removal of the supernatant. This was repeated twice using wash buffer, after which the E⁰ pellet was resuspended in 40µL/well of wash buffer, and 20µL transferred to each of two wells on round-bottom Nunc™ MicroWell™ plates. 20µL of 1 in 50 FITC-conjugated goat polyclonal IgG anti-human C3 antibody (Cappel™, USA, Product No. 0855167) in wash buffer was then added to one of the resuspended E⁰ pellets, and 1 in 50 FITC-conjugated goat polyclonal IgG anti-mouse IgG-Fc (Sigma-Aldrich, USA, Product No. F5387) added to the other. The plate was placed on ice for 1h and then centrifuged. After washing three times with 200µL/well of wash buffer, the E⁰ pellets were resuspended in 330µL of wash buffer and transferred to 1.2mL glass microtubes (Alpha Laboratories, UK, QS845) for fluorescence-activated cell sorting (FACS) analysis using a BD FACSVerse™ flow cytometer (BD Biosciences, USA) and FlowJo software version 7.6.5 (FlowJo, USA). C3 staining of E⁰ gated according to erythrocyte size and granulosity. Results are expressed in the text as MFI and on the histogram as Mean, Comp-FITC-A::FITC-A. Positive C3 staining for a small number of E⁰ incubated in C₅dplHS exceeded the measurement scale, and was excluded from the plot.

2.21 Detection of human proteins by western blot

SDS-PAGE was performed using a 10% polyacrylamide gel for separation of human C3 under reducing questions, and FH and FHR-5 under non-reducing conditions, and a 7.5% gel for separation of human C5 under non-reducing conditions. For the standardized assays, 0.28µL serum samples were used for detection of C3 and C5 detection. For the earlier (non-standardized) water bath incubation assays 0.5µL serum samples were used for C3 detection. Freshly prepared 1µL NHS, FHdplHS and FHdplHS+hFH⁰.5mg/mL samples were used...
for FH and FHR-5 detection (additionally, 0.1µL samples for FH detection). As controls, 0.28µg hC3, 0.03µg hFH and 0.01µg hC5 (Quidel, A403) were used. Following transfer, membrane blocking was performed using 5% NFDM/PBS for 1h at RT. Membranes were then incubated for 1h at RT in detecting antibody diluted in 5% NFDM as follows: 1 in 3,000 HRP-conjugated goat polyclonal IgG anti-human C3 antibody (Cappel™ 0855237); 1 in 2,500 goat anti-human C5 antisera (Quidel, A306) followed by washing three times with 0.1% PBST, reblocking in 5% NFDM/PBS for 1h at RT, and secondary detection with 1 in 5,000 HRP-conjugated mouse monoclonal IgG1 anti-goat/sheep IgG antibody (Sigma-Aldrich, USA, Product N° A9452) for 30 minutes at RT; 1 in 20,000 goat anti-human FH antisera (Quidel, A312), followed by washing three times and secondary detection with 1 in 20,000 HRP-conjugated mouse monoclonal IgG1 anti-goat/sheep IgG antibody (Sigma-Aldrich A9452) for 30 minutes at RT; or 1 in 1,000 rabbit polyclonal anti-human FHR-5 antibody (Abnova, Taiwan, Product N° H00081494-D01P, 1.08mg/mL), followed by washing three times and secondary detection with 1 in 2,000 HRP-conjugated swine polyclonal anti-rabbit Ig antibody (Dako, Denmark, Product N° P021702-2, 1.3mg/mL) for 45 minutes at RT. Western blots were developed as above.

2.2.2 Renal immunostaining following incubation with human sera

Kidneys for incubation in human sera/protein were obtained from C3/-/- mice with or without i.v. tail vein injection of 200µL 10% v/v sheep NTS in sterile Dulbecco’s PBS (Sigma-Aldrich D8537) 4h prior to sacrifice. For incubation in human sera/protein, sections were first blocked with 2% BSA for 30 minutes at RT. NHS, FHdplHS, FHdplHS and FHdplHS+hFH0.5mg/mL were prepared on ice at dilutions ranging from 1 in 10 to 1 in 80 in incubation buffer (PBS 10mM EGTA 5mM MgCl₂ pH 7.4) with or without 10mM EDTA. 5.4µL samples of 50% FHdplHS in incubation buffer underwent prior water bath incubation for 1h at 37°C before dilution in incubation buffer. hC3 was prepared in incubation buffer without serum at a concentration equivalent to 1.0mg/mL serum. The blocking solution was tapped off from the slides, and 60µL/section of each serum/protein preparation was applied to the sections. The closed chamber was placed into an incubator at 37°C. Additional samples were then added to EDTA-containing LDS buffer with MCE for storage at -80°C for western blot. After 1h, the slides were removed from the chamber and rinsed three times in PBS in a glass 200mL reservoir. Human C3 was detected using 1 in 500 FITC-conjugated goat polyclonal IgG anti-human C3 antibody (Cappel™, USA, Product N° 0855167) in PBS for 1h at RT.
2.23 Statistics

Data were analysed and $p$ values calculated using GraphPad Prism® 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). For comparison of two groups, Mann Whitney test (Chapters 3 and 4) or Student’s t-test (Chapter 5) were used. For comparison of three or more groups, Kruskal-Wallis test and Dunn’s multiple comparisons test for all comparisons (Chapters 3 and 4) or one-way ANOVA and Bonferroni’s multiple comparisons test for all comparisons (Chapter 5) were used, unless specified otherwise. Correlation coefficients ($r$) were determined using the Spearman test (Chapters 3 and 4) or Pearson test (Chapter 5). Survival curves were analysed using the log rank test. A $p$ value of $<0.05$ is reported as significant.
3. CHAPTER THREE: ROLE OF CD11B IN THE SPONTANEOUS PHENOTYPE OF FACTOR H-DEFICIENT MICE
3.1 Introduction

In this chapter, I assessed the role of iC3b in dense deposit disease from the standpoint of CD11b, its specific leukocyte receptor. I utilized the uniquely informative FH-deficient mouse model of experimental C3 glomerulopathy, in which spontaneous renal disease occurs due to accumulation of iC3b (± C3d) along the GBM [Pickering et al. 2002, Rose et al. 2008, Paixão-Cavalcante et al. 2009]. My hypothesis was that deficiency of CD11b would ameliorate experimental renal disease in FH-deficient mice (hypothesis 1) through abrogation of the iC3b-CD11b interaction. In order to test this, I evaluated the spontaneous renal phenotype in mice with homozygous deficiency of FH and/or CD11b. I monitored a cohort of ten female mice with combined homozygous deficiency of FH and CD11b (Cfh−/−.CD11b−/−). In addition, I monitored five female Cfh−/− mice and six female CD11b−/− mice, with comparison of the spontaneous renal phenotype in all three groups at 8 months. A similar timeframe has previously been sufficient for the development of MPGN and albuminuria in Cfh−/− mice [Pickering et al. 2002, Ruseva et al. 2013].
3.2 CD11b deficiency enhances albuminuria in 8-month old FH-deficient mice

Weights were well-matched between groups when first measured at four months of age (Table 6). One $Cfh^{-/-}.CD11b^{-/-}$ mouse developed 2+ dipstick haematuria and distress, necessitating humane sacrifice at 6 months. All other mice remained well and were haematuria-free until termination of the experiment, with survival at eight months being no different between groups ($p = 0.5769$).

Prior to sacrifice at 8 months of age, mice were placed into individual metabolic cages and urine collected for 16h overnight. 16h urinary albumin excretion was increased in $Cfh^{-/-}.CD11b^{-/-}$ mice compared to $Cfh^{-/-}$ mice (median urinary albumin excretion $Cfh^{-/-}.CD11b^{-/-}$ 173.2µg/16h versus $Cfh^{-/-}$ 74.8µg/16h; $p = 0.0475$, Fig. 6a) but not $CD11b^{-/-}$ mice (100.9µg/16h; $p$ versus $Cfh^{-/-}.CD11b^{-/-} = 0.6828$). The urine albumin concentration was increased in $Cfh^{-/-}.CD11b^{-/-}$ mice compared to $CD11b^{-/-}$ mice (median urinary albumin concentration $Cfh^{-/-}.CD11b^{-/-}$ 164.7µg/mL versus $CD11b^{-/-}$ 75.6µg/mL; $p = 0.0387$, Fig. 6b) but not $Cfh^{-/-}$ mice (118.6µg/mL; $p$ versus $Cfh^{-/-}.CD11b^{-/-} = 0.3745$). Urine volume in $Cfh^{-/-}$ mice was significantly reduced compared to $CD11b^{-/-}$ mice (median $Cfh^{-/-}$ 0.5mL/16h versus $CD11b^{-/-}$ 1.4mL/16h, $p = 0.0189$, Fig. 6c) but not $Cfh^{-/-}.CD11b^{-/-}$ mice (median 1.2mL/16h; $p$ versus $Cfh^{-/-} = 0.2982$). The median value for proteinuria in $CD11b^{-/-}$ of 100.9µg/16h clearly exceeded that in previous studies of WT mice (albumin excretion of 0µg/24h), although the range of values in those larger cohorts was similar [Pickering et al. 2002, Ruseva et al. 2013].
### Table 6 Spontaneous renal phenotype in 8-month old mice

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<tr>
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<th>Cfh-/-.CD11b/-</th>
<th>Cfh/-</th>
<th>CD11b/-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N° of mice assessed/sex</strong></td>
<td>10/F</td>
<td>5/F</td>
<td>6/F</td>
</tr>
<tr>
<td><strong>Weight at 4 months (g)</strong></td>
<td>22.3 (20.3-24.8)</td>
<td>24.0 (21.8-25.3)</td>
<td>23.9 (22.9-2.8.8)</td>
</tr>
<tr>
<td><strong>Survival to 8 months</strong></td>
<td>90%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Urine analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Haematuria (N° of mice)</strong></td>
<td>1/10</td>
<td>0/5</td>
<td>0/6</td>
</tr>
<tr>
<td><strong>Albumin excretion (µg/16hr)</strong></td>
<td>173.2* [72.5-987.2]</td>
<td>74.8 [33.8-141.2]</td>
<td>100.9 [76.2-176.8]</td>
</tr>
<tr>
<td><strong>Albumin concentration (µg/mL)</strong></td>
<td>164.7 † [70.5-1606.2]</td>
<td>118.1 [78.7-207.7]</td>
<td>75.6 [59.1-106.8]</td>
</tr>
<tr>
<td><strong>Urine volume (mL/16h)</strong></td>
<td>1.2 [0.5-1.6]</td>
<td>0.5 † [0.4-1.2]</td>
<td>1.4 [1.1-1.9]</td>
</tr>
<tr>
<td><strong>Plasma biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urea (mmol/L)</strong></td>
<td>13.3 †† [9.7-44.5]</td>
<td>11.5 [7.3-15.1]</td>
<td>4.4 [2.8-8.2]</td>
</tr>
<tr>
<td><strong>C3 (µg/mL)</strong></td>
<td>14.3 †† [1.1-43.5]</td>
<td>10.0 † [5.8-18.9]</td>
<td>268.1 [230.0-341.5]</td>
</tr>
<tr>
<td><strong>Glomerular Histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean cells (N°)</strong></td>
<td>53.4* †† [40.8-75.9]</td>
<td>36.6 [35.0-44.4]</td>
<td>35.5 [33.6-38.2]</td>
</tr>
<tr>
<td><strong>Crescents (N° of mice)</strong></td>
<td>1/10</td>
<td>0/5</td>
<td>0/6</td>
</tr>
<tr>
<td><strong>Mean PAS+ quadrants (N°)</strong></td>
<td>0.1 [0.0-0.2]</td>
<td>0.0 [0.0-0.1]</td>
<td>0.1 [0.0-0.1]</td>
</tr>
<tr>
<td><strong>Mean neutrophils (N°)</strong></td>
<td>0.3* [0.1-1.9]</td>
<td>0.0 [0.0-0.1]</td>
<td>0.1 [0.0-0.1]</td>
</tr>
<tr>
<td><strong>Glomerular IF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean CD68+ cells (N°)</strong></td>
<td>4.3 [0.9-9.9]</td>
<td>1.90 [1.2-3.1]</td>
<td>2.0 [0.2-3.4]</td>
</tr>
<tr>
<td><strong>Mean C3 (AFU)</strong></td>
<td>120.1 † [69.5-129.5]</td>
<td>131.1 †† [102.7-134.6]</td>
<td>34.3 [27.8-59.6]</td>
</tr>
<tr>
<td><strong>Mean C3d (AFU)</strong></td>
<td>37.0 [21.8-46.6]</td>
<td>41.4 [37.3-50.9]</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Mean IgG (AFU)</strong></td>
<td>36.9 [13.2-51.1]</td>
<td>37.2 [15.5-66.5]</td>
<td>43.4 [28.8-67.5]</td>
</tr>
</tbody>
</table>

Data expressed as median [range]. n/a not assessed.

* p < 0.05, ** p < 0.01 versus Cfh/-
† p < 0.05, †† p < 0.01 versus CD11b/-

Analysis by Kruskal-Wallis and Dunn’s tests except survival (log-rank test) and linear C3d (Mann-Whitney)
Figure 6 Albuminuria in 8-month old mice

(a) Urinary albumin excretion in female Cfh−/−.CD11b−/− (n=8), Cfh−/− (n=5) and CD11b−/− (n=6) mice was calculated in µg/16h on the basis of (b) albumin concentration in µg/mL and (c) urine volume in mL/16h. The sick Cfh−/−.CD11b−/− mouse sacrificed at 6 months is shaded green. Horizontal bars denote median values. *p < 0.05 (Kruskal-Wallis/Dunn’s).
3.3 CD11b deficiency does not influence plasma urea levels in 8-month old FH-deficient mice

Renal failure is not usually observed in 8 month-old Cfh-/- mice. Here an enzymatic ultraviolet assay was used to assess plasma urea levels, and showed no difference between Cfh-/-CD11b-/- and Cfh-/- mice (median plasma urea Cfh-/-CD11b-/- 13.3mmol/L versus Cfh-/- 11.5mmol/L; p = 0.0912, Fig. 7). The sick Cfh-/-CD11b-/- mouse alone had a markedly increased plasma urea level. As a result, plasma urea levels were increased only for the Cfh-/-CD11b-/- group in comparison to CD11b-/- mice (4.4mmol/L; p versus Cfh-/-CD11b-/- = 0.0012). This compares with a normal urea of 4.3-10mmol/L in mice based on a previous study [Wolfensohn & Lloyd 1998].
Figure 7 Plasma urea levels in 8-month old mice

Plasma urea levels are shown at 8 months in mmol/L. The sick mouse is shaded green. Horizontal bars denote median values. **p < 0.01 (Kruskal-Wallis/Dunn’s).
3.4 CD11b deficiency does not influence plasma C3 or C5 in FH-deficient mice

Uncontrolled C3 activation via the AP in Cfh-/ mice is associated with low plasma C3 and C5 levels. Plasma C3 levels were evaluated by an ELISA using a polyclonal anti-mouse C3 antibody, and showed no difference between Cfh-/ .CD11b-/ and Cfh-/ mice (median plasma C3 Cfh-/ .CD11b-/ 14.3mg/L versus Cfh-/ 10.0µg/mL, p > 0.9999, Fig. 8a). C3 levels were markedly lower in either group compared to CD11b-/ mice (median plasma C3 CD11b-/ 268.1mg/L; p versus Cfh-/ .CD11b-/ = 0.0039; p versus Cfh-/ = 0.0112).

The C3 activation state was assessed by western blot of plasma from three mice in each group using anti-mouse C3 antisera. In Cfh-/ .CD11b-/ mice, the α-chain of intact C3 and α'-chain of C3b were not detected, with only α'-chain fragments (and the β-chain) evident (Fig. 8b). An identical appearance was seen in three Cfh-/ mice. These findings are consistent with excessive C3 activation in both groups of FH-deficient mice. In three CD11b-/ mice, presence of the α-chain of intact C3 and absence of α'-chain fragments closely resembles the C3 profile in WT mice [Rose et al. 2008, Paixão-Cavalcante et al. 2009].

C5 western blot using cross-reactive anti-human C5 antisera has been used to assess differences in the C5 activation state in FH-deficient mice [Ruseva et al. 2013]. This showed an identical band for intact C5 to that of human recombinant C5 (MW 190kDa) in CD11b-/ mice but not Cfh-/ .CD11b-/ or Cfh-/ mice (Fig. 8c). These data are consistent with equivalent depletion of C5 in both these groups of FH-deficient mice. A second band present at ~75kDa in CD11b-/ mice only is likely to represent the β-chain of murine C5. This is absent from the corresponding blot using secondary antibody alone (Fig. 8d).
Figure 8 Plasma C3 levels and activation state in 8-month old mice

(a) C3 in μg/mL was measured in plasma from Cfh−/−.CD11b−/−, Cfh−/− and CD11b−/− mice by ELISA. The sick mouse is shaded green. Horizontal bars denote median values. *p < 0.05, **p < 0.01 (Kruskal-Wallis/Dunn’s). (b) C3 western blot under reducing conditions. (c) C5 western blot under non-reducing conditions. (d) Appearance of WB using the secondary antibody alone, as used for both the C3 and C5 western blots.
3.5 CD11b deficiency enhances the severity of MPGN in 8-month old FH-deficient mice

Previously, MPGN in Cfh-/- mice on the mixed 129/SvxC57BL/6 genetic background was characterized at 8 months by mesangial matrix expansion, mesangial hypercellularity, peripheral capillary loop thickening with accumulation of PAS-positive material and a double-contour appearance of the GBM [Pickering et al. 2002]. Here, LM in 8-month old Cfh-/-CD11b-/- mice revealed MPGN with prominent mesangial expansion and diffuse hypercellularity (Fig. 9a). Chronic renal injury in Cfh-/- mice was fairly mild and glomerular morphology in CD11b-/- mice was unremarkable (Fig. 9b,c). The sick Cfh-/-CD11b-/- mouse had a distinct glomerular lesion characterized by crescents in 100% of glomeruli, mesangial expansion and hypercellularity and GCW thickening (Fig. 9d). Thickening of the GCW was not prominent in any other mice on LM.
Figure 9 Renal PAS staining in 8-month old mice

Representative LM images (400×) are shown of glomeruli in (a) Cfh-/-.CD11b/-, (b) Cfh/- and (c) CD11b/- mice. (d) A circumferential crescent (indicated by arrow) in the sick Cfh-/-.CD11b/- mouse.
3.6 CD11b deficiency enhances glomerular hypercellularity in 8-month old FH-deficient mice

Histological assessment revealed increased glomerular cells in Cfh-/-,CD11b-/- mice compared to both Cfh-/- mice (median glomerular cells Cfh-/-,CD11b-/- 53.4 versus Cfh-/- 36.6; \( p = 0.0438 \), Fig. 10a) and CD11b-/- mice (35.5; \( p \) versus Cfh-/-,CD11b-/- = 0.008). Mesangial expansion was assessed by ranking the sections for severity, from 1 (least severe) to 21 (most severe), with no difference observed between groups (median rank Cfh-/-,CD11b-/- 15 versus Cfh-/- 11 versus CD11b-/- 8; \( p = 0.5375 \), Fig. 10b). PAS-positive material was virtually absent from glomerular capillary loops, with no difference between groups (median score for PAS-positive quadrants per glomerular cross-section Cfh-/-,CD11b-/- 0.09, Cfh-/- 0.04, CD11b-/- 0.05; \( p = 0.3571 \), Fig. 10c). Neutrophils were rare, albeit significantly increased in Cfh-/-,CD11b-/- compared to both other groups (median neutrophils per glomerular cross-section Cfh-/-,CD11b-/- 0.25 versus Cfh-/- 0.06, \( p = 0.0223 \); versus CD11b-/- 0.02; \( p = 0.0006 \), Fig. 10d).
Figure 10 Glomerular histological assessment in 8-month old mice

(a) Mean number of glomerular cells per glomerulus and (b) ranking of severity of mesangial expansion (from lowest, 1, to highest, 21) in Cfh-/-CD11b-/-, Cfh-/-, and CD11b-/- mice. Mean number of (c) glomerular quadrants containing PAS-positive material and (d) glomerular neutrophils. The sick mouse is shaded green. Horizontal bars denote median values. *p < 0.05, ***p < 0.001 (Kruskal-Wallis/Dunn’s).
3.7 Glomerular hypercellularity and albuminuria are correlated in Cfh-/- CD11b-/- mice

Glomerular hypercellularity and 16h urinary albumin excretion were the most prominent histological and functional markers, respectively, of progressive renal disease in Cfh-/- CD11b-/- mice. I therefore assessed the correlation of these parameters. In the Cfh-/- CD11b-/- group, correlation of hypercellularity and 16h urinary albumin excretion was very high (r = 0.92, p = 0.0005, Fig. 11). With the inclusion of the Cfh-/- and CD11b-/- groups, in which neither glomerular hypercellularity nor albuminuria were prominent, correlation of these parameters was reduced (r = 0.53; p = 0.0122).
Figure 11 Correlation of glomerular hypercellularity and urinary albumin excretion

Histological (glomerular hypercellularity) and functional (urinary albumin excretion in μg/16h) were correlated across all three groups (Spearman, *p < 0.05) and in the Cfh-/-.CD11b-/ group alone (Spearman, **p <0.001). The sick mouse is shaded green.
3.8 CD11b deficiency does not influence glomerular C3 or C3d staining in FH-deficient mice

In both Cfh-/-.CD11b/- and Cfh/- mice, renal immunostaining using a polyclonal anti-mouse C3 antibody revealed a linear C3 pattern in the glomerulus, consistent with C3 accumulation along the GBM, whereas extraglomerular C3 was absent (Fig. 12a,b). By contrast, in CD11b/- mice, a WT pattern of sparse, granular mesangial C3 together with C3 staining of Bowman’s capsule and the tubulointerstitium was seen (Fig. 12c). MFI of glomerular C3 staining was no different between Cfh-/-.CD11b/- and Cfh/- mice (median MFI Cfh-/-.CD11b/- 120.1 AFU versus Cfh/- 131.1 AFU; \( p = 0.7535 \), Fig. 12g), but was increased in either case compared to CD11b/- mice (median 34.3 AFU; \( p \) versus Cfh-/-.CD11b/- = 0.0123; \( p \) versus Cfh/- = 0.0015).

Renal immunostaining was also performed using an antibody against the C3d moiety of mouse iC3b and C3d(g) [Paixão-Cavalcante et al. 2009]. This revealed a linear C3d staining pattern Cfh-/-.CD11b/- and Cfh/- mice, corresponding to the pattern seen using the polyclonal anti-C3 antibody, whereas C3d was not detected in CD11b/- mice (Fig. 12d-f). The intensity of linear C3d staining was no different between Cfh-/-.CD11b/- and Cfh/- mice (median MFI Cfh-/-.CD11b/- 36.0 AFU versus Cfh/- 41.4 AFU; \( p = 0.0753 \), Fig. 12h).
Figure 12 Renal C3 and C3d staining in 8-month old mice.

