The therapeutic utility of Factor I in the treatment of complement dependent pathophysiological processes

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Abstract

The complement system is an important defence system of our body. Of the three complement activation pathways, the alternative pathway is continuously activated at a low rate by a mechanism called tick-over. The alternative pathway is governed by the relative rate of two competing cycles, the C3b feedback and breakdown cycle. Correct regulation of the alternative pathway is essential to prevent damage and polymorphisms in alternative pathway regulation are increasingly associated with (particularly age-related) diseases.

Factor I is unique in that it irreversible inactivates C3b. Raising the concentration of Factor I, slows down the rate of the C3b feedback cycle while the C3b breakdown cycle will be accelerated.

Renal ischemia is an inevitable, injurious event during renal transplantation but can also occur as a consequence of impaired kidney perfusion and it is known that the alternative pathway exacerbates injury although recent data highlight the importance of a lectin pathway-mediated activation mechanism in the reperfusion period. To test the effect of increased Factor I plasma concentration, recombinant Factor I was generated and tested for functional activity. Administration of mouse Factor I reduced mortality and renal injury in a mouse model of renal ischemia reperfusion injury when compared to administration of a control protein.

Factor I was also over-expressed *in vivo* as a gene therapy via an adeno-associated virus expression system. By titration of administrated virus particles, the levels of Factor I in mice could be raised up to 4x the normal concentration.

In order to diagnose and test therapeutic progress of a future therapy for early age-related macular degeneration, a new, fast and reliable method is required. A ScFv mini antibody was generated that specifically recognises iC3b, a major opsonin and marker of inflammation, and C3dg. This biomarker is intended for fluorescent detection of complement activation products in the retina.

Declaration

This accompanying dissertation submitted for the degree of PhD entitled “The therapeutic utility of Factor I in the treatment of complement dependent pathophysiological processes" is based on work conducted by the author at the University of Leicester mainly during the period between January 2012 and January 2013 and at the University of Cambridge during the period between January 2013 and November 2015.

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in these, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements.

This dissertation contains fewer than 50,000 words excluding bibliography and has fewer than 150 figures.

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Chapter 1

Introduction

The immune system represents a vital physiological component of living organisms, protecting them from diseases and infections. It is composed of an innate and adaptive arm, both of which rely on cellular and humoral effector components. The complement system is a major component of both innate and adaptive immune response and consists of a series of blood-borne factors.

The first description of the complement system goes back to the end of the 19th century, when bacteriocidal activity in normal serum was first observed. These studies of antimicrobial immunity followed the work of Pasteur and Koch. In 1895, Bordet found that the antibacterial activity in serum is heat-labile and that it is dependent upon but distinct from antibodies [1]. Bordet and Gengou first described complement fixation in 1901 [2] and Bordet soon found that complement was a substance rather than merely an activity in serum and even more importantly, first introduced the idea that it was not a single substance but consisted of several components [3]. A few years later, it was found that complement can also be activated by yeast and cobra venom [4, 5]. It soon became apparent that complement indeed consisted of more than one component, and by the 1930, these components had been described in the order C1, C2, C3, C4. [1] Up to the 1960, no attempt to purify these components was undertaken

[1] The complement components were numbered in the order of their discovery, before the pathway sequence was established. Thus, their numbers do not correspond to the actual sequence of events.

because the proposed "one hit theory" [2] of Mayer underestimated the concentration of complement by several orders of magnitudes which discouraged biochemical studies to undertake their purification [7, 3]. Nevertheless, the identification of C3 as β1c-globulin, a major plasma protein, by Muller-Eberhard et al. in 1960 changed the whole field [8] and isolation of complement proteins allowed characterisation of their biological activities and interactions. Soon the components of the terminal pathway were also purified and characterised.