Representative images (400×) of glomeruli in Cfh-/-, CD11b-/-, Cfh-/- and CD11b-/- mice showing (a-c) direct IF staining using FITC-labelled antibody against C3 (d-f) indirect IF staining using biotinylated anti-C3d antibody and FITC-labelled avidin. (g) Mean fluorescence intensity (MFI) for glomerular C3 staining in arbitrary fluorescence units (AFU). (h) MFI in AFU for glomerular C3d. The sick mouse is shaded green. Horizontal bars denote median values. *p < 0.05, **p < 0.01 (Kruskal-Wallis/Dunn's).
3.9 CD11b deficiency does not influence glomerular IgG staining in FH-deficient mice

A faint granular pattern of glomerular IgG accumulation was seen in all groups (Fig. 13a-c), with the exception of the sick *Cfh−/−.CD11b−/−* mouse, in which a striking linear IgG staining pattern was seen (Fig. 13d). No difference in intensity of glomerular IgG staining was seen between the three groups (median MFI of glomerular IgG *Cfh−/−.CD11b−/−* 36.85 AFU, *Cfh−/−* 37.3 AFU, *CD11b−/−* 43.4 AFU; *p* = 0.4577, Fig. 13e).
Figure 13 Renal IgG staining in 8-month old mice
(a-c) Representative images (400×) of glomeruli in *Cfh−/−.CD11b−/−, Cfh−/−* and *CD11b−/−* mice showing direct IF staining using FITC-labelled antibody against IgG. (d) A distinct IgG staining pattern in the sick *Cfh−/−.CD11b−/−* mouse. (e) MFI in AFU for glomerular IgG. The sick mouse is shaded green. Horizontal bars represent median values.
3.10 Renal CD11b+ cells are absent in CD11b-deficient mice

CD11b+ cells were identified in renal tissue through direct IF staining. Simultaneous indirect IF staining was performed using an anti-PNA antibody that has been used to stain proximal and/or distal tubules in several species, with negative glomerular staining [Phillips et al. 2001, Kakani et al. 2012]. CD11b+ cells were thus enumerated within glomeruli bordered by PNA-positive tubulointerstitial cells. Renal CD11b+ cells were not detected in either of the genetically CD11b-deficient groups, and were seen within the glomerulus only in Cfh−/− mice (median CD11b+ cells Cfh−/− 1.2 versus Cfh−/− CD11b−/− 0.0, p = 0.0001; versus CD11b−/− 0.0, p = 0.0006, Fig. 14a-d). This compares to a CD11b+ glomerular cell count in one previous study of unmanipulated, younger C57BL/6 mice of approximately 0.3 [Masaki et al. 2003].
Figure 14 Assessment of glomerular CD11b+ cells in 8-month old mice

Representative images (400x) of glomeruli in (a) Cfh-/-CD11b-/-, (b) Cfh-/- and (c) CD11b-/- mice showing direct IF staining with FITC-labelled (green) antibody against CD11b, with indirect IF staining using biotinylated anti-PNA antibody and AF555-labelled (red) streptavidin. (d) Assessment of glomerular CD11b+ cell number. Horizontal bars denote median values, ***p < 0.001 (Kruskal-Wallis/Dunn's).
3.11 **Cfh−/−.CD11b−/−** mice show a nonsignificant trend towards increased glomerular CD68+ cells compared to Cfh−/− and CD11b−/− mice at 8 months

Staining for CD11b is commonly used to identify glomerular macrophages in murine models of GN including accelerated nephrotoxic nephritis (ANTN) [Lee et al. 2014]. In order to compare groups, some of which had genetic CD11b deficiency, macrophages were instead assessed by staining for CD68. CD68 is a myeloid lysosomal glycoprotein expressed in all monocytes/macrophages and, to a lesser extent, DCs in both humans [Kelly et al. 1988, Holness & Simmons 1993] and mice (where it is sometimes called microsialin) [Ramprasad et al. 1996]. CD68 may in fact be more reliable than other macrophage markers including CD11b and F4/80 for numerical assessment of glomerular macrophages [Masaki et al. 2003]. There was a nonsignificant trend towards increased glomerular CD68+ cells in Cfh−/−.CD11b−/− mice (median CD68+ cells Cfh−/−.CD11b−/− 4.3, Cfh−/− 1.9, CD11b−/− 2.0; p = 0.2311, Fig. 15a-e). Similar CD68+ macrophages in the glomeruli of Cfh−/− and CD11b−/− suggest that CD68 expression is not upregulated on CD11b-negative macrophages. These values compare to a CD68+ glomerular cell count of approximately 0.5 in a previous study [Masaki et al. 2003].
Figure 15 Assessment of glomerular CD68+ cells in 8-month old mice.

Representative images (400×) of glomeruli in (a) Cfh-/-,CD11b-/- (median) (b) Cfh-/- and (c) CD11b-/- mice, and (d) the sick Cfh-/-,CD11b-/- mouse, showing direct IF staining with FITC-labelled (green) antibody against CD68, with indirect IF staining using biotinylated anti-PNA antibody and AF555-labelled (red) streptavidin. (e) Assessment of glomerular CD68+ cell number. The sick mouse is shaded green. Horizontal bars denote median values.
3.12 CD11b deficiency does not influence the renal phenotype in 2-4-month old FH-deficient mice

The spontaneous renal phenotype was compared in 2-4-month old Cfh-/-.CD11b-/-(n=31) and Cfh-/- (n=7) mice. Both groups contained a mix of male and female mice, none having haematuria or >1+ proteinuria on dipstick urinalysis. Plasma urea was not elevated in either group (median Cfh-/-.CD11b-/ 11.0mmol/L versus Cfh-/- 10.8mmol/L; p = 0.8850, Fig. 16a) and plasma C3 levels were uniformly low (median Cfh-/-.CD11b-/ 6.5μg/mL versus Cfh-/- 10.4μg/mL; p = 0.1366, Fig. 16b). The number of glomerular cells was no different between groups (median glomerular cells Cfh-/-.CD11b-/ 33.2 versus Cfh-/- 32.3; p = 0.2229 Fig. 16c). Glomerular neutrophils and PAS-positive material were virtually absent (Fig. 16e,f). A linear C3 staining pattern was seen in the glomeruli of all assessed mice, with no difference in intensity between groups (median MFI Cfh-/-.CD11b-/ 122.1 AFU, Cfh-/- 118.3 AFU; p = 0.1307, Fig. 16d).
Figure 16 Plasma biochemistry and LM in 2-4-month old mice

No difference was observed between Cfhl-/-.CD11b-/-. and Cfhl-/- mice in (a) plasma urea, (b) plasma C3 levels, (c) number of glomerular cells or (d) MFI in AFU of linear glomerular C3. Representative LM images (400×) of glomeruli in 2-4-month old (e) Cfhl-/-.CD11b-/-. and (f) Cfhl-/- mice.
3.13 Discussion

Progressive renal disease in Cfh-/- mice is associated with the accumulation of iC3b along the GBM. In this chapter, I have shown that accompanying deficiency of CD11b, the specific leukocyte receptor for iC3b, exacerbates the spontaneous renal phenotype of FH-deficient mice at 8 months. Cfh-/-CD11b-/- mice developed significantly increased urinary albumin excretion (in μg/16h) and glomerular hypercellularity compared to their Cfh-/- counterparts. The conclusion that MPGN was more severe in the Cfh-/-CD11b-/- group was strengthened by the high correlation of functional (urinary albumin excretion) and histological (glomerular hypercellularity) markers of glomerular injury. A non-significant trend towards increased mesangial expansion was also seen in Cfh-/-CD11b-/- mice (whereas thickening and double contours of the GBM were not prominent). These data provide experimental evidence negating hypothesis 1, that CD11b deficiency ameliorates experimental renal disease in FH-deficiency mice. Instead, accompanying CD11b deficiency produced the opposite effect in FH-deficient mice, indicating that CD11b plays a protective role in experimental C3 glomerulopathy. This finding of a protective effect due to CD11b in a spontaneous model of GN is a completely novel one. Furthermore, these results raise the intriguing possibility that partial renal protection in experimental C3 glomerulopathy is mediated specifically via the iC3b-CD11b interaction.

CD11b is expressed on a wide array of leukocytes possessing phagocytic (e.g. neutrophils, monocytes/macrophage) and/or antigen presenting (e.g. myeloid DCs, monocytes/macrophages) capabilities. As hypercellularity was the most prominent feature of MPGN in this study, glomerular leukocytes were counted. LM revealed scarce neutrophils in all groups, albeit significantly increased in Cfh-/-CD11b-/- compared to Cfh-/- and CD11b-/- mice. Neutrophils are short-lived cells, and are seen as a component of glomerular inflammation in only the most aggressive forms of GN [Holdsworth & Tipping 2007]. Hence it is possible that constant, low-grade neutrophil influx played an important role in functional glomerular injury in Cfh-/-CD11b-/- mice without contributing significantly to hypercellularity.

Glomerular recruitment of macrophages is a typical feature of proliferative GN in both human and murine studies [Holdsworth & Tipping 2007, Duffield 2010]. The functional role of macrophages in glomerular inflammation is unclear, since they are a heterogenous cell population comprising both proinflammatory and tissue reparative subtypes [Weisheit et al. 2015]. Here, glomerular macrophages were assessed by direct IF staining for mouse CD68 as a pan-monocyte/macrophage marker (although the possibility of CD68+ myeloid DCs is not excluded [Ferenbach & Hughes 2008]). Despite a greater than twofold increase in median glomerular CD68+ cells in the Cfh-/-CD11b-/- group compared to either Cfh-/- or
CD11b-/- mice, the difference between groups was not statistically significant. Nevertheless, macrophages clearly form a component of glomerular hypercellularity in Cfh-/-,CD11b-/- mice, and hence may contribute to glomerular injury. One possible mechanism involves iC3b ligation of CD11b on glomerular macrophages, mediating a protective, anti-inflammatory response to evolving glomerular injury. On this analysis, disease exacerbation could be due to functionally disabled reparative macrophages and/or disinhibited proinflammatory ones as a result of CD11b deficiency.

Additional macrophage immunophenotyping and functional studies were not performed as part of the experimental work for this thesis. However there is some evidence from the literature that iC3b ligation of CD11b on macrophages mediates suppression of proinflammatory signalling. Firstly, interaction of iC3b and macrophage-expressed CD11b has been shown to downregulate proinflammatory cytokines during phagocytosis of apoptotic cells [Mevorach et al. 1998, Ricklin et al. 2010]. Secondly, it has been suggested that CD11b is a negative regulator of proinflammatory responses mediated by Toll-like receptors (TLRs) [Means & Luster 2010]. One method by which CD11b-TLR crosstalk has been assessed involves stimulation of macrophages in vitro with TLR and CD11b ligands [Duffield 2010]. For example, work undertaken in my supervisor's laboratory has demonstrated that iC3b preligation of CD11b downregulates secretion by WT murine macrophages (but not DCs) of the proinflammatory cytokines IL-6 and CCL5 (also known as RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted) in response to lipopolysaccharide (LPS) stimulation [Ling et al. 2014]. Similar effects have been demonstrated using a variety of CD11b ligands including fibrinogen, with TLR4 stimulation of murine or human macrophages [Wang L. et al. 2010, Cao et al. 2010, Huynh et al. 2012]. Preligation with a synthetic CD11b agonist also inhibited human macrophage secretion of TNF in response to synthetic TLR7/8 ligands [Reed et al. 2013].

In another study, negative regulation of the TLR4-mediated proinflammatory response of macrophages due to CD11b in vitro was controversially attributed to inside-out signalling in the absence of an extracellular ligand [Han et al. 2010]. In vivo administration of LPS in CD11b-/- mice was associated with enhanced proinflammatory immune responses in comparison to CD11b+/- mice [Han et al. 2010]. However, the role of LPS as an extracellular ligand for CD11b [Van Strijp et al. 1993] was not addressed and thus the possibility of an exogenous CD11b ligand could not be excluded. That CD11b ligation by pathogen associated molecular patterns (PAMPs) is potentially important in CD11b-TLR crosstalk was highlighted in a later study in which polyinosinic:polycytidylic acid (poly:IC) was administered in CD11b-/- mice as a TLR3 ligand [Zhou et al. 2013]. Poly:IC was shown to be a novel ligand for CD11b on macrophages, enhancing both TLR3-dependent and TLR3-independent
proinflammatory pathways. Hence the divergent results of previous studies in which PAMP molecules were administered in CD11b-/− mice partly attest the different ligands, immune cell types and tissues assessed (Introduction, Table 3).

As increased glomerular hypercellularity in Cfh-/−.CD11b-/− mice is only partially accounted for by neutrophils and CD68+ macrophages, it appears certain that other cell types were increased in Cfh-/−.CD11b-/− mice. For example, glomerular T cell infiltration was not assessed by immunohistochemistry, and could theoretically have contributed to enhanced functional injury in Cfh-/−.CD11b-/− mice. However, in one recent biopsy study that included patients with MPGN (although not specifically C3 glomerulopathy), glomerular CD31+ neutrophils and, especially, CD68+ macrophages were prominent, whereas CD15+ T cells were much less numerous [Wu et al. 2012]. The role of T cells in models of GN is further discussed in Chapter 4. An additional component of glomerular hypercellularity in Cfh-/−.CD11b-/− mice (excluding those cells readily identified in glomerular capillary loops) may be due to proliferation of intrinsic glomerular cell types including mesangial and endothelial cells.

3.14 Conclusions

3.14.1 CD11b deficiency exacerbates the spontaneous renal phenotype in 8-month old FH-deficient mice (thus negating hypothesis 1).

3.14.2 This may be attributable to the anti-inflammatory effect of iC3b ligation of CD11b on glomerular macrophages, including suppression of TLR-mediated proinflammatory signalling.
4. CHAPTER FOUR: ROLE OF CD11B IN EXPERIMENTALLY INDUCED IMMUNE COMPLEX GLOMERULONEPHRITIS
4.1 Introduction

Induction of immune-mediated glomerular injury in experimental animals through the administration of exogenous antigens has elucidated disease mechanisms in human GN [Becker & Hewitson 2013, Alpers 2014]. One widely used model is nephrotoxic nephritis (NTN), reported as early as 1900 in rabbits [Lindemann 1900] and later as a model of human GN [Masugi & Tomizuka 1931]. In heterologous NTN, administration of an anti-GBM antibody preparation (termed nephrotoxic serum, NTS) produces biphasic renal injury [Hammer & Dixon 1963, Assmann et al. 1985]. The initial phase involves glomerular inflammation secondary to binding of the anti-GBM antibody. As the anti-GBM antibody is raised in a different species (typically rabbit or, as in my experiments, sheep) from the experimental animal (mouse or rat) this is referred to as the heterologous phase. Heterologous phase injury is dependent on glomerular neutrophil influx [Cochrane et al. 1965, Feith et al. 1996] and C3 activation [Hammer & Dixon 1963, Sheerin et al. 1997, Tang et al. 1997, Hebert et al. 1998]. Subsequently, the experimental animal develops an immune response to the heterologous anti-GBM antibody, acting ‘as a planted antigen in the glomerular capillary wall’ [Unanue et al. 1967]. This results in formation along the GBM of immune complexes comprising heterologous anti-GBM antibody and host IgG, and is termed the autologous phase. Progressive glomerular injury in the autologous phase mimics pathological features of human anti-GBM disease (or Goodpasture’s syndrome) [Sheerin et al. 2001]. NTS may also be administered in animals that have been sensitized to heterologous antibody through prior immunization with Ig from the same species used to generate the heterologous anti-GBM antibody. In this case, ‘accelerated’ autologous phase injury occurs due to rapid immune complex formation along the GBM (hence accelerated NTN, ANTN). The ANTN model produces significant renal injury at doses of NTS that would typically be subnephritogenic in unsensitized mice [Nagai et al. 1982]. In this chapter, I present ANTN experiments performed using an established protocol in which mice are sensitized using sheep IgG in adjuvant five days prior to injection of sheep anti-mouse GBM antibody [Pickering et al. 2002].

Studies in inbred strains of experimental mice have shown that the renal phenotype of ANTN is influenced by genetic background, possibly due to differences in T cell responses to antigen. My experiments are performed in C57BL/6 mice, in which ANTN has been extensively characterized as a model of human crescentic GN involving a delayed-type hypersensitivity (DTH)-like nephritogenic immune response directed by the Th1 subset of T helper cells [Holdsworth et al. 1999, Tipping & Holdsworth 2006]. Glomerular inflammation is characterized by infiltration of CD4-positive (CD4+) T cells and CD11b+ [Huang et al. 1997a] or CD68+ [Huang et al. 1997b] macrophages. Gene deletion studies in C57BL/6 mice have
shown the dependence of crescent formation on (1) CD4+ T helper 1 (T_h-1)-associated cytokines including interferon (IFN)-γ [Haas et al. 1995, Kitching et al. 1999] whereas T_h-2-associated interleukin [IL]-4 or IL-10 are protective [Kitching et al. 1998, Kitching et al. 2000]; (2) both αβ- and γδ-T cells [Rosenkranz et al. 2000]; (3) the IL-17-secreting CD4+ T cell (T_h-17) transcription factor, retinoid orphan receptor gamma t (RORγt) [Steinmetz et al. 2011]; and (4) macrophages (based on the requirement for granulocyte-macrophage colony-stimulating factor [GM-CSF] but not G-CSF [Kitching et al. 2002]).

In BALB/c mice, ANTN is instead characterized by a T_h-2-mediated response with predominant neutrophil influx and glomerular thrombosis (with infrequent crescents) [Huang et al. 1997b]. Both C3 activation and autologous Ig appear to contribute significantly to the severity of ANTN in BALB/c mice. By contrast, studies in C3-/− C57BL/6 mice have shown protection due to C3 activation [Sheerin et al. 2001]. Transplant studies indicated that circulating C3, rather than local renal C3 synthesis, mediated this protective effect against severe functional and histological renal injury [Sheerin et al. 2001]. Although a study in FB-deficient (Cfb−/−) mice, which are unable to activate C3 via the AP, showed reduced serum urea and albuminuria compared to WT mice, histological injury was very mild in both groups [Thurman et al. 2012]. The role of autologous Ig during ANTN in C57BL/6 mice is controversial. On one hand, FcRγ−/− mice lacking the gamma chain common to all Fc receptors, which mediate cellular responses to Ig, are protected from acute glomerular injury [Park et al. 1998, Tarzi et al. 2002]. Protection in CD40-deficient mice, which are unable to produce IgG, IgA or IgE due to defective Ig class switching, further indicates that antigen-specific IgM is insufficient to produce renal injury. On the other hand, Ig μ-heavy chain-deficient mice, which do not develop mature B cells or produce antibodies, were not protected in one study [Li et al. 1997], although proteinuria was reduced in another [Rosenkranz et al. 2000].

In this chapter, I present my results using the ANTN model in mice with homozygous deficiency of FH and CD11b. In previous studies, Cfh−/− mice have shown hypersensitivity to both heterologous NTN [Pickering et al. 2006] and ANTN [Pickering et al. 2002]. ANTN in Cfh−/− mice on the 129/Sv×C57BL/6 mixed genetic background was characterized by increased glomerular thrombosis and mortality compared to WT mice [Pickering et al. 2002]. This severe phenotype was abolished in Cfh−/− mice with accompanying deficiency of FB, indicating dependence of renal injury on C3 activation via the AP. Hence the ANTN model is a potentially informative one regarding disease mechanisms in human C3 glomerulopathy.
ANTN has not been reported in CD11b-/- mice. In heterologous NTN, CD11b deficiency was shown to be protective against transient glomerular neutrophil influx (at 2h) and albuminuria (at 4-8h) following a single injection of rabbit nephrotoxic serum (NTS). This protocol was later adapted to induce more severe glomerular injury via two NTS injections followed by LPS administration. CD11b-/- mice were protected from glomerular neutrophil influx (at 4-24h) and glomerular thrombosis and renal failure (at day 4) [Hirahashi et al. 2009]. Similar to conventional heterologous NTN, neutrophil depletion prior to disease induction attenuated glomerular thrombosis and renal failure in WT mice using the adapted model with LPS administration [Hirahashi et al. 2009]. These studies were reminiscent of earlier experiments showing a protective effect due to neutralizing anti-CD11b antibodies in heterologous NTN performed in WT rats [Wu et al. 1993, Mulligan et al. 1993, Wada et al. 1996, De Vriese et al. 1999].
### 4.2 Overview of ANTN experiments

My original research aim was to assess whether coexistent CD11b deficiency influences the renal phenotype of FH-deficient mice following induction of ANTN. Hence this chapter forms a component of my experimental work addressing hypothesis 1, that deficiency of CD11b ameliorates experimental renal disease in FH-deficient mice. I performed three ANTN experiments (Table 7). In the first experiment (ANTN1) I compared $\text{Cfh}^{-/-}\cdot\text{CD11b}^{-/-}$ and $\text{Cfh}^{-/-}$ mice (referred to as ANTN1a). I also compared $\text{CD11b}^{-/-}$ and WT mice in this experiment as control groups for the effect of CD11b deficiency on the ANTN phenotype independent of FH deficiency (ANTN1b). The entire first experiment (ANTN1) was terminated at day 2 on humane grounds following two deaths in the $\text{Cfh}^{-/-}\cdot\text{CD11b}^{-/-}$ group. Renal functional and histological data are presented for ANTN1a in detail below, revealing more severe renal injury in $\text{Cfh}^{-/-}\cdot\text{CD11b}^{-/-}$ compared to $\text{Cfh}^{-/-}$ mice. Owing to the severity of the renal phenotype in $\text{Cfh}^{-/-}\cdot\text{CD11b}^{-/-}$ mice, I did not repeat ANTN1a on humane grounds (in accordance with my animal licence).

ANTN1b is briefly summarized only, as renal failure and histological injury were absent at day 2 in both $\text{CD11b}^{-/-}$ and WT mice. However, a striking and unexpected finding of ANTN1 was of early (day 1) haematuria in both of the CD11b-deficient groups ($\text{Cfh}^{-/-}\cdot\text{CD11b}^{-/-}$ and $\text{CD11b}^{-/-}$ mice). I therefore moved to additional experiments addressing the effect of CD11b deficiency on the ANTN phenotype beyond day 2. In ANTN2 and ANTN3 severe crescentic nephritis was observed in $\text{CD11b}^{-/-}$ mice.

<table>
<thead>
<tr>
<th></th>
<th>Cfh$^{-/-}\cdot$CD11b$^{-/-}$</th>
<th>Cfh$^{-/-}$</th>
<th>CD11b$^{-/-}$</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTN1a (male mice)</td>
<td>n = 8</td>
<td>n = 8</td>
<td></td>
<td></td>
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<tr>
<td>ANTN1b (male mice)</td>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
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<td>ANTN2 (male mice)</td>
<td></td>
<td>n = 5</td>
<td>n = 5</td>
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<tr>
<td>ANTN3 (female mice)</td>
<td></td>
<td>n = 8</td>
<td>n = 10</td>
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</tbody>
</table>
4.3  CD11b deficiency increases early haematuria in ANTN (ANTN1)

ANTN was simultaneously induced in 8-10-week old male mice in four groups (all on a C57BL/6 background): Cfh-/- CD11b-/- (n=8), Cfh-/- (n=8), CD11b-/- (n=8), and WT (n=8). At this age, light microscopic features of MPGN are not yet evident in Cfh-/- mice [Vernon et al. 2011] or Cfh-/- CD11b-/- mice (Chapter 3.12). On day 1 after NTS injection, haematuria developed exclusively in 9 out of 15 assessed CD11b-deficient mice (3 out of 7 assessed Cfh-/- CD11b-/- mice and 6 out of 8 CD11b-/- mice, Fig. 17a,b) and none of the 16 Cfh-/- and WT mice (median dipstick haematuria 1+ in CD11b-deficient mice versus 0 in CD11b-sufficient mice; p = 0.0002). Proteinuria was also increased in CD11b-deficient mice (median dipstick protein 2+ versus 1+; p = 0.0028, Fig. 17a,c).

The experiment was terminated on humane grounds on day 2 because of two deaths in the Cfh-/- CD11b-/- group. Post-mortem examination in Cfh-/- CD11b-/- and CD11b-/- mice revealed injected material (water in oil emulsion of sheep IgG in CFA) within the peritoneal space (i.e. 7 days after i.p. injection). This material was absent in all CD11b-sufficient animals.
Figure 17 ANTN1: day 1 urinalysis

(a) Dipstick urinalysis on day 1 after simultaneous sheep NTS injection in sensitized Cfh-/-.CD11b-/-, Cfh-/-, CD11b-/ and WT mice. (b) Haematuria (0-3+) and (c) proteinuria (0-4+) with comparison of CD11b–deficient mice (CD11b-/-.Cfh-/ and CD11b-/-) to CD11b-sufficient mice (Cfh-/ and WT). Horizontal bars denote median values. **p < 0.01, ***p < 0.001 (Mann-Whitney).
4.4 CD11b deficiency enhances the susceptibility of FH-deficient mice to ANTN (ANTN1a)

In order to address my original research question regarding the effect of CD11b deficiency on experimental renal disease in FH-deficient mice, I compared the data in Cfh−/−.CD11b−/− and Cfh−/− mice (Table 8, ANTN1a). Weights were well-matched between groups at baseline (median weight Cfh−/−.CD11b−/− 20.3g versus Cfh−/− 22.2g; p = 0.8199). 24h after ANTN induction, 3 Cfh−/−.CD11b−/− mice only had developed haematuria (median dipstick haem 0.0 in both groups; p = 0.0769). Proteinuria was increased in Cfh−/−.CD11b−/− compared to Cfh−/− mice (median dipstick protein Cfh−/−.CD11b−/− 2+ versus Cfh−/− 0.5+; p = 0.0289). On day 2, there were two deaths in the Cfh−/−.CD11b−/− group, prompting termination of the experiment. Blood was not obtained from these two Cfh−/−.CD11b−/− mice, and their kidneys were processed for LM but not IF.

Plasma urea levels were higher in surviving Cfh−/−.CD11b−/− compared to Cfh−/− mice (median plasma urea Cfh−/−.CD11b−/− 16.2mmol/L versus Cfh−/− 11.1mmol/L; p = 0.0426; Fig. 18a). Plasma C3 levels were increased ~tenfold compared to those in the respective 2-4-month old groups in Chapter 3 (Fig. 16b). However, there was no difference between groups (median plasma C3 Cfh−/−.CD11b−/− 88.3µg/mL versus Cfh−/− 49.1µg/mL; p = 0.7413, Fig. 18b). In order to assess the murine IgG immune response to sheep NTS, plasma titres of mouse IgG against sheep IgG were assessed. This also revealed no difference between groups (median -log₂ titre Cfh−/−.CD11b−/− 12.6 versus Cfh−/− 13.1; p = 0.2035, Fig. 18c).
Table 8 ANTN1 in Cfh-/-, CD11b-/-, Cfh-/-, CD11b-/- and WT mice

<table>
<thead>
<tr>
<th></th>
<th>ANTN1 (2 days)</th>
<th>1a: Cfh-/-,CD11b-/- versus Cfh-/-</th>
<th>1b: CD11b-/- versus WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td>Cfh-/-,CD11b-/-</td>
<td>Cfh-/-</td>
<td>CD11b-/-</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td><strong>N° of mice assessed</strong></td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Age (weeks)</strong></td>
<td>9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td><strong>Survival to day 2</strong></td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Urine dipstick day 1</strong></td>
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<tr>
<td><strong>Haematuria (+)</strong></td>
<td>0.0 [0.0-3.0]</td>
<td>0.0 [0.0]</td>
<td>1.5** [0.0-3.0]</td>
</tr>
<tr>
<td><strong>Proteinuria (+)</strong></td>
<td>2.0* [1.0-2.0]</td>
<td>0.5 [0.0-2.0]</td>
<td>1.5 [0.0-2.0]</td>
</tr>
<tr>
<td><strong>Plasma results day 2</strong></td>
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<tr>
<td><strong>Schistocytes (%)</strong></td>
<td>4 [0-7]</td>
<td>0.5 [0-11]</td>
<td>0 [0-1]</td>
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<tr>
<td><strong>C3 (μg/mL)</strong></td>
<td>88.3 [11.7-107.6]</td>
<td>49.3 [12.8-160.5]</td>
<td>469.3* [310.2-511.4]</td>
</tr>
<tr>
<td><strong>Glomerular histology</strong></td>
<td></td>
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<tr>
<td>Mean PAS+ quadrants (N°)</td>
<td>0.7* [0.0-1.5]</td>
<td>0.0 [0.0-1.0]</td>
<td>0.1 [0.0-0.4]</td>
</tr>
<tr>
<td>Mean neutrophils (N°)</td>
<td>1.2 [0.1-4.3]</td>
<td>1.4 [0.7-3.0]</td>
<td>1.1 [0.0-1.68]</td>
</tr>
<tr>
<td><strong>Glomerular IF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean fibrin+ quadrants (N°)</td>
<td>0.7 [0.0-1.6]</td>
<td>0.0 [0.0-1.2]</td>
<td>0.2 [0.0-0.5]</td>
</tr>
<tr>
<td>Mean C3 (AFU)</td>
<td>95.0 [81.2-124.8]</td>
<td>93.3 [79.8-117.2]</td>
<td>53.2 [32.9-90.2]</td>
</tr>
<tr>
<td>Mean sheep IgG (AFU)</td>
<td>69.5 [51.4-79.0]</td>
<td>62.7 [38.1-78.6]</td>
<td>75.9 [58.2-118.0]</td>
</tr>
<tr>
<td>Mean mouse IgG (AFU)</td>
<td>45.3 [6.4-95.6]</td>
<td>34.4 [17.0-120.4]</td>
<td>86.1* [35.1-124.1]</td>
</tr>
</tbody>
</table>

Data expressed as median [range]. *p < 0.05, **p < 0.01 (Mann-Whitney test).

§6/8 mice assessed in Cfh-/-,CD11b-/- group.
Figure 18 ANTN1a: day 2 plasma biochemistry

(a) Plasma urea in mmol/L, (b) plasma C3 in μg/mL and (c) -log₂ plasma IgG titre against sheep IgG in surviving mice. Horizontal bars denote median values. *p < 0.05 (Mann-Whitney).
4.5 CD11b deficiency is associated with early glomerular TMA following induction of ANTN in FH-deficient mice (ANTN1a)

On LM, glomerular TMA was observed in Cfh-/-,CD11b-/- mice but not in Cfh-/- mice (Fig. 19a,b). This was characterized by PAS-positive material within glomerular capillary lumens, and thickening of GCWs with occasional double contours of the GBM in the most severely affected Cfh-/-,CD11b-/- mice. Increased glomerular PAS-positive material was seen in Cfh-/-,CD11b-/- compared to Cfh-/- mice (median PAS-positive quadrants Cfh-/-,CD11b-/- 0.7 versus Cfh-/- 0.0; p = 0.0376, Fig. 19c). The number of glomerular neutrophils was no different between groups (median neutrophils Cfh-/-,CD11b-/- 1.2 versus Cfh-/- 1.4; p = 0.6294, Fig. 19d).
Figure 19 ANTN1a: day 2 renal PAS staining

Representative LM images showing PAS-positive material (arrow) within glomerular capillary loops in (a) Cfh-/-, CD11b-/- but not (b) Cfh-/- mice. (c) PAS scoring based on number of glomerular quadrants (0-4) containing PAS-positive material. (d) Glomerular neutrophil count. The two dead Cfh-/-, CD11b-/- mice are highlighted green. Horizontal bars denote median values. *p < 0.05 (Mann-Whitney).
Glomerular TMA in ANTN1a was further assessed by staining for fibrin/fibronogen in the 6 surviving Cfh-/- CD11b-/- mice and the 8 Cfh-/- mice. A broken linear staining pattern due to subendothelial fibrin accumulation with translucent staining of intracapillary thrombogenic material has been reported to be characteristic of glomerular TMA [Lazsik 2015]. Fibrin staining was scored based on the number of glomerular quadrants in which it was identified (Fig. 20a). As a positive control, a renal section from a mouse with an established glomerular TMA phenotype was assessed. Fibrin scores were not significantly increased in the Cfh-/- CD11b-/- mice compared to Cfh-/- mice (median fibrin-positive quadrants Cfh-/- CD11b-/- 0.68 versus Cfh-/- 0.03; p = 0.2488, Fig. 20b). However a high correlation coefficient was seen with PAS scores (r = 0.97; p < 0.0001, Fig. 20c).
Figure 20 ANTN1a: day 2 glomerular fibrin/fibrinogen staining

Direct IF staining using a FITC-labelled antibody to fibrin/fibrinogen, with (a) a scoring system (0-4) based on number of glomerular quadrants with fibrin-positive staining, (b) Fibrin scores in (surviving) Cfh-/-.CD11b-/- and Cfh-/- mice, and (c) correlation of fibrin scores and PAS scores using LM (Spearman, ****p < 0.0001). Horizontal bars denote median values.
4.6 CD11b deficiency does not influence glomerular staining for sheep IgG or mouse IgG or C3 following induction of ANTN in FH-deficient mice (ANTN1a)

Heterologous (sheep) IgG was detected in a linear staining pattern, consistent with accumulation of injected sheep anti-GBM IgG (NTS) along the GBM (Fig. 21a,b). The intensity of glomerular sheep IgG was no different between groups (median MFI Cfh-/_.CD11b-/ 69.5 AFU versus Cfh-/ 62.7 AFU; p = 0.4855, Fig. 21c). The murine IgG immune response was further assessed by renal immunostaining for mouse IgG. This also revealed a linear staining pattern, consistent with formation of immune complexes along the GBM. No difference in intensity of glomerular mouse IgG was seen between groups (median MFI Cfh-/_.CD11b-/ 45.3 AFU versus Cfh-/ 34.4 AFU; p = 0.8352, Fig. 21d-f). A linear C3 staining pattern in both groups also showed no difference in intensity (median MFI Cfh-/_.CD11b-/ 95.0 AFU versus Cfh-/ 93.2 AFU; p = 0.9291, Fig. 21g-i).
Figure 21 ANTN1a: day 2 renal sheep IgG, mouse IgG and C3 staining

Representative images (400x) of glomeruli showing direct IF staining and MFI in AFU for (a-c) sheep IgG and (d-f) mouse IgG and (g-i) in Cfh/-./CD11b/-./ and Cfh/-./ mice. Horizontal bars denote median values.
4.7 CD11b deficiency exacerbates ANTN in FH-sufficient mice (ANTN2)

In ANTN1b, renal failure and histological injury were absent in the CD11b-/- and WT groups (Table 8, ANTN1b). However, due to the finding of a significant increase in haematuria at day 1 in CD11b-/-, I switched my research focus to the question: ‘does CD11b deficiency influence the ANTN phenotype?’ I performed a second ANTN experiment to assess whether CD11b-/- mice were more susceptible than WT mice to development of histological injury beyond day 2. 12-week-old male CD11b-/- (n=5) and WT (n=5) mice were followed for 10 days after NTS injection (Table 9, ANTN2). At baseline, there was no difference in weight between groups (median CD11b-/- 25.7g versus WT 26.5g; p = 0.6950). All mice survived to termination of the experiment on day 10.
Table 9 ANTN2 and ANTN3 in CD11b-/ and WT mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ANTN2 (10 days)</th>
<th>ANTN3 (10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b-/</td>
<td>CD11b-/</td>
<td>CD11b-/</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>N° of mice assessed</td>
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<td>8</td>
</tr>
<tr>
<td>Age (weeks)</td>
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<td>22</td>
</tr>
<tr>
<td>Baseline weight (g)</td>
<td>25.7 [25.2-28.3]</td>
<td>26.5 [25.1-29.0]</td>
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<tr>
<td></td>
<td>22.1 [22.0-24.2]</td>
<td>22.1 [20.5-24.0]</td>
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<tr>
<td>Urine dipstick day 1</td>
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<tr>
<td>Haematuria (+)</td>
<td>2.0* [0.0-3.0]</td>
<td>2.0*** [0-3.0]</td>
</tr>
<tr>
<td>Proteinuria (+)</td>
<td>2.0 [1.0-4.0]</td>
<td>3.0* [1.0-4.0]</td>
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<tr>
<td></td>
<td>1.0 [1.0-2.0]</td>
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<td>Urine dipstick day 10</td>
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<tr>
<td>Haematuria (+)</td>
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<td>0 [0-2.0]</td>
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<tr>
<td>Proteinuria (+)</td>
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<td>2.0 [1.0-4.0]</td>
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<td></td>
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<td>3.0 [0-3.0]</td>
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<tr>
<td>Plasma results day 10</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>38.8** [10.0-39.4]</td>
<td>43.2**** [12.7-43.5]</td>
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<td></td>
<td>6.4 [5.3-8.2]</td>
<td>10.6 [8.6-18.7]</td>
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<td>Haematocrit (%)</td>
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<td>n/a</td>
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<tr>
<td>C3 (μg/mL)</td>
<td>518 [443.2-676.9]</td>
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<td>407.6 [223.7-622.9]</td>
<td>298.1 [224.4-394.2]</td>
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<tr>
<td>IgG (-log₂ titre)</td>
<td>13.6** [12.6-14.6]</td>
<td>15.1 [12.6-17.6]</td>
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<tr>
<td>Glomerular LM</td>
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<tr>
<td>Crescents (%)</td>
<td>22** [16-52]</td>
<td>56** [0-64]</td>
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<tr>
<td></td>
<td>0 [0]</td>
<td>0 [0-6]</td>
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<tr>
<td>Mean PAS+ quadrants (N°)</td>
<td>0.8** [0.4-1.6]</td>
<td>0.4* [0.1-0.6]</td>
</tr>
<tr>
<td></td>
<td>0.1 [0.0-0.3]</td>
<td>0.1 [0.0-1.2]</td>
</tr>
<tr>
<td>Mean neutrophils (N°)</td>
<td>0.6 [0.3-1.2]</td>
<td>0.3* [0.1-0.6]</td>
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<td>0.3 [0.1-0.5]</td>
<td>0.2 [0.0-0.3]</td>
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<tr>
<td>Glomerular IF</td>
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<tr>
<td>Mean fibrin+ quadrants (N°)</td>
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<tr>
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<td>0.04 [0.0-0.7]</td>
<td>n/a</td>
</tr>
<tr>
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<td>5.3 [0.5-8.5]</td>
</tr>
<tr>
<td></td>
<td>3.6 [2.1-3.3]</td>
<td>5.0 [0.8-6.6]</td>
</tr>
<tr>
<td>Mean C3 (AFU)</td>
<td>70.0 [51.6-74.4]</td>
<td>73.8 [44.6-99.7]</td>
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<tr>
<td></td>
<td>58.7 [42.6-71.4]</td>
<td>79.6 [59.6-141.3]</td>
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<tr>
<td>Mean sheep IgG (AFU)</td>
<td>68.1 [58.1-76.1]</td>
<td>50.8 [33.0-85.5]</td>
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<td></td>
<td>87.8 [59.5-96.1]</td>
<td>50.2 [36.3-88.5]</td>
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<tr>
<td>Mean mouse IgG (AFU)</td>
<td>109.3 [87.0-132.5]</td>
<td>70.9 [54.3-95.6]</td>
</tr>
<tr>
<td></td>
<td>97.6 [81.1-149.3]</td>
<td>44.5 [27.9-73.1]</td>
</tr>
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</table>

Data expressed as median [range]. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Mann-Whitney test)
On day 1 of ANTN2, haematuria was again present exclusively in 4 of the 5 CD11b-/– mice (median dipstick CD11b-/– 2+ versus WT 0; \( p = 0.0476 \), Fig. 22a) whereas proteinuria was no different between groups (median dipstick CD11b-/– 2+ versus WT 1.0+; \( p = 0.3651 \), Fig. 22b). At this point, haematuria was still increased in CD11b-/– mice (median dipstick CD11b-/– 3+ versus WT 0.0; \( p = 0.0079 \)) whereas increased proteinuria from baseline in both groups was similar (median dipstick CD11b-/– 3+ versus WT 4+; \( p = 0.4921 \)). Urinary albumin excretion was not assessed in analogous fashion to the spontaneous cohort experiment (Chapter 3), as mice were not suitable for placement in metabolic cages. Day 10 plasma urea levels were increased in CD11b-/– compared to WT mice (median plasma urea CD11b-/– 38.8mmol/L versus WT 6.4mmol/L; \( p = 0.0079 \), Fig. 22c). Plasma titres of mouse IgG against sheep IgG were also increased in CD11b-/– compared to WT mice (median \(-\log_2 \) titre 13.64 versus WT 11.64; \( p = 0.0079 \), Fig. 22d). Plasma C3 levels were no different between groups (median CD11b-/– 517.7µg/mL versus WT 407.6µg/mL; \( p = 0.0952 \), Fig. 22e).
Figure 22 ANTN2: day 1 urine and day 10 plasma biochemistry

Dipstick (a) haematuria and (b) proteinuria on day 1 following NTS injection in CD11b/- and WT mice. Plasma (c) urea levels in mmol/L, (d) -log₂ IgG titre against sheep IgG and (e) C3 levels in µg/mL at sacrifice on day 10. Horizontal bars denote median values. *p < 0.05, **p < 0.01 (Mann-Whitney).
4.8 CD11b deficiency is associated with crescentic nephritis in ANTN (ANTN2)

LM in ANTN2 showed circumferential crescents exclusively in CD11b-/- mice (median crescents CD11b-/- 22% of glomeruli versus WT 0% of glomeruli; \( p = 0.0079 \), Fig. 23a-c). Increased PAS-positive material was present in glomerular capillary loops in CD11b-/- compared to WT mice (median PAS-positive quadrants CD11b-/- 0.8 versus WT 0.1; \( p = 0.0079 \), Fig. 23d). A non-significant trend was seen towards increased glomerular neutrophils in CD11b-/- mice (median neutrophils 0.62 versus WT 0.26, \( p = 0.0873 \), Fig. 23e).
Figure 23 ANTN2: day 10 renal PAS staining

Representative LM images (400x) of glomeruli in (a) CD11b/- mice, showing a circumferential crescent, and (b) WT mice. (c) Glomeruli with crescents as a percentage of total glomeruli, and mean number of (d) glomerular quadrants containing PAS-positive material and (e) glomerular neutrophils. Horizontal bars denote median values. **p < 0.01 (Mann-Whitney).
4.9 CD11b deficiency does not influence glomerular staining for sheep IgG or mouse IgG or C3 in ANTN (ANTN2)

Renal immunostaining showed increased glomerular fibrin/fibrinogen deposition in CD11b−/− compared to WT mice (median fibrin-positive quadrants CD11b−/− 1.6 versus WT 0.0; p = 0.0317). Fibrin and PAS scores were correlated (r = 0.77; p = 0.0125). A linear pattern of sheep IgG deposition was seen in both groups (i.e. 10 days after NTS administration), with a non-significant trend towards reduced intensity in CD11b−/− compared to WT mice (median MFI CD11b−/− 68.1 AFU versus WT 87.8 AFU; p = 0.0952, Fig. 24a-c). By contrast, intensity of linear glomerular mouse IgG staining showed a non-significant increase in CD11b−/− compared to WT mice (median MFI CD11b−/− 70.9 AFU versus WT 44.5 AFU; p = 0.0952, Fig. 24d-f). A granular mesangial pattern of C3 staining was of similar intensity in groups (median MFI CD11b−/− 70.0 AFU versus WT 58.7 AFU; p = 0.3095, Fig. 24g-i).
Figure 24 ANTN2: day 10 glomerular sheep IgG, mouse IgG and C3 staining

Representative images (400×) of glomeruli in CD11b−/− and WT mice showing direct IF staining in and MFI in AFU of (a-c) linear sheep IgG and (d-f) linear mouse IgG and (g-i) granular C3. Horizontal bars denote median values.
4.10 CD11b deficiency is associated with a non-significant increase in glomerular CD68+ macrophages in ANTN (ANTN2)

Glomerular CD68+ macrophages were prominent in the glomeruli and, especially, the tubulointerstitium of some CD11b-/- mice. However, no difference in glomerular CD68+ macrophages was seen between groups overall (median score CD11b-/- 5.9 versus WT 3.6; \( p = 0.1667 \), Fig. 25a-c). This compares to a previous study of day 10 ANTN in C57BL/6 mice showing a CD68+ glomerular cell count of 7 [Masaki et al. 2003].
Figure 25 ANT2: day 10 renal CD68 staining
Representative images (400×) of glomeruli in (a) CD11b−/− and (b) WT mice showing direct IF staining of CD68+ cells (green), with indirect IF staining of PNA (red). (c) Mean number of glomerular CD68+ cells. Horizontal bars denote median values.
4.11 CD11b deficiency exacerbates ANTN in FH-sufficient mice (ANTN3)