Early studies already indicated that complement can also be activated independently from antibodies, for instance by yeast or cobra venom [5], but the significance of these results was not appreciated until Pillemer and his lab discovered a further factor, Properdin, and claimed that this was an essential factor of another complement pathway. This "Properdin" pathway required two further components, Properdin Factor A and Properdin Factor B, to enable activation on (typically) zymosan and some other carbohydrates [9].[3] The latter discovery was made in the 1950ies and resulted in great controversy in the complement field (especially between Nelson and Pillemer), whether the proposed new "Properdin" pathway is an artefact or a physiological distinct mechanism of complement activation. Unfortunately, Pillemer died before it became clear that there was indeed a pathway that functioned without the classical complement components, i.e. C1, 2 and 4. This was made possible with the discovery of animals with deficiencies in the classical pathway in the late 1960s [12]. The original pathway was then called classical pathway and the new mechanism alternative pathway.

In the late 60s, patients, usually children, with a defect in phagocytosis where described which is due to lack of opsonisation and these observations were eventually linked to low levels of Mannose-Binding Lectin (MBL) [13]. In 1982, researchers in Japan observed that carbohydrate residues on invading microorganisms can initiate complement activation in the absence of antibodies by a mechanism unrelated to the alternative pathway [14]. A few years later, Ikeda et al. suggested that rat MBL was able to activate the classical pathway [15] and

[2] If "one hit" (i.e. one complete complement reaction) is sufficient to lyse a cell, far less complement would be required than if complement is dependent on multiple hits [6].

[3] Properdin factor A and B were later found to be C3 and Factor B, respectively [10, 11].

in 1992, a C1s-like protease, MASP, was co-purified with MBL [16]. The exact activation mechanism of the lectin pathway is still somewhat controversial and will be introduced in the section below (1.1.2).

Classically, the complement system can be initiated by these three main pathways which use different recognition molecules and its sequence can be subdivided into several steps which are activation of complement, formation of a C3 convertase and terminal pathways.

1.1 Activation of the complement system

Complement activation is traditionally described as occurring via three different mechanisms, the classical, lectin and alternative pathway, with the latter being mentioned being ubiquitous and recruited by the other two pathways once complement is activated. As depicted in Fig. 1.1, the classical and lectin pathway are activated via a similar mechanism that takes the form of a cascade of proteolytic complexes, while the alternative pathway is based on a horizontal feedback mechanism. All three pathways converge at the cleavage of the abundant plasma protein C3 which enables activation of the terminal pathway as well as other complement effector mechanisms. The next sections will describe these mechanisms and a schematic representation of all three activation pathways is shown in Fig. 1.1.

The majority of complement proteins is synthesised by hepatocytes and released in the bloodstream. By comparison of allotypes of *C3*, *C6* and *Factor B* (*FB*) from donors and recipients after a liver transplantation, Chester Alper was able to demonstrate that hepatocytes synthesise the majority of circulating complement proteins. He found that in the recipient a total and permanent conversion to the donor allotype occurred [17]. Complement components which are not synthesised by hepatocytes are C7, which is made by Kupffer cells [18] and Factor D (FD) which is expressed by adipocytes. C7 therefore, unlike C6, C3, C4, C5 and C9, is not an acute phase reactant and its levels do not rise during acute inflammation. However, in some tissues with limited access to the circulation, an extrahepatic system of complement biosynthesis exists, e.g. in the brain or the RPE/Choroid tisue complex of the eye [19, 20]. Anderson et al. performed quantitative analyses of the neural retina