In order to validate the findings in CD11b-/- mice, I performed a repeat ANTN experiment in 22-week old female CD11b-/- (n=8) and WT (n=10) mice (Table 9, ANTN3). Baseline weights were no different between groups (median weight 22.1g; p = 0.8108). On day 2, haematuria developed exclusively in 7 out of 8 CD11b-/- mice (median dipstick CD11b-/- 2+ versus WT 0.0; p = 0.0003, Fig. 26a) and proteinuria was also increased in CD11b-/- mice (median dipstick CD11b-/- 3+ versus WT 1.5+; p = 0.0178, Fig. 26b). The experiment was terminated on day 10 with 100% survival in both groups. At this point haematuria was still increased in CD11b-/- mice despite its appearance in 4 out of 10 WT mice (median dipstick CD11b-/- 2.5+ versus WT 0.0; p = 0.0062), whereas no difference was seen in proteinuria between groups (median CD11b-/- 2+ versus WT 3+; p = 0.6589). Haematocrit was reduced, with no difference between groups (median haematocrit CD11b-/- 23% versus WT 28.5%; p = 0.6486). Renal failure was present exclusively in 7 out of 8 CD11b-/- mice (median plasma urea CD11b-/- 43.2mmol/L versus WT 10.6mmol/L; p<0.0001, Fig. 26c). High plasma titres of mouse IgG against sheep IgG were no different between groups (median -log2 titre CD11b-/- 15.14 versus WT 15.39; p = 0.9541, Fig. 26d). Plasma C3 levels were increased in CD11b-/- compared to WT mice (median CD11b-/- 450.7µg/mL versus WT 298.1µg/mL; p = 0.0494, Fig. 26e).
Figure 26 ANTN3: day 2 urine and day 10 plasma biochemistry

(a) Dipstick haematuria and (b) dipstick proteinuria on day 2 following NTS injection in CD11b-/- and WT mice. (c) Plasma urea levels in mmol/L, (d) -log2 plasma IgG titres against sheep IgG and (e) plasma C3 levels in µg/mL on day 10. Horizontal bars denote median values. *p < 0.05, ****p < 0.0001 (Mann-Whitney).
4.12 CD11b deficiency is associated with crescentic nephritis in ANTN (ANTN3)

LM in ANTN3 revealed the presence of circumferential crescents exclusively in 7 out of 8 CD11b/- mice, more extensive than in ANTN2 (median crescents CD11b/- 56% of glomeruli versus WT 0% of glomeruli; \( p = 0.0017 \), Fig. 27a-c). PAS-positive material in glomerular capillary loops was very minor compared to ANTN2, albeit with a slight increase in CD11b/- compared to WT mice (median PAS-positive score CD11b/- 0.38 versus WT 0.08; \( p = 0.0264 \), Fig. 27d). Glomerular neutrophils were very rare, although slightly increased in CD11b/- compared to WT mice (median CD11b/- 0.34 versus WT 0.17; \( p = 0.0308 \), Fig. 27e).
Figure 27 ANTN3: day 10 renal PAS staining

 Representative LM images (400×) of glomeruli in (a) CD11b-/- mice, showing a circumferential crescent and (b) WT mice, with a normal glomerular appearance. (c) Glomeruli with crescents as a percentage of total glomeruli, and mean number of (d) glomerular quadrants containing PAS-positive material and (e) glomerular neutrophils. Horizontal bars denote median values. *p < 0.05, **p < 0.01 (Mann-Whitney).
4.13 CD11b deficiency does not influence renal immunostaining for sheep or mouse IgG in ANTN (ANTN3)

Renal immunostaining showed a linear pattern of glomerular sheep IgG with similar IF intensity in both groups (median MFI $CD11b^{-/-}$ 50.8 AFU versus WT 50.2 AFU; $p = 0.5557$, Fig. 28a-c). Linear mouse IgG was also no different between groups (median $CD11b^{-/-}$ 109.3 AFU versus WT 97.6; $p = 0.3564$, Fig. 28d-f).
**Figure 28 ANTN3: day 10 renal immunostaining for sheep and mouse IgG**

Representative images (400×) of glomeruli in CD11b−/− and WT mice and MFI in AFU for (a-c) sheep IgG and (d-f) mouse IgG. Horizontal bars denote median values.
4.14 Circumferential C3 staining is observed in CD11b/- mice following induction of NTN (ANTN3)

C3 staining in ANTN3 revealed a striking circumferential glomerular C3 staining pattern exclusively in CD11b/- mice (median glomeruli CD11b/- 94% versus WT 0%; $p = 0.0002$, Fig. 29a-c). Excluding circumferential C3, scanty intraglomerular C3 was seen in a granular pattern in both groups, with no difference in IF intensity (median CD11b/- 73.8 versus WT 79.6 AFU; $p = 0.5557$, Fig. 29d).
Figure 29 ANTN3: day 10 renal immunostaining for C3

Representative images (200x) show circumferential glomerular C3 in (a) CD11b-/− but not (b) WT mice. (c) Number (%) of glomeruli with circumferential C3 and (d) MFI of intraglomerular C3 (i.e. excluding circumferential C3 staining). Horizontal bars denote median values. ***p < 0.001 (Mann-Whitney).
4.15 CD11b deficiency does not influence the number of glomerular CD68+ macrophages in ANTN (ANTN3)

Glomerular CD68+ macrophages were no different in number between $CD11b^{-/-}$ and WT mice (median score $CD11b^{-/-}$ 5.3 versus WT 5.0; $p = 0.5576$, Fig. 30a-c).
Figure 30 ANTN3: day 10 renal CD68 staining

Representative images (200×) show glomerular CD68+ cells (in green, with staining of PNA in red) in (a) CD11b−/− and (b) WT mice. (c) Number of glomerular CD68+ cells. Horizontal bars denote median values.
4.16 CD11b deficiency does not enhance the plasma IgG immune response in heterologous NTN

At the conclusion of ANTN2, plasma IgG titres against sheep IgG were higher in CD11b-/- mice compared to WT mice (with increased glomerular mouse IgG seen at day 2 in ANTN1b). In order to assess whether NTS induces a greater IgG immune response in CD11b-/- compared to WT mice, I performed heterologous NTN. Weight-matched, 13-week old male CD11b-/- (n=8) and WT (n=8) mice received a single i.v. tail vein injection of an identical dose of sheep NTS to that used in the ANTN experiments.

Haematuria remained absent and proteinuria did not exceed 1+ over an 8-week observation period, at the end of which 100% survival was observed in both groups. Plasma urea was no different between groups (median plasma urea CD11b-/- 9.3mmol/L versus WT 9.2mmol/L; \( p = 0.6606 \), Fig. 31a). Plasma C3 levels were also no different between groups (median plasma C3 CD11b-/- 157.2µg/mL versus WT 147.0µg/mL; \( p = 0.4245 \), Fig. 31b). Mouse IgG against sheep IgG rose from baseline titres in both groups, but with no difference between CD11b-/- and WT mice at any assessed time-point (\( p > 0.05 \), Fig. 31c).
Figure 31 Heterologous NTN: plasma biochemistry

(a) Plasma urea levels in mmol/L and (b) plasma C3 levels in µg/mL at week 8. Horizontal bars denote median values. (c) -log₂ plasma IgG titres against sheep IgG prior to NTS injection (PRE) and over an 8-week evaluation period. Points represent median values with bars showing range.
4.17 Renal histological injury is absent in heterologous NTN

On LM, features of glomerular injury/inflammation were absent (Fig. 32a,b). PAS-positive material was absent in glomerular capillary loops (median PAS-positive score 0.0 in both groups; $p = 0.2308$, Fig. 32c) and glomerular neutrophils were also virtually absent (median $CD11b^{-/-}$ 0.1 versus WT 0.0; $p = 0.0$, Fig. 32d).
Figure 32 Heterologous NTN: renal PAS staining

Representative LM images (400×) of glomeruli in (a) CD11b−/− mice and (b) WT mice. (c) PAS staining of intracapillary material in glomerular quadrants and (d) glomerular neutrophils. Horizontal bars denote median values.
4.18 CD11b does not influence glomerular deposition of mouse IgG in heterologous NTN

On renal immunostaining, linear-pattern glomerular sheep IgG was of similar intensity in both groups (median MFI CD11b/- 56.74 AFU versus WT 52.82 AFU; \( p = 0.9319 \), Fig. 33a). Intensity of granular C3 staining in the glomerulus was also similar in both groups (median MFI C3 CD11b/- 76.0 AFU versus WT 63.4 AFU; \( p = 0.3231 \), Fig. 33b). Intensity of linear mouse IgG staining was also no different between groups (median MFI CD11b/- 102.04 AFU versus 92.83; \( p = 0.8541 \), Fig. 33c). This was correlated with week 8 plasma IgG titres \( (r = 0.79; p = 0.0006, \text{Fig. 33d}) \), with no separation of the CD11b/- and WT groups.
Figure 33 Heterologous NTN: glomerular scoring based on immunostaining

MFI in AFU of (a) linear sheep IgG, (b) granular C3 and (c) linear mouse IgG. Horizontal bars denote median values. (d) Correlation of MFI of glomerular mouse IgG with plasma IgG titres against sheep IgG (Spearman, ***p < 0.001).
4.19 Enhanced IgG response in CD11b-/− mice correlates with antigen persistence

In order to examine whether the IgG immune response to antigen/CFA was influenced by CD11b deficiency, I performed an alternative immunization protocol. This involved s.c. base-of-tail injection of female CD11b-/− (n=6) and WT (n=6) mice with NP₄-haptenated chicken gammaglobulin (CGG) in CFA. CD11b-/− mice were older than WT mice (22.4 weeks versus 13.7 weeks) and weighed more (median weight CD11b-/− 24.8g versus WT 17.3g; \( p = 0.0022 \), Fig. 34c). 10 days after injection, ulcerating lesions were noted at the injection site in 4 out of 6 CD11b-/− mice, whereas WT mice were unaffected (Fig. 34a,b). The injected material was present in the wounds, which received daily topical antibiotic treatment with 2% fusidic acid cream (Fusiderm, Eva Pharma, Egypt) before healing completely at around weeks 4-5. The clinical course correlated with the anti-hapten IgG response in CD11b-/− mice. Titres of low and high affinity IgG against haptenated CGG were significantly increased in CD11b-/− compared to WT mice at week 2 (median titre CD11b-/− 14.3 versus WT 6.8; \( p = 0.0312 \), Fig. 34d), peaking at week 4 (median titre CD11b-/− 17.2 versus WT 4.6; \( p<0.0001 \)). At week 6, titres had fallen in 5 out of 6 CD11b-/− mice to levels similar to WT (median titre CD11b-/− 7.5 versus WT 5.8; \( p = 0.2656 \)). At sacrifice, plasma C3 levels were also no different between groups (median plasma C3 CD11b-/− 295.3 versus WT 321.6, \( p = 0.8550 \)).
Figure 34 S.c. injection of NP₄-haptenated chicken gammaglobulin in CFA

Representative images obtained 3 weeks after s.c. base-of-tail injection with CGG/CFA showing an ulcerating lesion at the injection site in (a) CD11b⁻/- but not in (b) WT mice. (c) Baseline weights. (d) Anti-hapten IgG titres in both groups prior to CGG/CFA injection (PRE) and over a 6-week evaluation period (points represent median values with bars showing range). **p < 0.01 (Mann-Whitney)
4.20 Discussion

In this chapter, deficiency of CD11b was shown to enhance the susceptibility of FH deficient mice to ANTN. In ANTN1, dipstick proteinuria at day 1 and plasma urea and glomerular thrombosis at day 2 were increased in Cfh-/-, CD11b-/- compared to Cfh-/- mice, with a non-significant trend towards increased day 2 mortality in the Cfh-/-, CD11b-/- group. Due to early termination of the experiment on day 2, the severity of renal injury was only mild to moderate in surviving Cfh-/-, CD11b-/- mice. Nevertheless, these data are consistent with the increased severity of experimental C3 glomerulopathy in the absence of CD11b demonstrated in chapter 3. Hence they provide additional evidence that CD11b deficiency exacerbates experimental renal disease in Cfh-/- mice.

An unexpected finding in ANTN1 was that CD11b deficiency was associated with day 1 haematuria irrespective of the FH genotype. This implies that CD11b may mediate protection against ANTN outside the setting of FH deficiency, and was therefore investigated in two additional ANTN experiments that compared CD11b-/- and WT mice only. Early haematuria was again observed exclusively in CD11b-/- mice, and was associated with the development of crescentic nephritis at day 10. Crescents were more extensive in ANTN3 than ANTN2, possibly related to the use of female mice in ANTN3 [Park et al. 1998]. This protective effect of CD11b in ANTN could again be dependent on ligation of CD11b on glomerular macrophages, as important immune effector cells in the ANTN model. Moreover CD11b ligation specifically due to iC3b appears likely on the basis of (1) generation of abundant C3 activation products using the ANTN model; (2) previous demonstration of increased severity of ANTN in C3-/- mice [Sheerin et al. 2001]; and (3) the finding that deficiency of other CD11b ligands including ICAM-1 [Janssen et al. 1998] and fibrinogen [Drew et al. 2001] is associated with reduced severity of ANTN.

An additional explanation for renal protection due to CD11b involves the role of T cell stimulation in ANTN, which in C57BL/6 mice is conventionally viewed as a model of Th1 CD4+ T cell-directed DTH (and more recently recently of Th17 immune differentiation). An important role for CD11b in suppressing both DTH and Th17-mediated immunity has previously been demonstrated using a model of low-dose oral antigen (ovalbumin) exposure followed by high-dose immunization with antigen in CFA [Ehirchiou et al. 2007]. Whereas ovalbumin-specific DTH following the high-dose immunization was no different in CD11b-/- and WT PBS-fed mice, it was increased in CD11b-/- compared to WT antigen-fed mice. Passive transfer of naive WT splenic APCs in CD11b-/- mice prior to antigen feeding was effective in re-establishing orally induced peripheral immune tolerance (i.e. strongly reducing antigen-specific DTH). After completion of the experiment, single cell suspensions from
draining lymph nodes (LNs) were restimulated *in vitro* with ovalbumin, revealing enhanced production of IL-2, IL-6 and IL-17 in LNs from *CD11b/-* compared to WT mice (only under antigen feeding conditions). The *CD11b/-* LN CD4+ T cell population was also shown to be enriched for the T_{H}17 subset. IL-17 administration in WT mice was shown to abrogate ovalbumin-specific oral tolerance. On this basis, the authors attributed enhanced DTH in *CD11b/-* mice to the suppressive effects of CD11b on T_{H}17 immune differentiation. Hence susceptibility of *CD11b/-* mice to crescentic ANTN in the present study may also be attributable to enhanced DTH and T_{H}17-mediated immunity.

Secondly, absence of renal injury 8 weeks after induction of heterologous NTN in unsensitized *CD11b/-* mice suggests a critical role of the sensitization using CFA in the ANTN model. One possible explanation for this is the crosstalk between CD11b and CFA-induced proinflammatory signalling. CFA is a recognised TLR agonist [Billiau & Matthys 2001, Lim 2003] and has also been shown to induce a CD11b+ splenic cell population with (controversial) immunosuppressive effects on T-cell-mediated immunity [Matthys et al. 2001, Wang Z. et al. 2010]. Deficiency of CD11b could therefore result in a more severe nephritogenic immune response in CFA-sensitized mice via either of these mechanisms. In particular, ANTN is one of several experimental models that have highlighted the deleterious role of TLRs in GN [Couser 2012]. The importance of TLR stimulation at sensitization was demonstrated in an adapted ANTN protocol in which aluminium hydroxide was substituted for CFA as the immune adjuvant. A synthetic TLR2 ligand [Brown et al. 2006] or LPS [Giorgini et al. 2010] was co-administered with sheep IgG/adjuvant at sensitization. Following NTS injection, severe crescentic nephritis developed, from which mice with genetic deficiency of the corresponding TLRs were protected.

The antigen-specific IgG immune response was not different between *CD11b/-* and WT mice in the heterologous NTN model, which does not use immune adjuvant. I investigated the effects of CFA using a CFA-based protocol in which sheep IgG was substituted as the T-dependent antigen by haptenated CGG. Following s.c. base-of-tail injection of CGG/CFA, local inflammation and ulceration developed exclusively in *CD11b/-* mice, with injected material persistently present in the wounds. This was reminiscent of the retained intraperitoneal sheep IgG/CFA observed exclusively in CD11b-deficient mice in ANTN1. Inflammation at local injection sites is a recognised complication of CFA administration [Billiau & Matthys 2001]. Infiltration of s.c. CFA injection sites by CD11b+ leukocytes has also previously been described [Wang Z. et al. 2010], with possible implications for antigen/CFA clearance in *CD11b/-* mice. Here, persistent s.c. CGG/CFA and local inflammation were temporally associated with increased anti-hapten IgG titres in affected
CD11b/- mice at 2-4 weeks. Hence loss of CD11b-dependent clearance of antigen/CFA, potentiating the nephritogenic immune response, may be an additional mechanism leading to increased severity in ANTN in CD11b/- mice. This impaired antigen clearance in the setting of CD11b deficiency could further exacerbate TLR-mediated inflammation. In addition, generation of complement activation products by APCs and T cells during antigen presentation within the peritoneum may become deranged in the setting of impaired antigen clearance, effectively priming a proinflammatory immune response that is then targeted to the GBM following the administration of NTS. This mechanism is compatible with recent evidence showing a role for immune cell-derived complement in directing the differentiation of T helper 1 (Th1) and Th17 cells, and potentiating TLR-driven APC maturation and the secretion of pro-inflammatory cytokines [Kolev et al. 2014].

4.21 Conclusions

4.21.1 CD11b deficiency exacerbates ANTN in FH-deficient mice. This corroborates the findings of Chapter 3 that CD11b is protective in experimental renal disease in FH-deficient mice (thus negating hypothesis 1).

4.21.2 CD11b deficiency exacerbates ANTN. This is consistent with CD11b-mediated suppression of potentially important mechanisms in GN including DTH, Th17-mediated immunity and TLR-mediated proinflammatory signalling.
CHAPTER FIVE: EFFECT OF AN ENGINEERED NOVEL MINI-FH PROTEIN ON THE SPONTANEOUS PHENOTYPE OF FACTOR H-DEFICIENT MICE
5.1 Introduction

Cfh-/- mice demonstrate low plasma C3 levels and a linear pattern of C3 staining in the glomerulus [Pickering 02]. Murine experimental C3 glomerulopathy thus provides a useful model for studying the pathogenetic mechanisms and response to therapeutics in DDD. Previously, i.p. injection of 0.5mg of plasma-derived human FH in Cfh-/- mice was shown to increase plasma C3 levels at 2h, reaching WT plasma C3 levels at 24h [Fakhouri et al. 2010]. This was accompanied by reduced intensity and extent of linear C3 staining, similar to the effect reported following administration of murine FH in Cfh-/- mice [Paixão-Cavalcante et al. 2009]. Accordingly, human recombinant FH has been proposed as a logical treatment for establishing physiological AP control and halting disease progression in patients with C3 glomerulopathy. However, due to the size and complexity of the FH protein, production of therapeutic quantities represents a significant challenge [Sharma & Pangburn 1994, Sanchez-Corral et al. 2002, Buttner-Mainik et al. 2011]. For this reason a number of smaller human recombinant FH proteins expressing only key functional domains are now entering preclinical evaluation. Two human recombinant proteins comprising N-terminal complement regulatory SCR domains 1-4 and the C-terminal surface recognition SCR domains 19-20 have been studied in vitro [Hebecker et al. 2013, Schmidt et al. 2013]. It was proposed that C-terminal binding to surface-bound iC3b and C3dg was in fact superior to that of full-length FH, effectively targeting the novel reagent to ‘sites of high opsonic turnover’ [Schmidt et al. 2013]. A putative advantage of this class of recombinant proteins may therefore be surface targeting, whereby some of the systemic effects (specifically, increased risk of infection) associated with therapeutic complement inhibition are circumvented [Thurman et al. 2013].

Administration of murine and human FH in Cfh-/- mice was also associated previously with the appearance at 24h of tubulointerstitial C3. The presence of tubulointerstitial C3 in WT mice [Park et al. 2001, Thurman et al. 2003] and C4-deficient mice [Quigg et al. 1998], but not Cfb-/- mice [Lenderink et al. 2007], indicates a requirement for an intact AP. As discussed in Chapter 1, in vitro studies indicate that the apical surface of proximal renal tubular cells provides an AP-activating surface (section 1.2.8.2). It has also been suggested based on studies in sub-total nephrectomized rats that tubulointerstitial C3 deposition occurs due to accumulation of ammonia in the renal tubules, providing an AP-activating surface [Nath et al. 1985]. Studies in mice with deficiency of Crry indicate that this rodent-specific regulatory protein normally provides partial protection against C3 activation via the AP on the PTEC surface. IF microscopy of WT murine renal tissue [Thurman et al. 2006a, Miao et al. 2014] indicates that Crry is the only membrane-bound complement regulatory protein expressed on PTEC, where it is localized to the basolateral surface (with a similar finding for MCP on human PTEC [Ichida et al. 1994]). Reconstitution of Cfb-/- mice with purified murine
FB led to increased deposition of C3 along the proximal tubule in those with accompanying Crry deficiency compared to those without [Renner et al. 2010]. PTEC-specific deficiency of Crry was also associated with increased C3 on the basolateral PTEC surface in unmanipulated mice [Miao et al. 2014]. Finally, in the ischaemia/reperfusion (I/R) model, enhanced C3 deposition on the basolateral surface of PTEC and associated tubular injury are AP-dependent [Thurman et al. 2003, Thurman et al. 2006b, Miwa et al. 2013]. A study in WT mice concluded that loss of Crry from the basolateral tubular surface as a result of I/R injury ‘permits uncontrolled activation of the alternative pathway on the surface of tubular epithelial cells’ [Thurman et al. 2006a]. Mice with PTEC-specific Crry deficiency also show increased severity of tubular injury using the I/R model [Miao et al. 2014].

Paradoxically, given uncontrolled C3 activation via the AP, Cfh/-/- mice lack tubulointerstitial C3. The appearance of tubulointerstitial C3 24h after murine FH administration in Cfh/-/- mice has thus been attributed to a requirement for ‘the presence of intact circulating C3’ [Paixão-Cavalcante et al. 2009]. Similarly, transplantation of kidneys from Cfh/-/- mice into WT recipients resulted in the appearance of discontinuous tubular C3 staining within 30 minutes, and more prominent tubular and Bowman’s capsule staining at 5 weeks [Alexander et al. 2007]. The authors concluded that ‘activation of C3 onto the basolateral aspects of tubules requires an intact systemic alternative pathway, including its main regulator, Cfh’ [Alexander et al. 2007]. The authors of a study in which tubulointerstitial C3 was absent in Cfh/-/- mice even after induction of I/R injury concluded that ‘uncontrolled fluid phase activation of C3 in these mice does not leave sufficient intact C3 to support complement activation on the TEC surface after I/R’ [Renner et al. 2011]. The interaction of FH with PTEC was assessed in WT mice through administration prior to I/R induction of a recombinant murine FH protein fragment comprising SCR domains 19-20 as a ‘dominant negative inhibitor of the native protein’ [Coleman et al. 2008]. Although this led to increased tubular injury, indicating that ‘factor H is important for limiting injury after renal I/R’, C3 staining was not reported [Coleman et al. 2008].

In this chapter, I describe the administration in Cfh/-/- mice of a novel engineered protein comprising SCR domains 1-5 and 18-20 of human FH, hereafter referred to as ‘mini-FH’. My experimental aim was to assess whether the C-terminal surface recognition domains of FH are necessary for therapeutic effect in experimental C3 glomerulopathy. Hence these experiments address hypothesis 2, that FH protects the GBM from surface phase C3 activation via the AP. I show that mini-FH reduces glomerular C3 reactivity in Cfh/-/- mice similar to full-length, serum-derived human FH (hFH), with partial restoration of plasma C3 levels. This effect was not reproduced using a recombinant human FH protein comprising only SCR domain 1-5 (FH1-5). These results indicate a plausible therapeutic role for mini-FH
in patients with DDD and other disorders of AP regulation. A second observation was that, unlike plasma-derived FH, administration of mini-FH did not lead to the appearance of tubulointerstitial C3 staining at 24h. As the presence of tubulointerstitial C3 is potentially informative regarding surface phase C3 activation via the AP, an additional in vivo experiment was performed to address its relationship to C3 accumulation along the GBM in experimental C3 glomerulopathy. Here, my experimental aim was to assess whether glomerular and tubulointerstitial C3 staining could be triggered concurrently in FH-deficient mice. I injected F1-containing mouse serum in Cfh−/−.Cfi−/− mice, which has previously been shown to result in the appearance of linear C3 staining in the glomerulus [Rose et al. 2008]. As this was not associated with the appearance of tubulointerstitial C3 staining at any of the assessed time-points, it appears that C3 accumulation occurs via distinct mechanisms along the GBM and on the renal tubular surface.
5.2 Overview of the mini-FH experiments

Mini-FH was assessed for therapeutic effect following i.p. injection in Cfh−/− mice. In the first experiment (Mini-FH Exp. 1, Table 10), comparison was made with a 3nmol (0.50mg) dose of hFH, as this was the dose used to positive effect in the previous study in Cfh−/− mice [Fakhouri et al. 2010]. A 6nmol dose (0.35mg) of mini-FH was assessed, double the molar dose of hFH, in order to allow for potentially increased clearance of the smaller, recombinant protein from plasma. Plasma C3 levels were assessed at 2h, 6h and 24h and glomerular C3 staining at 24h, as in the earlier study.

In the second experiment (Mini-FH Exp. 2), the effect of mini-FH on renal C3 staining in Cfh−/− was assessed 6h after administration (timed to coincide with the maximal effect of mini-FH on plasma C3 in the first experiment). In order to assess whether mini-FH produces a dose-dependent effect on plasma C3 levels and renal C3 staining, two doses of mini-FH were assessed, a 6nmol dose identical to that used for the first experiment, and a double (12nmol) dose. As in the first experiment, 3nmol hFH was used as a positive control. A negative control group comprising PBS-injected mice was also included in this experiment. Additional mice were injected with 12nmol FH1-5. As this protein lacks the surface recognition SCR domains 18-20 present on both mini-FH and hFH, my experimental aim was to assess the role of these domains specifically on glomerular C3 staining.

In the third experiment (Mini-FH Exp. 3), the effect of split doses of mini-FH at 6-hourly intervals was assessed over a 24-hour period. Limited availability of the mini-FH reagent meant that only 3nmol mini-FH could be used per injection. Mice received four 6-hourly injections of 3nmol mini-FH, equimolar (3nmol) FH1-5 or PBS. Additional mice received a single injection of 3nmol hFH, used as in the previous experiments as a positive control, or a single equimolar (3nmol) dose of mini-FH or FH1-5, or PBS. In the four-injections groups, the last dose was administered at the 18h time-point, with termination of the experiment in all mice at 24h.
Table 10 Doses of human FH reagents used in mini-FH experiments 1-3

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Protein concentration (mg/mL)</th>
<th>MW (kDa)</th>
<th>Molarity (μM)</th>
<th>Total protein injected (μg)</th>
<th>Moles per injection (x10^-9)</th>
<th>Total volume in sterile PBS (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1 (24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hFH</td>
<td>11.00</td>
<td>155</td>
<td>71</td>
<td>500</td>
<td>3</td>
<td>1000</td>
</tr>
<tr>
<td>mini-FH</td>
<td>0.65</td>
<td>59</td>
<td>11</td>
<td>350</td>
<td>6</td>
<td>1000</td>
</tr>
<tr>
<td>Exp. 2 (6h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hFH</td>
<td>2.88</td>
<td>155</td>
<td>18.6</td>
<td>465 or 930</td>
<td>3</td>
<td>900</td>
</tr>
<tr>
<td>mini-FH</td>
<td>0.88</td>
<td>59</td>
<td>14.9</td>
<td>355 or 710</td>
<td>6 or 12</td>
<td>900</td>
</tr>
<tr>
<td>FH1-5</td>
<td>1.00</td>
<td>36</td>
<td>28.2</td>
<td>430</td>
<td>12</td>
<td>900</td>
</tr>
<tr>
<td>Exp. 3 (24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hFH</td>
<td>1.60</td>
<td>155</td>
<td>10.3</td>
<td>465</td>
<td>3</td>
<td>340 (x1 dose) or 200 (x4 doses)</td>
</tr>
<tr>
<td>mini-FH</td>
<td>1.00</td>
<td>59</td>
<td>17.0</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH1-5</td>
<td>1.75</td>
<td>36</td>
<td>48.6</td>
<td>110</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3 6nmol mini-FH transiently increases plasma C3 levels in Cfh-/- mice (Mini-FH Exp. 1)

Mini-FH Exp. 1 was terminated 24h after administration of 6nmol mini-FH (n=4, 2M/2F) or 3nmol hFH (n=4, 2M/2F) in weight-matched, 8-12-week old mice. Plasma C3 levels rose in mice injected with mini-FH, peaking at 6h (median plasma C3 59.2 μg/mL, Fig. 35). However, a much greater rise was observed in hFH-injected mice, peaking at 24h (median plasma C3 254.5μg/mL versus 42.3 μg/mL for mini-FH; p = 0.0020).
**Figure 35 Mini-FH Exp.1: 24h plasma C3 levels**

Plasma C3 levels in μg/mL before (PRE) and after (at 2h, 6h and 24h) injection of 6nmol mini-FH or 3nmol hFH in Cfh−/− mice. Horizontal bars represent median values. **p < 0.01 (t-test).**
5.4 6nmol mini-FH alters the glomerular C3 pattern at 24h (Mini-FH Exp. 1)

In the glomerulus, a mixed granular mesangial and linear C3 staining pattern was seen at 24h in mice injected with 6nmol mini-FH (Fig. 36a), whereas the pattern in 3nmol hFH-injected mice was predominantly mesangial (Fig. 36b). In the tubulointerstitium, prominent C3 staining was observed within the renal cortex and outer medulla in 3nmol hFH-injected mice, whereas minimal tubulointerstitial C3 was seen in mice injected with 6nmol mini-FH.
Figure 36 Mini-FH Exp.1: 24h glomerular C3 staining

Representative images (400×) following direct IF staining for C3 24h after administration in Cfh−/− mice of (a) 6nmol mini-FH, with a mixed linear and mesangial C3, and (b) 3nmol hFH, showing a granular mesangial C3 staining pattern with some tubulointerstitial C3. (c) A pure linear C3 pattern is shown from a naïve Cfh−/− mouse for comparison.
5.5 Mini-FH is rapidly cleared from plasma after 2h (Mini-FH Exp. 2)

Mini-FH Exp. 2 was terminated 6h after administration of 6nmol mini-FH (n=3; 2M/1F) or 12nmol mini-FH (n=3; 2M/1F), 3nmol hFH (n=3; 2M/1F), 12nmol FH1-5 (n=3, 2M/1F) or PBS (n=3; 2M/1F) in weight-matched, 9-10-week old Cfh/- mice. An ELISA was developed by Dr Kevin Marchbank to detect the injected proteins in plasma. This assay did not give a signal in the PBS-injected animals indicating that it was not detecting mouse FHR proteins, which are still present in Cfh/- mice. Using this assay, mini-FH was detectable 2h after injection (Fig. 37). After a single 12nmol dose of mini-FH, the 2h median plasma level was 203.1µg/mL. After a single 12nmol dose of FH1-5, the 2h median plasma level was 189.1µg/mL. At 6h, plasma levels had fallen for the recombinant proteins, with only hFH detectable.
Figure 37 Plasma levels of injected proteins

FH ELISA performed by Dr K. Marchbank using OX24 antibody shows plasma levels of injected proteins in μg/mL 2h and 6h after injection of 6nmol or 12nmol mini-FH, 3nmol hFH, 12nmol FH1-5 or PBS in Cfh/- mice. Horizontal bars denote median values. *p < 0.05, **p < 0.01, ****p < 0.0001 versus PBS (one-way ANOVA/Bonferroni).
5.6 6-12nmol mini-FH increases plasma C3 levels at 2-6h (Mini-FH Exp. 2)

Compared to PBS, mini-FH produced a significant rise in plasma C3 levels at 2h at both the 6nmol dose (median plasma C3 mini-FH 41.2µg/mL versus PBS 9.9µg/mL; \( p = 0.0360 \), Fig. 38) and the 12nmol dose (median plasma C3 mini-FH 41.5µg/mL; \( p = 0.0369 \) versus PBS). This effect at 2h was comparable to that of 3nmol hFH (median plasma C3 47.8µg/mL; \( p = 0.0128 \) versus PBS). By contrast, 12nmol FH1-5-treated animals did not have significantly elevated plasma C3 levels at the 2h time-point (median plasma C3 29.6µg/mL; \( p = 0.2044 \) versus PBS). At 6h, plasma C3 levels were significantly increased compared to PBS for the 12nmol dose of mini-FH (median plasma C3 mini-FH 88.5µg/mL versus PBS 33.4; \( p = 0.0268 \)) and 3nmol hFH (median plasma C3 139.3µg/mL; \( p = 0.0005 \) versus PBS) only.
Figure 38 Mini-FH Exp.2: plasma C3 levels

Plasma C3 levels in μg/mL before and after injection of 6nmol or 12nmol mini-FH, 3nmol hFH, 12nmol FH1-5 or PBS in Cfh−/− mice. Horizontal bars denote median values. *p < 0.05, **p < 0.01 versus PBS (one-way ANOVA/Bonferroni).
5.7 6-12nmol mini-FH reduces glomerular C3 staining at 6h (Mini-FH Exp. 2)

Compared to PBS, mini-FH reduced the intensity of linear C3 staining in the glomerulus at both the 6nmol dose (median MFI of mini-FH 62.4 AFU versus PBS 110.3 AFU; \( p = 0.0174 \)) and 12nmol dose (median MFI 58.1 AFU versus PBS 110.3 AFU; \( p = 0.0098 \), Fig. 39a,d,e). Intensity of glomerular C3 staining was also reduced in mice injected with 3nmol hFH (median MFI 73.5 AFU versus PBS 110.3 AFU; \( p = 0.0974 \), Fig. 39b). That this did not reach statistical significance in comparison to PBS is attributable to the presence of some mesangial C3 staining only in mice injected with hFH, increasing the total glomerular C3 intensity in comparison to the mini-FH-injected mice. Slightly increased glomerular C3 intensity was observed for PBS and especially 12nmol FH1-5 (Fig. 39c) compared to a naïve Cfh-/- mouse. This likely reflects glomerular accumulation of increased plasma C3 due to acute phase response. Tubulointerstitial C3 was absent at 6h in all mice.
Figure 39 Mini-FH Exp.2: 24h renal cortical C3 staining

Representative images (400x) of direct IF staining for C3 in Cfh-/- mice 6h after administration of (a) 12nmol mini-FH, (b) 3nmol hFH, (c) 12nmol FH1-5, (d) PBS. (e) MFI in AFU for glomerular C3 in Cfh-/- mice at 6h. Horizontal bars denote median values. *p < 0.05, **p < 0.01 versus PBS (one-way ANOVA/Bonferroni).
5.8 hFH and mini-FH are detected in the glomerulus at 6h in Cfh-/- mice (Mini-FH Exp. 2)

Glomerular deposition of injected human FH reagents was assessed. In a previous study in Cfh-/- mice injected with full-length human FH, goat anti-human FH antisera (Quidel) used to detect the injected human FH protein were found to cross-react with murine FHR proteins in a linear staining pattern [Paixão-Cavalcante et al. 2009]. Accordingly, I developed an alternative FH staining protocol using OX24, a mouse monoclonal IgG1 anti-human FH antibody that recognises an epitope within human FH SCR 5 [Sim et al. 1983, Jokiranta et al. 1996]. Dr Marchbank has demonstrated that OX24 recognises both the mini-FH and FH1-5 proteins *in vitro* (capture antibody for FH ELISA, Fig. 37, and also western blot).

6h after administration of 12nmol mini-FH, this reagent was faintly detected in a linear staining pattern in the glomerulus using biotinylated OX24 followed by phycoerythrin (PE)-labelled streptavidin (Fig. 40a). A much brighter linear pattern was seen following injection of 3nmol hFH (Fig. 40b), with absence of linear staining using the PE-labelled streptavidin alone (Fig. 40d). Linear staining was also absent in mice injected with 12nmol FH1-5 (Fig. 40c).
Figure 40 Mini-FH Exp.2: OX24 staining with PE secondary

Representative images (400×) of direct IF staining using biotinylated OX24 and PE-conjugated streptavidin 6h after injection of human proteins. Shown are (a) 12nmol mini-FH, (b) 3nmol hFH, and (c) 12nmol FH1-5. (d) PE-labelled streptavidin without primary antibody in a 3nmol hFH-injected mouse.
A similar profile of glomerular staining was obtained using OX24 with a different secondary antibody (AF555-labelled streptavidin). This showed a faint linear appearance for 12nmol mini-FH, bright linear staining for 3nmol hFH, and absent glomerular staining for 12nmol FH1-5 (Fig. 41a-c). By contrast, when OX24 was used with a third secondary antibody (AF488-labelled avidin), faint linear staining only was observed for 3nmol hFH, with absent glomerular staining for 12nmol mini-FH (Fig. 42a,b).

These data are consistent with accumulation of mini-FH along the GBM and its persistence 6h after administration in Cfh-/- mice. However, the binding of OX24 to the glomeruli is reduced after mini-FH administration in comparison to hFH despite a fourfold higher molar dose of mini-FH. This suggests that binding of mini-FH to the GBM and/or GBM-bound C3 fragments may be less efficient than that of hFH. Glomerular staining was absent for mini-FH using OX24 with the AF488-labelled secondary antibody. This is likely to be due to the AF488-labelled secondary antibody, which also produced comparatively faint linear staining for hFH compared to the other secondary antibodies tested. As OX24 was unable to detect 12nmol FH1-5 using the PE-labelled or AF555-labelled secondary antibodies, both of which detected 12nmol mini-FH and 3nmol hFH, this is attributed to absence of FH1-5 within the glomerulus 6h after injection.
**Figure 41 Mini-FH Exp.2: OX24 staining with AF555 secondary**

Representative images (400×) of direct IF staining using biotinylated OX24 and AF555-conjugated streptavidin 6h after injection of human proteins. Shown are (a) 12nmol mini-FH, (b) 3nmol hFH, and (c) 12nmol FH1-5.
Figure 42 Mini-FH Exp.2: OX24 staining with AF488 secondary

Representative images (400×) of direct IF staining using biotinylated OX24 and AF488-conjugated streptavidin (green) 6h after injection of human proteins. Shown are (a) 12nmol mini-FH and (b) 3nmol hFH.
5.9 3nmol mini-FH is detectable in plasma 2-6h after administration in Cfh-/− mice (Mini-FH Exp. 3)

Mini-FH Exp. 3 was terminated at 24h, following four 6-hourly injections 3nmol mini-FH (n=4), 3nmol FH1-5 (n=4) or PBS (n=4) in 8-week old female mice. In the single injection group, 13-week old female mice received 3nmol mini-FH (n=4), 3nmol hFH (n=1), 3nmol FH1-5 (n=4) or PBS (n=4). An FH western blot of mouse plasma using polyclonal anti-FH antibody (Quidel) reveals the presence of the injected proteins in plasma 2h after a single injection (Fig. 43a). 24h after a single injection of 3nmol hFH, this protein was still detectable in plasma (Fig. 43b). The recombinant proteins mini-FH and FH1-5 were detectable in plasma at the 24h time-point in mice that had received four 6-hourly 3nmol doses (i.e. six hours after the last of the four doses, which was administered at the 18 time-point).
Figure 43 Mini-FH Exp.3: detection of injected human FH reagents in mouse plasma

FH western blot using 1μL Cfh-/- mouse plasma under non-reducing conditions, with detection of injected reagents using primary anti-human FH antisera (Quidel). Predicted MWs are 155kDa (hFH), 59kDa (mini-FH) and 36kDa (FH1-5). (a) 2h after a single 3nmol dose of hFH (n=1), mini-FH (n=4) or FH1-5 (n=4). (b) 24h after a single 3nmol dose of hFH (n=1) or four 6-hourly 3nmol doses of mini-FH (n=4) or FH1-5 (n=4), with longer exposure of the film than for (a).
5.10 3nmol mini-FH has little or no effect on plasma C3 at 2-6h (Mini-FH Exp. 3)

Notwithstanding a rise in plasma C3 levels from baseline in all mice injected with mini-FH, FH1-5 and PBS, no difference was seen between these proteins at any of the assessed time-points (with the analysis at 24h time-point divided between one and four injection groups, Fig. 44). This is likely attributable to the lower dose of mini-FH (3nmol) used for this experiment. A marked rise was seen in plasma C3 levels in mice injected with 3nmol hFH, peaking at 24h as in Mini-FH Exp. 1.
Figure 44 Mini-FH Exp. 3: plasma C3 levels

Plasma C3 levels in μg/mL before and at 2 h and 6h after a single injection of 3nmol mini-FH, 3nmol hFH, 3nmol FH1-5 or PBS in Cfh−/− mice, and at 24h after a single injection or four 6-hourly injections of 3nmol mini-FH, 3nmol FH1-5 or PBS. Horizontal bars denote median values. Arrows indicate injections.
5.11 Four 6-hourly injections of 3nmol mini-FH reduce glomerular C3 staining at 24h (Mini-FH Exp. 3)

In the glomerulus, the intensity of linear C3 staining was reduced in mice that received four injections of 3nmol mini-FH compared to four doses of PBS (median MFI mini-FH 65.5 AFU versus PBS 138.9 AFU; \( p < 0.0001 \); Fig. 45a,d,e). Some mesangial C3 staining was also evident in the mini-FH-injected mice, with a similar glomerular appearance in mice that received a single injection of 3nmol hFH (Fig. 45b). Four injections of 3nmol FH1-5 produced no effect, with a bright linear C3 pattern identical to four injections of PBS (Fig. 45c). Neither 3nmol mini-FH nor 3nmol FH1-5 administered as a single dose produced any effect on linear C3 staining at 24h in comparison to PBS.
Figure 45 Mini-FH Exp.3: 24h glomerular C3 staining

Representative images (400×) of direct IF staining for C3 24h after (a) four 6-hourly injections of 3nmol mini-FH, (b) a single injection of 3nmol hFH, or four 6-hourly injections of (c) 3nmol FH1-5 or (d) PBS. (e) MFI of glomerular C3 staining is reduced following four 6-hourly injections of 3nmol mini-FH, but not 3nmol FH1-5, compared to PBS. Horizontal bars denote median values. ****p < 0.0001 versus PBS (one-way ANOVA/Bonferroni).
5.12 Mini-FH does not affect glomerular C3d staining at 24h in Cfh-/- mice (Mini-FH Exp. 3)

The linear staining pattern for C3d was no different following four injections of 3nmol mini-FH, 3nmol FH1-5 or PBS (Fig. 46a-c). This indicates that the reduction in linear C3 staining and appearance of mesangial staining following four injections of mini-FH may be due to cleavage of GBM-bound iC3b. This could release C3c to the mesangium (detected using the anti-C3 antibody but not the anti-C3d antibody), leaving C3d attached to the GBM, as surmised previously at 24h using human FH [Fakhouri et al. 2010].
Figure 46 Mini-FH Exp.3: 24h glomerular C3d staining

Representative images (400×) at 24h of indirect IF staining of the glomerulus using biotinylated anti-C3d antibody and PE-labelled streptavidin after four 6-hourly injections in Cfh/- mice of (a) 3nmol mini-FH, (b) 3nmol FH1-5, or (c) PBS.
5.13 24h plasma C3 levels are correlated with tubulointerstitial C3 scores (Mini-FH Exp. 3)

As noted in Mini-FH experiment 1, prominent 24h tubulointerstitial C3 staining was seen only in the 3nmol hFH-injected mouse. Tubulointerstitial C3 staining in all mice was assigned a score between 0 (negative C3 staining) and 3 (maximal C3 staining, Fig. 47). In contrast to the mouse injected with a single dose of 3nmol hFH (24h tubulointerstitial C3 score of 3), mice receiving a single injection of 3nmol mini-FH, 3nmol FH1-5 or PBS all had 24h tubulointerstitial C3 scores of 0. Mice receiving four 6-hourly injections of 3nmol mini-FH, 3nmol FH1-5 or PBS had 24h tubulointerstitial C3 scores of 0 or 1. Correlation of 24h plasma C3 levels and tubulointerstitial C3 scores shows the distinct effect of a single 3nmol dose of hFH on both these parameters compared to four doses of 3nmol mini-FH, 3nmol FH1-5 or PBS (r = 0.84, p = 0.0004, Fig. 48).
Figure 47 Mini-FH Exp.3: scoring system used for 24h tubulointerstitial C3 staining
Tubulointerstitial C3 scoring (0-3) is based on direct IF staining for C3, as represented by low original magnification (200×) images.
Correlation of 24h plasma C3 levels and tubulointerstitial C3 scores enables separation of the 3nmol hFH-injected mouse from mice injected four times with 3nmol doses of either of the recombinant FH reagents, or PBS (Pearson, ***p < 0.001)

Figure 48 Mini-FH Exp. 3: Correlation of 24h plasma C3 levels and tubulointerstitial C3 scores
5.14 FI is detected in plasma of Cfh-/- Cfi-/- mice injected with Cfh-/- C3-/- serum

In Mini-FH Exp. 1 and Mini-FH Exp. 3, administration of hFH produced opposite effects at 24h on glomerular C3 staining (reduced) and plasma C3 levels/tubulointerstitial C3 staining (increased). The apparent dissociation of glomerular and tubulointerstitial C3 staining raises the possibility that C3 accumulation along the GBM and in the tubulointerstitium arise via distinct mechanisms. In order to assess whether in fact they could be triggered concurrently in FH-deficient mice, an alternative in vivo approach was adopted. In Cfh-/- Cfi-/- mice, excessive C3 activation via the AP is characterized by low plasma levels of C3, circulating in the form of C3b, and by mesangial C3 accumulation [Rose et al. 2008]. Previously FI reconstitution of Cfh-/- Cfi-/- mice led to the appearance of florid linear C3/C3d staining from 24h in association with rapid cleavage of plasma C3b [Rose et al. 2008, Paixão-Cavalcante et al. 2009]. In the present study, a single injection of pooled Cfh-/- C3-/- serum was performed, with assessment of plasma and renal histology at serial time-points. 2h after injection, FI was detected in plasma of Cfh-/- Cfi-/- mice by western blot, falling below the detection limit after 8h (Fig. 49).
Figure 49 Fl western blot of Cfh-/-.Cfi-/ mouse plasma before and after Fl reconstitution

Western blot under non-reducing conditions of plasma from Cfh-/-.Cfi-/ mice before (PRE) and after injection of Cfh-/-.C3-/ serum (denoted as Fl), with detection of mouse Fl (88kDa) using primary anti-human Fl antisera.
5.15 FL reconstitution of Cfh-/-, Cfi-/- mice is associated with rapid cleavage of plasma C3b

Prior to FL reconstitution of Cfh-/-, Cfi-/- mice, C3b and high MW C3b complexes were detected in plasma. 2h after administration of Cfh-/-, C3-/- serum, cleavage of total plasma C3b had occurred (Fig. 50). This was associated with the appearance of abundant C3b cleavage products, which gradually diminished before the reappearance of C3b after 96h.
Figure 50 C3 western blot of Cfh-/-Cfi-/- mouse plasma

Western blot under reducing conditions of plasma from Cfh-/-Cfi-/- mice before and after injection of Cfh-/-C3-/- serum (FI), with C3 detection using a polyclonal anti-mouse C3 antibody. Prior to injection, the α'-chain of C3b is seen together with high MW C3b aggregates. 2h after FI administration, the α'-chain is absent and α'-chain fragments are seen. 8 days after injection, the C3 profile has returned to baseline. WT serum shows the slightly higher, uncleaved α-chain of intact C3. C3 is not detected in Cfh-/-C3-/- serum.
5.16 FI reconstitution of Cfh-/-.Cfi/- mice leads to C3/C3d accumulation along the GBM but not renal tubules

C3 staining in Cfh-/-.Cfi/- mice prior to injection showed a mesangial C3 pattern without linear or tubulointerstitial C3 [Rose et al. 2008] (Fig. 51a). 8h after injection of Cfh-/-.C3/- serum, mesangial C3 was less prominent, with the appearance of linear C3 staining (Fig. 51b). At 24-48h, linear C3 was more prominent (Fig. 51c,d). Thereafter the intensity of linear C3 staining gradually reduced, and at 192h some mesangial C3 staining had reappeared (Fig. 51e). Glomerular C3d staining was absent in naïve Cfh-/-.Cfi/- mice, as previously shown [Paixão-Cavalcante et al. 2009] (Fig. 51f). Following administration of Cfh-/-.C3/- serum, glomerular C3d staining was seen in a linear pattern at 8h, intensifying at 24h (Fig.51g-i). Thereafter it became less prominent although it was still faintly visible at 192h (Fig. 51j). Tubulointerstitial C3 staining was not apparent in Cfh-/-.Cfi/- mice at any of the assessed time-points after FI reconstitution. As a positive control, a Cfh/- mouse received an i.p. injection of 3nmol hFH, which was associated with reduction in linear C3 and the appearance of mesangial and prominent tubulointerstitial C3 at 24h (Fig. 51k,l). I.p. injection of hFH in Cfh-/-.Cfi/- mice did not alter the C3 staining pattern at 24h (Fig. 51m). This is consistent with an identical renal phenotype in FI-deficient mice independent of the presence or absence of FH [Rose et al. 2008].
Figure 51 Renal immunostaining in Cfh−/−.Cfi−/− mice
(a-e) Direct IF for C3 and (f-j) indirect IF for C3d (red) in Cfh−/−.Cfi−/− mice before (PRE) and after i.v. injection of Cfh−/−.C3−/− (denoted as FI). (k,l) C3 staining in a Cfh−/− mouse before and after i.p. injection of hFH, and (m) in a Cfh−/−.Cfi−/− mice after i.p. injection of hFH. All images at magnification 400×.
5.17 Discussion

Accumulation of C3 along the GBM is the earliest pathological renal abnormality observed in Cfh−/− mice [Pickering et al. 2002]. Accordingly, reduction in glomerular C3 staining at 24h has previously been used as a surrogate marker for therapeutic effect in experimental C3 glomerulonephropathy following administration of plasma-derived murine or human FH [Paixão-Cavalcante et al. 2009, Fakhouri et al. 2010]. Here, in collaboration with colleagues at the Institute of Cellular & Genetic Medicine, Newcastle University, I have demonstrated that administration in Cfh−/− mice of a novel engineered mini-FH protein comprising SCR domains 1-5 and 18-20 leads to reduced linear C3 staining in the glomerulus at 24h (Mini-FH Exp. 1 and Exp. 3) and at 6h (Mini-FH Exp. 2). This effect was comparable to that of full-length, serum-derived hFH, used at lower molar doses as a positive control. One implication of these data is that the 'middle' SCR domains 6-17, which include the GAG-binding site in SCR 7, are not essential to the therapeutic effect of FH reagents.

The therapeutic effect of both mini-FH and hFH was associated with the detection of these human reagents in a linear staining pattern within the glomerulus 6h after injection (Mini-FH experiment 2). This glomerular staining pattern is consistent with binding to the GBM and/or to GBM surface-attached iC3b ± C3d(g). The demonstration of FH surface-binding on the GBM and/or GBM-attached C3 fragments in association with therapeutic effect in Cfh−/− mice is a novel one. In the previous studies, neither murine nor human FH could be detected in the glomerulus 24h after injection of Cfh−/− mice [Paixão-Cavalcante et al. 2009, Fakhouri et al. 2010]. Daily administration of human FH for 10 days led to the development of proliferative GN, in association with glomerular deposition of C3, IgG and human FH in a non-linear staining pattern. This was attributed to formation of immune complexes containing human FH and murine anti-human FH antibodies, which were demonstrated in the circulation in conjunction with rapid clearance of human FH. In another study, murine FH was detected only within the mesangium of Cfh−/− kidneys that had been transplanted into WT recipients [Alexander et al. 2007].

One interpretation of these data is that the mini-FH and FH must bind to the surface in order to reduce glomerular C3. This is ostensibly supported by the observation that FH1-5, which lacks the C-terminal SCR domains 18-20 present on both mini-FH and hFH, could not be detected in the glomerulus and also did not reduce linear C3 staining. Hence these data provide evidence in support of hypothesis 2, that FH protects the GBM from surface phase C3 activation via the AP.
However, an alternative explanation for the lack of therapeutic effect of FH1-5 is its inefficiency at restoring fluid phase AP control. For example, in mini-FH experiment 2, plasma C3 levels were significantly increased for 6nmol or 12nmol mini-FH, but not 12nmol FH1-5, compared to PBS. As the presence of C-terminal domains 18-20 would not be expected to confer increased FH complement regulatory activity in the fluid phase, these data could be explained by more rapid clearance of FH1-5 compared to mini-FH from the circulation. Factors that might favour more rapid clearance of FH1-5 include its lower MW and the absence of SCR 18 as the only natural N-glycosylation site present on mini-FH. Both mini-FH and FH1-5 were more rapidly cleared from plasma than hFH based on Dr Marchbank’s ELISA. However, a difference in half-life between mini-FH and FH1-5 was not demonstrated, and more detailed pharmacokinetic studies would be required to answer this question. In summary, restoration of fluid phase AP control was insufficient at the doses of FH1-5 assessed in these experiments for this to be discounted as the principle mechanism by which hFH and mini-FH reduced glomerular C3. Accordingly, hypothesis 2, that FH protects the GBM from surface phase C3 activation via the AP, is not excluded on the basis of Mini-FH Experiments 1-3.

Mini-FH merits further evaluation for therapeutic use in DDD and potentially other AP-mediated conditions. Although the reduction in glomerular C3 achieved using mini-FH was comparable to that of hFH, mini-FH has a lesser effect than hFH on plasma C3 levels. This may indicate superior targeting of the therapeutic effects of mini-FH to the glomerular site of C3 activation. As it lacks SCR domains 6 and 7, which are important binding sites for microorganisms as part of complement evasion [Blom et al. 2009, Caesar et al. 2014], mini-FH may also confer a favourable infection risk profile. However, a clear role for therapeutic FH administration in AP-mediated conditions associated with normal plasma FH levels has yet to be defined.

The dissociation of linear C3 staining and tubulointerstitial C3 staining 24h after administration of hFH in Cfh/- mice prompted an additional experiment in Cfh-/--Cfi-/- mice. FI reconstitution led to accumulation of C3/C3d in a linear staining pattern in the glomerulus, in conjunction with cleavage of circulating C3b, as previously reported [Rose et al. 2008, Paixão-Cavalcante et al. 2009]. This was not associated with the appearance of tubulointerstitial C3 at any of the assessed time-points up to 192h, at which point the plasma C3 profile had returned to baseline. These data suggest that C3 accumulates along the GBM via a distinct mechanism from that of tubulointerstitial C3, for which strong evidence exists in vitro and in vivo implicating surface phase C3 activation via the AP. Nevertheless these observations in vivo provide insufficient grounds for rejecting hypothesis 2.
5.18 Conclusions

5.20.1 Mini-FH reduces glomerular C3 staining in Cfh−/− mice at 6h and 24h, indicating a plausible therapeutic role in patients with DDD.

5.20.2 A modest effect on plasma C3 levels, and detection of mini-FH in a linear staining pattern in the glomerulus, indicate that its therapeutic effect may be related to binding to the GBM and/or GBM-bound C3 activation products.

5.20.3 The inability of FH1-5, at the doses assessed here, to be detected deposit along the GBM or reduce glomerular C3 staining further suggest that surface recognition C-terminal SCR domains 18-20 may be critical to the therapeutic effect of mini-FH.

5.20.4 Nevertheless the minor effects of mini-FH on plasma C3 levels cannot be ruled out as a critical determinant of its therapeutic effect; hence hypothesis 2 is not excluded.

5.20.5 Absence of tubulointerstitial C3 in Cfh−/− mice and in FI-reconstituted Cfh−/−.Cfi−/− mice provides circumstantial evidence in vivo that C3 accumulation along the GBM is not due to surface phase C3 activation. Nevertheless, this is insufficient to exclude hypothesis 2.
6. CHAPTER SIX: THE RELATIONSHIP BETWEEN COMPLEMENT FACTOR H AND SUSCEPTIBILITY TO C3 ACCUMULATION ALONG THE GLOMERULAR BASEMENT MEMBRANE
6.1 Introduction

The strong association of DDD with FH deficiency/dysfunction implies that the FH protein normally protects against pathogenic C3 accumulation along the GBM. An unresolved question is whether FH-mediated protection is due to prevention of C3 activation in the circulation or on the GBM surface [Barbour et al. 2015]. This distinction has implications for therapeutic drug design, for example systemically acting versus surface-targeted C3 inhibitors. The fluid phase hypothesis states that C3b cleavage products generated in the circulation become associated with the GBM in patients with DDD. This is consistent with several clinical observations: (1) abundant circulating C3b cleavage products in patients with DDD [Schwertz et al. 2001, Appel et al. 2005, Zhang et al. 2014]; (2) proteomic studies showing C3 fragments in the glomerular tissue of patients with DDD, without detection of FB (required for AP C3 convertase formation) [Sethi et al. 2010]; (3) low serum C3 serum in a case of C3NeF-associated MPGN persisting weeks after bilateral nephrectomy [Vallota et al. 1971], indicating that ‘hypocomplementemia could be the result almost entirely of a complement reaction occurring in the circulation rather than...in the kidneys’ [West et al. 1973]; (4) a human DDD renal biopsy study suggesting that deposition of ‘circulating iC3b’ mediated renal pathology [West et al. 2001]; and (5) the demonstration that excessive C3 activation via the AP due to FH-resistant C3 in a family study of DDD was restricted to the fluid phase in vitro [Martinez-Barricarte et al. 2010]. In addition, the appearance of linear C3 staining in Cfh-/-,Cfi-/- mice following administration of murine FI-containing serum provides experimental evidence that C3 accumulation in DDD is critically dependent on FI-mediated cleavage of circulating C3b [Rose et al. 2008]. However, the tropism of systemically generated C3 fragments for the GBM has not been adequately explained for the fluid phase hypothesis [West & McAdams 1995].

The surface phase hypothesis states that covalent binding of C3b to the GBM results in C3b amplification on the GBM surface, with subsequent conversion to surface-bound C3b cleavage products. In this case, protection due to FH would be attributable to the ability of the FH to bind surface-attached C3b, abrogating surface phase C3b amplification. A key argument in support of this hypothesis has been that the GBM, which lacks intrinsic membrane-bound AP regulators, is highly susceptible to surface phase C3 activation via the AP in the setting of FH deficiency/dysfunction [Jansen et al. 1998, Zipfel 2006]. Thus in the original family study of ‘atypical’ DDD associated with homozygous FH deficiency, disease causation was explained in terms of ‘locally uncontrolled alternative pathway activation’. There, low plasma C5 levels in the affected brothers were attributed to the presence of ‘an alternative pathway activating surface (possibly in the glomerulus) allowing the formation on its surface of a C3/C5 convertase, which is unusually stable in the absence of [FH]’ [Levy et
The demonstration in vitro of enhanced FH deregulation using serum fractions containing abnormal FHR proteins from patients with familial C3GN also provides experimental evidence that FH normally protects the GBM from surface phase C3 activation [Tortajada et al. 2013, Goicoechea de Jorge et al. 2013].

In this chapter, I address hypothesis 2, that FH protects the GBM from surface phase C3 activation via the AP. My approach was to assess the ability of normal human serum (NHS) and FH-depleted human serum (FHdplHS) to trigger C3 activation and/or deposition on purified GBM components. These components were collagen type IV, fibronectin and laminin. As a positive control for C3 activation in my assays, I used zymosan as the canonical AP activating surface [Pillemer et al. 1953, Lay et al. 2014]. I also examined the ability of murine renal cryosections to activate C3. Notably, a number of studies have shown that human GBM preparations [Lambert et al. 1974, Williams et al. 1984] or purified GBM-like proteins [Hindmarsh & Marks 1998] provide an AP activating surface (i.e. in the presence of FH). However, strict AP dependence has not generally been demonstrated, nor in some cases appropriate experimental controls used (as discussed below). Additional studies have suggested that the binding affinity of intact C3 or its activation products is increased for some of these purified GBM components, without proposing surface phase C3 activation per se [Hautanen & Keski-Oja 1983, Leivo & Engvall 1986].
6.2 Development of a plate assay to assess surface phase C3 activation via the alternative pathway

Purified GBM proteins were assessed for their ability to activate C3 via the AP in vitro. I used a zymosan-coated surface as a positive control in this assay, and detected surface C3 deposition using a polyclonal anti-human C3 antibody.

A standard curve for surface phase C3 activation via the AP was developed by incubating zymosan-coated wells in 5% v/v NHS in EGTA/Mg²⁺-containing incubation buffer at 37°C for 45 minutes. As expected, detection of surface-bound C3 using the polyclonal anti-human C3 antibody was increased for zymosan-coated wells compared to wells incubated with coating buffer (CB) alone in a dose-dependent manner (maximal mean OD 1.54 for zymosan 12.5µg/mL versus mean OD 0.28 for CB, Fig. 52a). The zymosan-dependent C3 activation was inhibited when EDTA was added to incubation buffer (maximal mean OD 0.14 for zymosan 25µg/mL versus mean OD 0.10 for CB alone). C3 western blot using buffer from the wells showed evidence of C3 α'-chain fragments in zymosan 6.25µg/mL-coated wells (Fig. 52c). The appearance of these fragments was EDTA-inhibitable. Hence, in my assay, zymosan-coated wells triggered the appearance of surface C3 and fluid-phase C3 fragments both of which were inhibited by EDTA. Slightly increased C3 detection in CB-coated wells following incubation with NHS compared to NHS in the presence of EDTA (NHS<sup>EDTA</sup>) indicates minor C3 activation via the AP not specifically due to zymosan. Based on my dose-response data (Fig. 52a,b), zymosan 6.25µg/mL was used as the upper limit for surface phase C3 activation via the AP in this assay.
Figure 52 Zymosan plate assay to detect surface phase C3 activation via the alternative pathway using NHS

Wells coated with zymosan up to 25µg/mL in coating buffer (CB) were incubated using NHS in Mg²⁺/EGTA-containing incubation buffer with or without 10mM EDTA for 45 minutes at 37°C. (a) Surface-bound C3 was detected using HRP-labelled anti-human C3 antibody, and (b) the OD plotted against zymosan concentration on a logarithmic scale, yielding a sigmoid curve. (c) The corresponding C3 western blot reveals C3 activation in the presence of zymosan 6.25 µg/mL (with EDTA inhibition). Data are shown as mean values with vertical bars representing the standard error of the mean (SEM).
6.3 Assessment of GBM proteins for C3 activation in normal human serum

I next examined whether purified collagen type IV, fibronectin and laminin could trigger surface phase C3 activation. Using NHS, there was no evidence that purified collagen type IV, fibronectin or laminin could activate C3 in my plate assay (Fig. 53a-c). In addition, there was no evidence of activation of C3 within the well buffer with the exception (as previously seen, Fig. 52c) of the wells containing zymosan (Fig. 54). To ensure that each of these proteins was bound to the well following coating, I performed an ELISA assay using antibodies specific for each protein. This confirmed dose-dependent binding of the purified proteins to the wells (Fig. 55). I next examined the effect of using sera that did not contain FH in these assays. First, I characterized the FH-depleted serum that I would use in the assays.
Figure 53 Plate assay to assess the ability of fibronectin, laminin and collagen type IV to activate C3 through the alternative pathway using NHS

Wells were coated with each of the purified GBM components up to a concentration of 25µg/mL, or CB alone. Zymosan-coated wells were used as a positive control. C3 detection due to (a) collagen type IV, (b) fibronectin and (c) laminin. Data are shown as mean with horizontal bars representing the SEM.
Figure 54 Assessment of C3 activation in NHS due to purified GBM components: collagen type IV, fibronectin and laminin

C3 western blot using buffer from wells incubated with 6.25µg/mL zymosan (Zym), collagen type IV (Coll), fibronectin (Fibro) and laminin (Lam) in the presence of NHS.
Figure 55 Detection of plated-protein used in C3 plate assay

ELISA was used to detect collagen type IV, fibronectin and laminin after coating at increasing concentrations.
6.4 Rapid activation of C3 in the fluid phase in FH-depleted serum

In order to study the ability of FH to protect surfaces from C3 activation and to control C3 activation in the fluid phase, I utilized FH\textsuperscript{dpl}HS, which I purchased from CompTech. I wanted to ensure that this FH\textsuperscript{dpl}HS contained intact C3, since it has long been appreciated that removal of FH in sera results in rapid and complete C3 activation via the AP. In other words, there is a difference between FH-deficient sera (in which there is complete consumption of C3 and the AP activation protein, FB and variable consumption of terminal pathway components) and experimentally generated FH-depleted serum (in which the only missing serum component is FH). To prevent C3 activation, the FH\textsuperscript{dpl}HS is supplied in the absence of cations. I expected to see intact C3 at the time cations were restored, followed by rapid C3 activation after incubation in EGTA/Mg\textsuperscript{2+}-containing buffer at 37°C. Serum C3 was assessed by western blot using the polyclonal anti-human C3 antibody. C3 $\alpha$-chain and $\beta$-chain were seen in both NHS and FH\textsuperscript{dpl}HS before incubation (Fig. 56a). The presence of a faint lower band corresponding to $\alpha'$-chain fragments is consistent with minor C3 activation during handling prior to incubation. After incubation of NHS for 60 minutes, the intact C3 $\alpha$-chain was still evident, with a minor increase in $\alpha'$-chain fragments. As expected, the intact C3 $\alpha$-chain was absent from FH\textsuperscript{dpl}HS after only 10 minutes, with the concomitant appearance of the $\alpha'$-chain of C3b and prominent $\alpha'$-chain fragment bands. After 60 minutes, the $\alpha'$-chain of C3b had also disappeared from FH\textsuperscript{dpl}HS, with a 38kDa $\alpha'$-chain fragment band being prominent. Rapid activation of total intact C3 and C3b thus occurred spontaneously during incubation of FH\textsuperscript{dpl}HS, generating C3b (transiently) and C3b cleavage products. Purified hC3 in incubation buffer had an unchanged appearance after incubation for 60 minutes. However, addition of hC3\textsuperscript{1.0mg/mL} to FH\textsuperscript{dpl}HS prior to a second incubation assay again resulted in activation of the exogenous C3 after 60 minutes (Fig. 56b).
Figure 56 Western blots of C3 using normal serum, FH-depleted serum and purified C3 during incubation in Mg$^{2+}$/EGTA-containing buffer at 37°C

Samples taken before (PRE) and 10 and 60 minutes after incubation of (a) NHS, FH$^{dpl}$HS, hC3$^{1.0mg/mL}$ and (b) FH$^{dpl}$HS reconstituted with hC3$^{1.0mg/mL}$ in Mg$^{2+}$/EGTA-containing buffer at 37°C. Western blot was performed under reducing conditions using 0.5µL serum, with C3 detection using primary polyclonal anti-human C3 antibody. This reveals the α-chain of intact C3 (MW 110kDa), β-chain (MW 75kDa), α'-chain of C3b (MW 108kDa) and ~38kDa/42kDa α'-chain fragments (C3b cleavage products). High MW bands (>230kDa) likely represent aggregates.
C3 activation in FH<sup>dp</sup>iHS was prevented through reconstitution of FH<sup>dp</sup>iHS with either hFH<sup>0.05mg/mL</sup> (Fig. 57a) or hFH<sup>0.5mg/mL</sup> (Fig. 57b) prior to adding EGTA/Mg<sup>2+</sup>-containing buffer. Addition of EDTA to incubation buffer prevented C3 activation in FH<sup>dp</sup>iHS, confirming dependency on Mg<sup>2+</sup>-dependent formation of the AP C3 convertase (Fig. 57b).

Despite rapid activation of C3 in FH<sup>dp</sup>iHS, only intact C5 was demonstrable after 60 minutes using both NHS and FH<sup>dp</sup>iHS (Fig. 57c). This is consistent with the comparatively slow physiological rate of C5 activation compared to C3 activation [Rawal & Pangburn 1998] and could be further explained in FH<sup>dp</sup>iHS by the rapid conversion of C3b to fragments, preventing the formation of a fluid-phase C5 convertase.
Figure 57 Western blots of C3 using FH-depleted serum with and without purified FH or C3 during incubation in Mg$^{2+}$/EGTA-containing buffer at 37°C

Human serum samples taken before and 10 and 60 minutes after incubation in Mg$^{2+}$/EGTA-containing buffer at 37°C. (a) Incubation of FH$^{\text{dpl}}$HS with or without hFH 0.05mg/mL reconstitution, and reducing C3 western blot using 0.5µL serum. (b) Standardized incubation (see Materials and Methods 2.17) of FH$^{\text{dpl}}$HS with or without hFH 0.5mg/mL reconstitution and/or addition of 10mM EDTA, and reducing C3 western blot using 0.28µL serum. (c) Standardized incubation method and C5 western blot with C5 detection using anti-human C5 antisera. Comparison is with purified human C5 (hC5, MW 190kDa).
6.5 Characterization of commercially sourced FH-depleted serum

It might be expected that cleavage of C3b would not occur in FH-depleted serum since FH is considered to be an essential cofactor for the factor I-mediated cleavage of C3b to iC3b in plasma [Pangburn et al. 1977, Harrison & Lachmann 1980]. Clearly in my assays C3b cleavage did occur in the FH\textsuperscript{dpl}HS and I considered that this might be due to residual FH, FHR or FHL-1 proteins or possibly to cofactor activity of C4BP [Nagasawa & Stroud 1977, Fujita & Nussenzweig 1979, Blom et al. 2003] or CR1 [Lachmann 2010].

In NHS used for these assays I could detect both of the glycosylated forms of FHR-1 with or without FHL-1 using the primary anti-human FH antibody (Fig. 58a). None of these proteins was seen in the FH\textsuperscript{dpl}HS. Western blot using secondary antibody only was negative (Fig. 58b). Importantly I could not detect any FH in the FH\textsuperscript{dpl}HS using this assay. FH was clearly detectable, as expected, following reconstitution of FH\textsuperscript{dpl}HS with hFH\textsuperscript{0.5mg/mL}. FHR-1/FHL-1 was not detected in FH\textsuperscript{dpl}HS reconstituted with hFH\textsuperscript{0.5mg/mL}, indicating that the FH preparation did not contain FHR-1/FHL-1.

FHR-5 was detected in NHS, with FHR-1 also detected using this anti-human FHR-5 antibody (Fig. 58c). None of these proteins were detected in FH\textsuperscript{dpl}HS or FH\textsuperscript{dpl}HS reconstituted with hFH\textsuperscript{0.5mg/mL}.

I concluded that (1) neither the FH\textsuperscript{dpl}HS nor the purified FH contained FHR-1, FHL-1 or FHR-5; and (2) the FH\textsuperscript{dpl}HS did not contain residual FH.
Figure 58 FH and FHR western blots using FH<sup>δpl</sup>HS

Western blot under non-reducing conditions using 1μL (or 0.01μL, denoted as 1%) of NHS, and FH<sup>δpl</sup>HS with or without FH<sup>0.5mg/mL</sup> reconstitution. (a) Detection of FH (MW 155kDa) using primary anti-human FH antisera. A double band at ~35 and 37kDa is slightly below the predicted MW of the two isoforms for FHR-1 (37kDa and 43kDa) with or without FHL-1 (43kDa). A large amount of protein was loaded to ensure that the FHR proteins (present at lower concentrations than FH) could be detected. (b) FH western blot with secondary antibody only. (c) Detection of FHR-5 (MW 55kDa) and FHR-1 using polyclonal anti-human FHR-5 antibody. (d) FHR-5 western blot with secondary antibody only.
6.6 Surface deposition of C3 on GBM proteins using FH-depleted serum

Using the plate assay for C3 activation, increased C3 deposition was detected in 6.25 zymosan-coated wells following incubation of FH\textsuperscript{dpl}HS compared to FH\textsuperscript{dpl}HS\textsuperscript{EDTA} (Fig. 59a). However, there was also significant C3 detected (based on similar OD readings) in wells that did not contain zymosan (referred to as CB only, Fig. 59b). This was consistent with rapid activation of C3 in the FH\textsuperscript{dpl}HS on restoration of cations, with non-specific deposition of C3 within the well. This C3 activation in FH\textsuperscript{dpl}HS was, as previously, EDTA-inhibitable (Fig. 59a). When I coated the purified GBM components (at a concentration of 6.25µg/mL) and incubated with FH\textsuperscript{dpl}HS I again noted significant OD readings that were comparable to those for uncoated wells (Fig. 59a).

C3 western blot showed an identical pattern of C3 activation in FH\textsuperscript{dpl}HS after the ELISA irrespective of surface coating with zymosan, the GBM components, or CB alone (Fig. 60). This was characterized by the disappearance of the α-chain of intact C3 and appearance of the α´-chain and α´-chain fragment bands. Based on the presence of the α´-chain, C3b cleavage appeared to be less efficient in the ELISA compared to water bath incubation. Nevertheless, activation of total intact C3 using FH\textsuperscript{dpl}HS for the ELISA contrasts with the findings using NHS (Fig. 54).

These results created a difficulty in answering my original question, which was ‘do components of the GBM (fibronectin, laminin, collagen type IV) activate C3 in the absence of FH?’ In the complete absence of FH, but in the presence of intact C3 (the situation that exists in the FH\textsuperscript{dpl}HS), allowing the AP to proceed (through the restoration of cations i.e. the Mg\textsuperscript{2+}/EDTA buffer) resulted in rapid C3 activation beyond C3b in the fluid phase together with ‘indiscriminate’ C3 deposition on surfaces. This suggests that, if there is sufficient C3 in proximity to a surface, then the absence of FH will result in indiscriminate surface opsonization.

I next performed a functional assay to determine the effect of FH depletion of human serum on both C3 opsonization and surface phase C3 activation via the AP. I chose to utilize the erythrocyte surface, as haemolysis provides a functional readout of surface phase C3 activation (in the presence of C5).
Figure 59 C3 activation in plate assay is EDTA-inhibitable but indiscriminate when using FH<sub>dp</sub>HS.

C3 activation ELISA shows (a) reduced C3 deposition on 6.25μg zymosan-coated wells using FH<sub>dp</sub>HS compared to either NHS or FH<sub>dp</sub>HS reconstituted with hFH<sub>0.5mg/mL</sub>. C3 activation was similar using FH<sub>dp</sub>HS for zymosan and the purified GBM components at a concentration of 6.25μg/mL or (b) CB only. C3 deposition on zymosan-coated wells was EDTA-inhibitable using NHS or FH<sub>dp</sub>HS (a).
Figure 60 Assessment of GBM-like proteins using C3 activation ELISA in FH^{dpl}HS

C3 western blot before and after 45 minute incubation of FH^{dpl}HS in wells coated with 6.25µg/mL zymosan (Zym), collagen type IV (Coll), fibronectin (Fibro) and laminin (Lam) or CB alone.
6.7 Assessment of FH-depleted serum for lysis of erythrocytes

In my haemolysis assay, 0.6% rabbit erythrocytes (E<sup>R</sup>) were incubated with human sera/proteins in EGTA/Mg<sup>2+</sup>-containing lysis buffer for 1h at 37°. Serum concentration-dependent haemolysis was observed after a single incubation of E<sup>R</sup> in NHS but not FH<sup>dpl</sup>HS (Fig. 61a). At the maximal NHS concentration tested (20%), mean haemolysis was 104.3% of that due to the positive control (water), compared to 8.5% due to lysis buffer alone (negative control). Mean haemolysis in 10% and 5% NHS also exceeded that in lysis buffer alone. By contrast, FH<sup>dpl</sup>HS even at the maximal 20% concentration produced only 6.9% mean haemolysis. However, following reconstitution of 20% FH<sup>dpl</sup>HS with hFH, haemolysis was observed proportional to hFH concentration (Fig. 61b). Addition of EDTA to lysis buffer prevented haemolysis using either 20% NHS or 20% FH<sup>dpl</sup>HS reconstituted with hFH<sub>0.5mg/mL</sub>, confirming AP dependence of E<sup>R</sup> lysis in this assay (Fig. 61a). C3 western blot of pre-assay (sera/proteins) and post-assay (supernatant) specimens revealed complete loss of the α-chain of intact C3 in FH<sup>dpl</sup>HS, but not NHS or FH<sup>dpl</sup>HS reconstituted with hFH<sub>0.5mg/mL</sub> (Fig. 61c).

In summary, FH depletion of human serum abrogated AP-dependent E<sup>R</sup> lysis during a single serum incubation. This was associated with rapid activation of total intact C3/C3b in FH<sup>dpl</sup>HS. I hypothesized that some C3 was nevertheless deposited on the E<sup>R</sup> surface, and that this would render E<sup>R</sup> susceptible to lysis during a second incubation with FH<sup>dpl</sup>HS.
**Figure 61 Rabbit haemolysis assay using human sera**

E° lysis following incubation in human sera/proteins in in Mg²⁺/EGTA-containing lysis buffer for 1h at 37°C, with results expressed as percent haemolysis due to water. (a) Haemolysis due to 2.5-20% NHS, and FH\(^{dpl}\)HS with or without hFH\(^{0.125mg/mL}\) reconstitution. (b) hFH concentration-dependent haemolysis in reconstituted 20% FH\(^{dpl}\)HS, with EDTA inhibition. Dotted lines show haemolysis in 20% NHS and NHS\(^{EDTA}\) (c) C3 western blot of human sera before, and supernatant after, incubation with E°. Data are shown as mean values with vertical bars representing the SEM.
6.8 Erythrocyte lysis during sequential incubation in FH-depleted serum

Following an initial incubation in FH$_{dpl}$HS, which I had shown did not result in lysis, E$^R$ were gently washed before undergoing a second incubation in one of NHS, FH$_{dpl}$HS, FH$_{dpl}$HS$_{EDTA}$ or C3-depleted human sera (C3$_{dpl}$HS). Serum concentration-dependent haemolysis was observed following the second incubation of E$^R$ in either NHS or FH$_{dpl}$HS (Fig. 62a). Haemolysis using two sequential incubations of 20% FH$_{dpl}$HS was equivalent to sequential incubation in 20% FH$_{dpl}$HS followed by 20% NHS. Mean haemolysis was 92.6% and 90.2%, respectively, of that due to water (substituted for the second incubation).

As a single incubation of E$^R$ in FH$_{dpl}$HS is associated with activation of total intact C3/C3b, the effect of C3 replenishment was assessed. Substitution of 20% FH$_{dpl}$HS$_{EDTA}$ for the second incubation produced only 18.0% mean haemolysis (compared to 11.9% for lysis buffer alone), confirming AP-dependence (Fig. 62a). Substitution of 20% C3$_{dpl}$HS for the second incubation also produced 18.8% haemolysis, indicating that replenishment of intact C3 (and possibly additional AP factors) during the second incubation with FH$_{dpl}$HS is critical to haemolysis.

Next, the functional importance of surface C3 accumulation during the initial incubation of E$^R$ in FH$_{dpl}$HS was assessed. Compared to 85.8% mean haemolysis following two incubations in FH$_{dpl}$HS, substitution of 20% C3$_{dpl}$HS for the first incubation produced only 18.7% mean haemolysis (compared to 11.1% haemolysis using 20% C3$_{dpl}$HS for both incubations, Fig. 63a). In a separate assay, an initial incubation of E$^R$ in either 20% FH$_{dpl}$HS or 20% C3$_{dpl}$HS was followed by a second incubation using 20% C3$_{dpl}$HS with hC3 reconstitution. Approximately four times the hC3 concentration was required for equivalent E$^R$ lysis during the second incubation if the initial incubation was performed using C3$_{dpl}$HS compared to FH$_{dpl}$HS (Fig. 63b).

Dependence of E$^R$ lysis on the availability of intact C3 was confirmed by demonstrating that the inability of C3$_{dpl}$HS to mediate lysis was restored after the addition of hC3 up to 1.0mg/mL serum (Fig. 62b)
Figure 62 Rabbit haemolysis assay on repeated incubation with FH\textsuperscript{dpl}HS

(a) Following an initial incubation of E\textsuperscript{b} in FH\textsuperscript{dpl}HS where lysis does not occur, cells were incubated a second time in (1) NHS, (2) FH\textsuperscript{dpl}HS, (3) FH\textsuperscript{dpl}HS containing EDTA and (4) C3-depleted human sera and then haemolysis assessed. (b) C3-dependent haemolysis in reconstituted 20% C3\textsuperscript{dpl}HS, with EDTA inhibition. Data are shown as mean values with vertical bars representing the SEM.
Figure 63 Rabbit haemolysis assay: effect of initial incubation in FH\textsuperscript{dpl}HS

(a) Haemolysis due to 2.5-20% FH\textsuperscript{dpl}HS following an initial incubation in FH\textsuperscript{dpl}HS is compared to that following an initial incubation in 20% C3\textsuperscript{dpl}HS. NHS is used as a positive control for lysis of E\textsuperscript{a} following an initial incubation in C3\textsuperscript{dpl}HS. (b) Haemolysis following a second incubation in reconstituted C3\textsuperscript{dpl}HS has ~fourfold greater hC3 requirement if the first incubation is performed in C3\textsuperscript{dpl}HS compared to FH\textsuperscript{dpl}HS. Data are shown as mean values with vertical bars representing the SEM.
6.9 C3 deposition on the erythrocyte surface following exposure to FH-depleted serum

That E\textsuperscript{R} lysis in FH\textsuperscript{dpl}HS occurred only with two sequential incubations in FH\textsuperscript{dpl}HS suggested that the initial incubation resulted in sub-lytic deposition of C3 on the E\textsuperscript{R} surface. Thus during the first incubation of FH\textsuperscript{dpl}HS, rapid activation of C3 and cleavage of C3b in the fluid phase produced a C3 deficiency state in which surface phase C3 activation could not proceed. On re-incubation with FH\textsuperscript{dpl}HS, which contains intact C3 initially, I hypothesized that C3b deposited on the E\textsuperscript{R} surface as a result of the first incubation would, in the presence of additional C3, facilitate surface phase C3 activation sufficient to trigger lysis. I next examined C3 deposition on E\textsuperscript{R} exposed to FH\textsuperscript{dpl}HS using FACS. E\textsuperscript{R} were incubated in 2.5% human sera followed by C3 detection using the polyclonal anti-human C3 antibody. As control sera I used C5-depleted human sera (C5\textsuperscript{dpl}HS), in which I expected normal C3 deposition but absent lysis due to the lack of C5, enabling me to analyse intact cells. Surface C3 was detected on E\textsuperscript{R} after incubation with FH\textsuperscript{dpl}HS: MFI 338 AFU (Fig. 64a). This was much lower than that seen after incubation with the C5-depleted control sera: MFI 1739 AFU. Hence these data are consistent with ‘inefficient’ surface opsonization in FH\textsuperscript{dpl}HS: surface C3 positivity was 81\% lower using FH\textsuperscript{dpl}HS compared to C5\textsuperscript{dpl}HS. Increased C3 detection using C3\textsuperscript{dpl}HS compared to buffer only was attributed to detection of non-specific human antigens in serum, similar to detection of the negative control protein (mouse IgG) following incubation in 10\% human sera (Fig. 64b).

In summary, my sequential haemolysis and flow cytometry data show that FH depletion of human serum is associated with inefficient opsonization and surface phase C3 activation via the AP. Nevertheless, C3 deposited on the E\textsuperscript{R} surface as a result of the first incubation in FH\textsuperscript{dpl}HS was sufficient to mediate E\textsuperscript{R} lysis during a second incubation with FH\textsuperscript{dpl}HS (provided C3 was also replenished). This implies that some of the surface C3 deposited on the E\textsuperscript{R} surface during the first FH\textsuperscript{dpl}HS incubation must be in the form of C3b. This is because only C3b is functionally capable of forming the AP C3 convertase on the E\textsuperscript{R} surface leading to surface phase C3 activation via the AP, and to E\textsuperscript{R} lysis during the second FH\textsuperscript{dpl}HS incubation.
Figure 64 Surface C3 detection on E8 following incubation in FH<sup>dp</sup>HS sera using flow cytometric analysis

(a) Detection of C3 following a single incubation in human sera. C3 due to FH<sup>dp</sup>HS is intermediate between that due to C3<sup>dp</sup>HS (negative control serum) and C5<sup>dp</sup>HS (positive control serum).

(b) Corresponding assay with detection of mouse IgG (negative control protein).
6.10 C3 deposition on renal tissue ex vivo

I next examined the ability of renal tissue to activate C3 in NHS or FH<sup>dpl</sup>HS. In my experimental approach, C3 activation was assessed by incubation of C3-deficient mouse renal sections ex vivo with human sera/protein in EGTA/Mg<sup>2+</sup>-containing incubation buffer for 45 minutes at 37°C. C3 deposition on the PTEC apical surface following serum incubation ex vivo of renal cryosections is generally attributed to surface phase C3 activation via the AP (Chapter 1.2.8.2). The use of C3-deficient mouse tissue avoided the concern that any detected C3 could be endogenous murine C3 rather than derived from the human sera.

Following incubation in 10% NHS, C3 detection using the polyclonal anti-human C3 antibody was most prominent in the corticomedullary junction (Fig. 65a). At higher magnification of this area, intense, granular C3 staining followed the pattern of proximal tubular cells. This is most clearly visualized using 5% NHS (Fig. 65c). Negative staining using the equivalent concentrations of NHS<sub>EDTA</sub> confirmed AP dependence (Fig. 65b,d). Glomerular C3 staining was less bright than proximal tubular staining using 5-10% NHS, and at a lower 1.25% concentration of NHS was almost absent from the cortical areas that contain numerous glomeruli (Fig. 66a).

C3 staining following incubation of FH<sup>dpl</sup>HS was much fainter than for NHS, and was no different on proximal tubules from other anatomical structures (Fig. 65e,g). That some AP-dependent C3 activation had nevertheless occurred using FH<sup>dpl</sup>HS is indicated by comparison with absent C3 staining using FH<sup>dpl</sup>HS<sub>EDTA</sub> (Fig. 65f,h). In contrast to sparse glomerular staining with NHS, 1.25% FH<sup>dpl</sup>HS produced intense C3 staining of glomeruli (Fig. 66b). High magnification of glomeruli revealed sparse C3 using NHS, with EDTA inhibition (Fig. 66c,d), and bright granular C3 with some staining of capillary loops using FH<sup>dpl</sup>HS. with EDTA inactivation (Fig. 66e,f).

Reconstitution of FH<sup>dpl</sup>HS with hFH<sup>0.5mg/mL</sup> restored the scanty glomerular C3 pattern observed using NHS (Fig. 66g). Glomerular C3 staining was absent using 1.25% FH<sup>dpl</sup>HS that had undergone prior water bath incubation for 1h at 37°C thereby resulting in complete C3 activation before incubation on the renal tissue (Fig. 66h). The glomerular pattern of C3 staining due to FH<sup>dpl</sup>HS is unchanged following incubation on a section from a C3-deficient mouse injected with sheep NTS (Fig. 66i). Glomerular C3 staining is absent using purified hC3<sup>1.0mg/mL</sup> (at the equivalent 1.25% serum concentration, Fig. 66j).

In summary, these data show that FH depletion of human serum reduces the degree and specificity of C3 activation on mouse cryosections. This is consistent with my other assays showing that surface opsonization using the FH<sup>dpl</sup>HS was both inefficient and indiscriminate.
5-10% human sera were incubated on cryosections in Mg²⁺/EGTA-containing buffer for 45 minutes at 37°C, followed by C3 detection using polyclonal anti-human C3 antibody. (a) Using 10% NHS, bright C3 staining is seen along the inner cortex/outer medulla at low magnification (20×), with (b) EDTA inhibition. (c) At higher magnification of this area (200×), bright C3 staining follows the pattern of proximal tubular cells, and is most intense on their internal (luminal) aspect, with (d) EDTA inhibition. (e) In the corresponding low power image using FH^{del}HS, C3 staining is not visualized, being no different to (f) incubation in the presence of EDTA. (g) At higher power, FH^{del}HS produces some C3 staining in the corticomedullary junction. This is less bright than for NHS, and is similar for non-tubular as well as tubular structures, with (h) EDTA inhibition.
Figure 66 Glomerular C3 staining following incubation of C3-deficient mouse renal sections with human sera.

Representative images (200×) of C3 staining of renal cortex following incubation of C3-deficient renal cryosections with 1.25% (a) NHS and (b) FH^{dpi}HS. High power images (400×) of glomeruli following incubation with (c) NHS with (d) EDTA inhibition, and (e) FH^{dpi}HS with (e) EDTA inhibition. Glomerular C3 staining is also shown using (g) FH^{dpi}HS reconstituted with hFH^{0.125mg/mL} or (h) preincubated FH^{dpi}HS. (i) The glomerular C3 pattern following incubation of FH^{dpi}HS on a cryosection from a C3−/− mouse injected with sheep NTS (anti-GBM Ig). (j) Glomerular C3 staining using hC3^{1.0mg/mL} in buffer only.
6.11 Discussion

In this chapter, GBM components (collagen type IV, fibronectin and laminin) were assessed for their ability to activate C3 via the AP in human sera. Compared to zymosan, none of these proteins produced significant C3 activation in the presence of FH. Hence these components do not confer the properties of an AP-activating surface on the GBM. When I assessed the ability of the GBM components to activate C3 in the absence of FH, my results were notable in two respects: (1) rapid activation of total intact C3/C3b occurred in the fluid phase; and (2) surface deposition of C3 was similar for each of the proteins, zymosan, and CB alone. This suggested that FH depletion of human serum produces indiscriminate C3 opsonization. However, a limitation of this assay using the purified GBM components was that the specific contribution of surface phase C3 activation to the total signal for C3 surface deposition could not be accurately assessed. To determine whether surface phase C3 activation could still occur in this setting of FH depletion, I moved to an alternative assay in which E^R were used as the AP-activating surface in human sera.

The sequential haemolysis and flow cytometry assays show that, whereas C3 activation in the fluid phase occurs rapidly in FH^{dpl}HS, opsonization and surface phase C3 activation via the AP are reduced in comparison to FH-replete serum. AP-dependent E^R lysis was absent during a single incubation of FH^{dpl}HS despite levels of intact C3 sufficient to mediate lysis if FH^{dpl}HS was instead reconstituted with hFH protein prior to incubation. Flow cytometry confirmed that C3 deposition was reduced on the E^R surface following incubation in FH^{dpl}HS compared to C5^{dpl}HS. These flow cytometry data recapitulate the original findings of Fearon & Austen that surface deposition of C3 is inefficient in the absence of FH [Fearon & Austen 1977b]. These authors further showed that C3 opsonization occurred indiscriminately, being similar on both AP-activating and non-activating surfaces in the absence of FH (0.14% and 0.16% C3 uptake for E^R and E^S, respectively). However, they did not address the implications of this finding for surface phase C3b amplification, whereas I used haemolysis as a functional readout. In my assays, AP-dependent E^R lysis could be effectively restored via a second incubation using fresh FH^{dpl}HS. C3 deposited on the E^R surface as a result of the first incubation in FH^{dpl}HS was a critical requirement of haemolysis, as was the provision of intact C3, during the second FH^{dpl}HS incubation. Hence these data show that (1) surface accumulation of C3b does occur despite rapid C3 activation in the fluid phase using FH^{dpl}HS; and (2) this facilitates unequivocal surface phase C3 activation via the AP, albeit insufficient for E^R lysis during a single incubation. My data showing attenuation, but not abrogation, of surface phase C3 activation via the AP thus challenge the view that ‘the presence of a regulated fluid phase reaction is essential for fixation of C3b on the activating principle’ [Fearon & Austen 1977b].
Finally, FH depletion of human serum was shown to attenuate renal tubular C3 deposition following *ex vivo* incubation of murine kidney sections. This finding recapitulates the observation *in vivo* in Chapter 5 of absent tubulointerstitial C3 staining in Cfh−/− mice, restored only through administration of hFH. As intact C3 is present in both FH*ds*HS and NHS prior to incubation on mouse renal sections, these data suggest that failure to deposit C3 on renal tubules (as an AP activating surface) is due to a functional defect in surface phase C3 activation using FH*ds*HS, consistent with the *in vitro* assays presented in this chapter. Hence absence of tubulointerstitial C3 in the setting of FH deficiency *in vivo* may equally be due to a functional defect in surface phase C3 activation via the AP (rather than being merely the result of chronic serum depletion of C3). Notably, at lower serum concentrations, FH depletion had the opposite effect on AP-dependent glomerular C3 staining *ex vivo*, which was enhanced. These data are reminiscent of the dissociation *in vivo* of glomerular and tubulointerstitial C3 staining in Cfh−/− mice discussed in Chapter 5.

The mechanism underlying preferential glomerular (as opposed to tubular) C3 staining following *ex vivo* incubation of mouse renal sections using low concentration FH*ds*HS remains unclear. Notably, the C3 staining pattern was not strictly a linear one even in the presence of GBM-bound IgG as a putative AP-activating surface in NTS-injected mice [Reiter & Fishelson 1989]. Nevertheless some C3 detected on glomerular structures may have accumulated preferentially along the GBM. On one hand, surface phase C3 activation on the GBM and/or other glomerular structures using FH*ds*HS appears unlikely given (1) the absence of C3 staining on tubular structures (as an established AP-activating surface); and (2) the demonstration *in vitro* using the haemolysis assay that FH depletion of human serum attenuates surface phase C3 activation via the AP on a classic AP activating surface (E5). Moreover, surface phase C3 activation specifically on the GBM appears unlikely since purified proteins similar to those found in the GBM did not provide an AP activating surface *in vitro*. However, these data do not exclude some other property of the GBM favouring surface phase C3 activation in the absence of FH. In theory, this could be assessed though incubation with FH*ds*HS of purified human or murine GBM and comparison with basement membranes obtained from other tissues. In practice, difficulties would probably arise regarding accurate assessment of surface phase C3 activation, similar to those encountered due to indiscriminate and nonspecific C3 activation in FH*ds*HS using the ELISA presented in this chapter.

An alternative explanation for preferential glomerular C3 staining following *ex vivo* incubation of murine sections using FH*ds*HS is that abundant C3b cleavage products generated in the fluid phase during serum incubation accumulate preferentially on the GBM and/or other glomerular structures. It is possible that binding of FH to the GBM and/or GBM-bound C3
fragments normally prevents ongoing deposition of serum C3b metabolites along the GBM (irrespective of whether FH is also preventing surface phase C3 activation). Limitations to those studies purportedly showing detection of FH along the GBM in human kidney sections were discussed in Chapter 1.3. However, the detection of hFH along the GBM following administration in Cfh-/- mice could be consistent with a role in preventing deposition. Additional assays to assess binding of FH to the GBM, with comparison to other basement membranes, are clearly warranted. Of note, however, glomerular C3 staining was not observed following incubation of murine sections using FH(dpl)HS that had undergone prior water bath incubation. As this serum contains abundant C3b metabolites, it might have been expected to produce a similar C3 staining pattern in the glomerulus to fresh FH(dpl)HS if accumulation of C3b metabolites alone was required. Finally, as glomerular C3 accumulation occurred following incubation of murine sections in FH(dpl)HS, this might suggest that physiological filtration across the GBM, which does not occur using the ex vivo staining method, is not required for glomerular C3 accumulation in vivo. However, as noted above, the C3 staining pattern was not strictly a linear one, and further assessment of C3 accumulation specifically along the GBM would be required.

In summary, these data indicate that ongoing deposition of minor amounts of C3b and consequent surface C3b amplification could plausibly account for C3 accumulation along the GBM in patients with DDD. Hence hypothesis 2, that FH protects the GBM from surface phase C3 activation, is not excluded. At the same time, my experiments demonstrate that, in human plasma depleted of FH, C3 activation in vitro occurs predominantly in the fluid phase. This suggests that accumulation of iC3b ± C3d fragments along the GBM as a consequence of their generation in the fluid phase, is a more likely explanation in DDD. A third possibility, that C3 accumulation occurs via both these mechanisms in the setting of FH deficiency, is also not excluded. For example, whereas fluid phase C3 activation may be the predominant cause of C3 accumulation in DDD, surface phase C3 activation may play a minor role. This pattern would be reversed in those cases of C3 glomerulopathy (e.g. CFHR5 nephropathy) in which abnormal FHR proteins mediate FH deregulation.

6.12 Conclusions

6.12.1 FH depletion of human serum in vitro is associated with inefficient and indiscriminate C3 opsonization of surfaces. This could also explain surface accumulation of C3 activation products in vivo, including along the GBM, in individuals with FH deficiency/dysfunction.

6.12.2 FH depletion of serum attenuates (but does not abrogate) surface phase C3 activation via the AP, in association with rapid fluid phase C3 activation in the fluid phase. Hence hypothesis 2 cannot be excluded.
7. CHAPTER SEVEN: FINAL DISCUSSION

In Chapter 3, I demonstrated increased severity of the spontaneous renal phenotype due to accompanying CD11b deficiency in 8-month-old Cfh-/− mice. This was characterized by increased albuminuria and glomerular hypercellularity, with a nonsignificant trend towards increased mesangial expansion. A minor increase in glomerular neutrophils was observed in Cfh-/−.CD11b-/− compared to Cfh-/− mice, together with a nonsignificant trend towards increased glomerular CD68+ macrophages. The finding that CD11b ameliorates experimental C3 glomerulopathy is a novel and unexpected one. It is especially intriguing since progressive renal disease in Cfh-/− mice is attributable to accumulation of iC3b, a major CD11b ligand, along the GBM. Hence one possible explanation for my results is that CD11b ligation by abundant glomerular iC3b in Cfh-/− mice mediates partial protection. This would reconcile the observation in a previous study of spontaneous GN in SLE-prone MRL/MpJ-Fas− mice of no difference in severity between those with and without CD11b deficiency [Kevil et al. 2004]. iC3b is not implicated in the lupus model, in which severe GN in older mice was characterized by crescents, hypercellularity, mesangial expansion and neutrophil accumulation. In Cfh-/− mice, I have suggested that CD11b+ macrophages are likely to play a key role in glomerular protection. This is based on a body of previous work showing that ligation of CD11b on macrophages by iC3b and other molecules suppresses proinflammatory immune responses, including via negative regulation of TLR signalling.

In Chapter 4, further evidence in support of a protective effect due to CD11b was provided using the ANTN model of immune complex GN. Cfh-/−.CD11b-/− mice showed enhanced susceptibility to glomerular thrombosis at day 2 compared to their Cfh-/− counterparts. Following the completion of the experimental work presented in this thesis, a different model of immune complex GN was reported that showed increased severity in Cfh-/− mice that received a CD11b-/− bone marrow (BM) transplant compared to a Cfh-/− or WT BM transplant [Alexander et al. 2015]. The chronic serum sickness (CSS) model used in that study involves repeated daily i.p. administration of horse spleen apoferitin for 5 weeks. This leads to the appearance of anti-horse apoferitin IgG and immune complexes in plasma and mouse IgG within the glomerulus. Cfh-/− mice (on a C57BL/6 background) had previously been shown to be susceptible to proliferative GN using this model, as characterized by diffuse glomerular hypercellularity and crescents in ~6% of glomeruli [Alexander et al. 2005]. BM transplantation studies were also suggestive of protection against glomerular immune complex deposition, but not proliferative GN, due to FH expressed on platelets [Alexander et al. 2006]. This was attributed to the role of platelet FH as the immune adherence receptor, analogous to human erythrocyte-expressed CR1.
Induction of CSS in Cfh-/- mice 4 weeks after BM transplant led to increased plasma anti-apoferritin IgG titres, circulating immune complexes and extension of mesangial immune complexes in CD11b/-/- chimaeras compared to Cfh-/- or WT chimaeras [Alexander et al. 2015]. This was associated with increased albuminuria, plasma urea and glomerular hypercellularity in the CD11b/-/- chimaeric mice. Hence these results were broadly consistent with my data showing exacerbation of experimental GN due to CD11b deficiency in Cfh-/- mice. Glomerular macrophages were not specifically assessed in the CSS model, but immunophenotyping of tubulointerstitial macrophages revealed an increase in classic, pro-inflammatory (M1) macrophages in the CD11b/-/- chimaeras.

An unexpected finding in my first ANTN experiment was that CD11b deficiency was significantly associated with day 1 haematuria irrespective of the FH genotype of the mice. I therefore switched the focus of my study away from experimental C3 glomerulopathy, to study the phenotype of CD11b deficiency in ANTN. I showed that ANTN in CD11b/-/- mice is characterized by severe crescentic nephritis at day 10. I concluded that CD11b could mediate a protective effect in ANTN not only via its interaction with iC3b on macrophages, but also via its reported role in suppression of DTH and Th17 immune activation. It is perhaps surprising, therefore, that glomerular macrophages, as major effector cells of DTH-like immune responses, were not significantly increased in CD11b/-/- mice. However previous ANTN studies (in C57BL/6 mice) have also shown no difference in CD68+ glomerular macrophage recruitment in Fas-ligand-deficient mice [Tarzi et al. 2012] and leukocyte specific FcRγ/-/- mice (both of which were protected from crescentic GN induced in WT mice). In the latter study, the authors concluded that ‘without FCRγ stimulation, the infiltrating macrophages were not activated to induce glomerular injury’ [Tarzi et al. 2002].

Similarly, my results do not exclude the possibility that, in the absence of CD11b ligation, macrophages recruited to the glomerulus following induction of NTN are unable to mediate important protective effects. It is recognised that macrophages are a heterogeneous cell population. That their deleterious effects in experimental models of GN including ANTN are more familiar may be partly explained by methods of immune induction that favour distinct macrophages subsets/functions. This is exemplified by a study in transgenic mice that express the human diphtheria toxin receptor under the control of the CD11b promoter. Macrophage ablation through administration of diphtheria toxin on day 15 after ANTN induction was associated with a subsequent reduction in crescents and renal failure [Duffield et al. 2005]. As glomerular macrophages appeared to comprise both proinflammatory and reparative subtypes, the authors concluded that the effects of macrophage depletion were dependent on its timing in relation to initiation and recovery of glomerular injury.
I proposed one additional mechanism by which CD11b may prevent severe glomerular injury in ANTN, namely suppression of CFA-mediated proinflammatory signalling, including that due to TLR stimulation. One other CFA-based protocol showed increased severity of immune complex GN due to CD11b deficiency in lupus-prone transgenic mice using the lupus passive transfer model [Rosetti et al. 2012]. Of note, increased day 14 albuminuria was also observed in 2 out of 7 sensitized CD11b-/- mice compared to none out of 3 WT mice (or a total of 16 CD11b-sufficient mice) [Rosetti et al. 2012]. This difference was not statistically significant, whilst other parameters of glomerular injury were not reported.

In my ANTN and heterologous NTN experiments, I did not find any difference in the antigen-specific IgG immune response between CD11b-deficient and CD11b-sufficient mice. A recent study has suggested that CD11b plays a role in negative regulation of B-cell receptor (BCR) signalling [Ding et al. 2013]. CD11b deficiency was associated with increased B cell proliferation and survival following BCR ligation in vitro using B cells from anti-snRNP Ig transgenic mice. In vivo, weekly i.v. administration of apoptotic thymocytes for 9 weeks was associated with increased serum IgG antinuclear antibody (ANA) and anti-snRNP antibody levels, and increased glomerular mouse IgG deposition, in the transgenic mice with CD11b deficiency compared to those without. In the CSS model, increased severity of proliferative GN in Cfh-/- mice receiving a CD11b-/- BM transplant correlated with increased anti-apoferritin IgG titres [Alexander et al. 2015]. Both the thymocyte and CSS studies utilized repeated antigen administration (without adjuvant) and a longer course (5-13 weeks) than the ANTN experiments, and hence may be more sensitive models for assessing IgG immune responses. Notably, however, CSS (without BM transplantation) did not produce proliferative GN in CD11b-/- mice (notwithstanding significantly increased albuminuria compared to WT mice), nor were differences observed in the anti-apoferritin IgG immune response between CD11b-/- and WT mice.

In summary, the results of these ANTN experiments are in agreement with several previous studies showing protection due to CD11b in severe, immune complex GN [Rosetti et al. 2012, Shi et al. 2013, Alexander et al. 2015]. By contrast, earlier hNTN studies indicated that CD11b plays a proinflammatory role in acute GN [Tang et al. 1997, Hirahashi et al. 2009]. However, those studies specifically addressed early, neutrophil-dependent glomerular injury, which was either mild [Tang et al. 1997] or mimicked features of HUS, in the setting of LPS co-administration with NTS [Hirahashi et al. 2009]. Hence the protective effects of CD11b in my ANTN experiments occurred within the specific immune context of DTH and Th17 immune differentiation, which are of undoubted importance in GN, and of CFA-mediated proinflammatory responses.
In Chapter 5, I showed for the first time that a mini-FH protein is able to regulate the AP in vivo, effectively reversing glomerular C3 accumulation and transiently increasing plasma C3 levels in *Cfh/-* mice. Given the robust association of FH deficiency/dysfunction with severe, progressive renal disease, therapeutic evaluation of full-length recombinant FH has long seemed merited. However this has been technically challenging due to the size and complexity of the FH protein. By contrast, production of recombinant mini-FH is technically feasible and may offer therapeutic advantages over full-length FH. For example, targeting of the therapeutic effects of mini-FH to the glomerular site of C3 activation may be superior. As mini-FH lacks SCR domains 6 and 7, which are important binding sites for microorganisms, particularly meningococcal strains, as part of complement evasion [Blom et al. 2009, Caesar et al. 2014], it may also confer a favourable infection risk profile.

A novel finding in my mini-FH experiments is that the reduction in glomerular C3 staining was associated with detection of administered FH or mini-FH in a linear staining pattern within the glomerulus. This could be consistent with a requirement for surface recognition domains, as an FH1-5 protein lacking these domains had no effect on glomerular C3. However, mini-FH was also shown to have a modest effect on fluid phase AP control, which therefore cannot be excluded in glomerular protection. That FH1-5 demonstrated neither significant plasma nor glomerular C3 effects may thus have been simply a matter of insufficient doses. Importantly, both mini-FH and FH1-5 were rapidly cleared in vivo, which could potentially limit their clinical utility.

In chapter 6, I showed that the purified GBM components (collagen type IV, fibronectin and laminin) do not provide an AP-activating surface in vitro. Several previous studies have suggested that GBM constituents may induce C3 activation. One early study used a purified human GBM preparation to assess ‘the possibility of a local activation of complement by components of renal glomeruli…in vitro with glomerular basement membranes’ [Lambert et al. 1974]. C3 activation was demonstrated in NHS only if the GBM preparation first underwent partial proteolytic digestion with pronase. After collagen type IV-like soluble glycoproteins had been isolated from the GBM preparation, protein concentration-dependent C3 activation was also demonstrated, with dependency on both the AP and CP. The authors concluded that ‘GBM altered in vivo by some proteolytic processes or solubilized GBM fragments may act as local activators of the complement system, proceeding through both the classical or alternate pathways of the complement system’. In a later study, incubation of a human GBM preparation in NHS or C2-depleted human serum was associated with a ~threefold increase in AP-dependent C3 deposition compared to FD-depleted human serum [Williams et al. 1984]. This could be inhibited in C2-depleted serum through the addition of supraphysiological purified human FH and FI. Immuno-EM revealed C3 on the epithelial, but
not endothelial, aspect of the GBM. The authors concluded that ‘isolated and intact human GBM serves as a surface activator of the human alternative C [complement] pathway, as assessed by the deposition of C3b...compatible with an endogenous activating capacity of the native surface’. However, C3 deposition was not compared using any other surface, and C3 activation attributed to the GBM was modest (‘2.2% of the activating activity of E’ [E9] and 0.09% of the activating activity of zymosan’ [Williams et al. 1984]). Hence, the possibility that C3 tickover was solely responsible for C3 deposition was not excluded. By contrast, one other study showed that incubation of a rat renal tubular fraction in either rat serum or NHS resulted in C3 activation via the AP, whereas this was absent using a glomerular fraction [Camussi et al. 1982].

In a widely cited study, AP-dependent C3 deposition following incubation of NHS was demonstrated on subendothelial ECM from which the HUVEC monolayer had been removed [Hindmarsh & Marks 1998]. C3 deposition on the ECM surface co-localized with the ECM components fibronectin and collagen type IV. NHS was then incubated in culture wells coated with HUVEC, cell-free subendothelial ECM, Matrigel (a soluble matrix preparation containing collagen type IV, laminin, entactin and heparan sulfate proteoglycan) or purified human ECM components. ELISA-based detection of surface-bound C3 was increased for ECM compared to either HUVEC or gelatin (negative control). C3 detection was also increased for Matrigel or vitronectin, with a slight increase for fibronectin, but not for laminin or collagen type IV. Finally, C3 detection was increased for ECM but not HUVEC following incubation with purified hC3 if a prior incubation was performed using purified hC3 and AP components rather than NHS. This was attributed to formation of a stable C3 convertase in the absence of serum-derived regulatory proteins. Nevertheless, two incubations using NHS led to increased C3 detection for ECM (but not HUVEC) compared to a single NHS incubation. An important weakness of the study is that gelatin was not also assessed using the purified AP components. For example, had this shown similar C3 activation to ECM, then the increased C3 activation due to ECM in comparison to both HUVEC and gelatin using NHS would not have been replicated. The authors’ conclusion that ‘spontaneous activation of complement on ECM suggested that the alternative pathway was responsible, and this interpretation was supported by the finding that purified alternative pathway components could reproduce the complement activation observed with whole serum’ [Hindmarsh & Marks 1998] is therefore unsatisfactory.

I further showed in chapter 6 that FH depletion of human serum attenuates, but does not abolish, surface phase C3 activation via the AP. The implication in patients with DDD is that C3 accumulation could plausibly be due to chronic, low-level opsonization and C3b amplification along the GBM. However, the finding that C3 activation in the absence of FH
predominantly occurs in the fluid phase suggests that this may also be the source of glomerular C3 fragments including iC3b. If surface phase C3 activation is indeed attenuated in the in vivo setting of FH deficiency/dysfunction, this could provide an additional explanation for those surface-based assays (classically, rabbit haemolysis) showing AP dysfunction in patients with DDD. It may also account for the absence of tubulointerstitial C3 in Cfh−/− mice, resolving the paradox wherein ‘activation of C3 onto the basolateral aspects of tubules requires an intact systemic alternative pathway, including its main regulator, Cfh’ [Alexander et al. 2007]. A similar solution concerns the Cfh−/−.FHΔ16-20 mouse model, which provides evidence that ‘FH mutations specifically impairing surface recognition can result in spontaneous aHUS’ [Pickering et al. 2007]. The absence of spontaneous renal TMA in both Cfh−/−.FHΔ16-20low mice, in which plasma FHΔ16-20 levels were only 2% of WT FH levels, and Cfh−/− mice was attributed to a requirement for ‘effective plasma C3 regulation’ if spontaneous renal TMA was to occur [Pickering et al. 2007]. Absence of a renal lesion (TMA) that is dependent on surface phase C3 activation via the AP could thus be attributable to a relative surface phase defect in these strains.

The presence of a surface phase defect sufficient to prevent tubulointerstitial C3 deposition and renal TMA in Cfh−/− mice would suggest that C3 accumulation along the GBM is equally unlikely to be due to surface phase C3 activation. In my in vivo experiments in Chapter 5, I initially noted this dissociation of glomerular and tubulointerstitial C3 following hFH administration in Cfh−/− mice. I further demonstrated that tubulointerstitial C3 staining was not apparent at any stage during the evolution of experimentally triggered C3 accumulation along the GBM in FI-reconstituted Cfh−/−.Cfi−/− mice. I then assessed the dissociation of glomerular and tubulointerstitial C3 deposition ex vivo through serum incubation of murine cryosections. Here again, FH depletion of human serum markedly attenuated tubular C3 deposition, as a marker of surface phase C3 activation. At the same time, it enhanced glomerular C3 accumulation, although the mechanism by which this occurred remains unexplained. As discussed above, these data do not exclude the contribution of a gradual process of surface phase C3 activation to C3 accumulation along the GBM in patients with DDD.
8. CHAPTER EIGHT: FUTURE WORK

No treatment is proven to be effective in DDD. My experimental results in \textit{Cfh-/-} mice indicate that pharmacological potentiation of CD11b could be an effective strategy in patients with DDD. Preclinical evaluation of small-molecule, synthetic CD11b agonists (so-called leukadherins) has shown promising results in a number of animal models of glomerular disease [Kanagavelu et al. 2013, Khan et al. 2014]. This includes murine heterologous NTN where, paradoxically, leukadherin administration was more effective than an inhibitory anti-CD11b antibody in reducing peak glomerular neutrophil influx at 3 days in C57BL/6 mice [Maiguel et al. 2011]. This may have been related to the ability of leukadherins to trap neutrophils in a ligand-binding conformation during adhesion, preventing de-adhesion and migration. In my future work I would aim to assess the therapeutic effect of leukadherins in the FH-deficient mouse model of C3 glomerulopathy. Administration of leukadherins prior to induction of ANTN in \textit{Cfh-/-} mice would be one valid experimental approach.
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