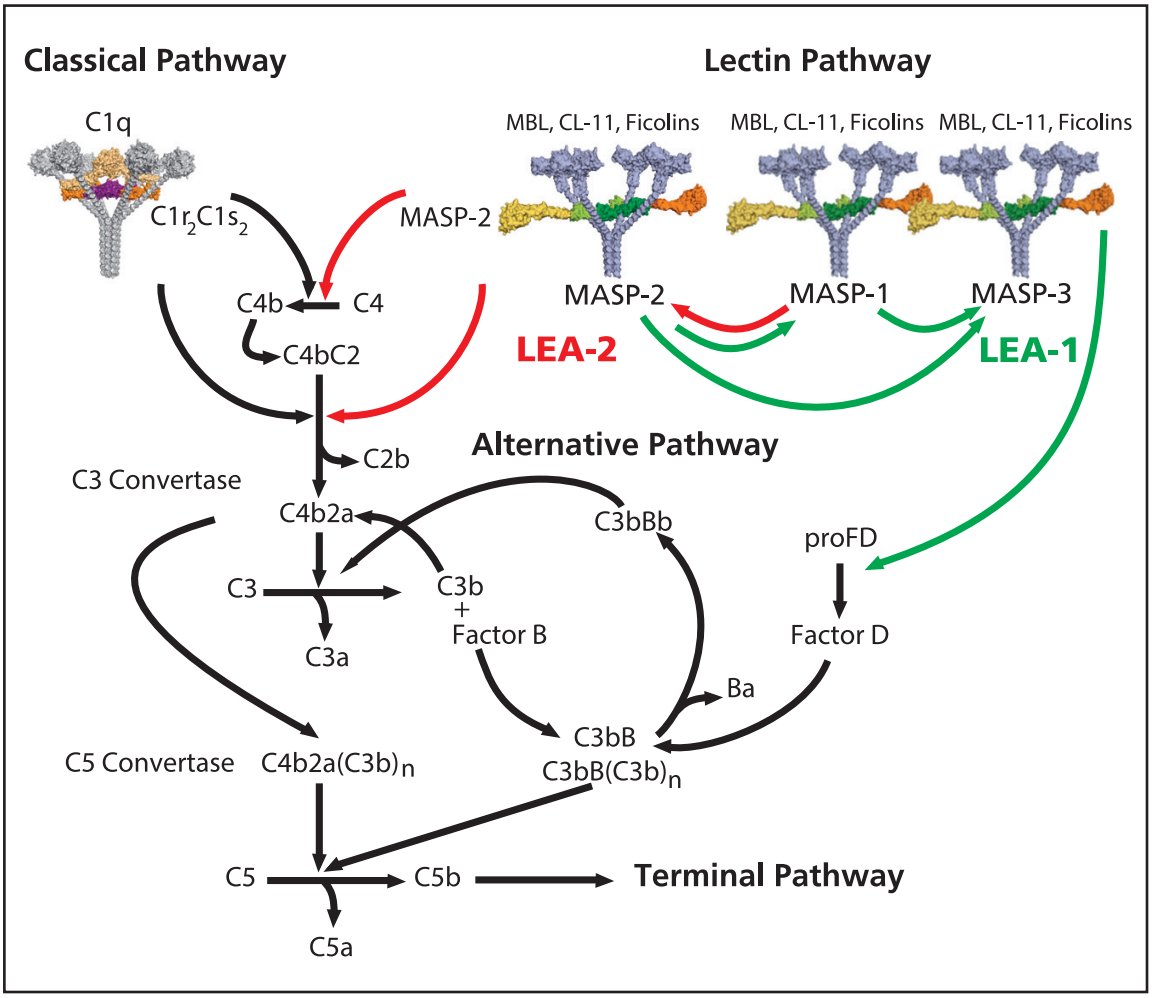

Fig. 1.1 Steps of complement activation. Figure provided by Professor Wilhelm Schwaeble.

and RPE-choroid complement gene expression profiles and found that cells in the human RPE/Choroid complex are able to express the complete set of proteins and regulators required for the classical and alternative pathway, while only little evidence for lectin and terminal pathway gene expression [21]. Tang et al. further reported that around 10% of C3 cannot be attributed to hepatic synthesis [22].

1.1.1 The classical pathway

Although the classical pathway was the first to be discovered, evolutionary it was developed most recently, because it requires immunoglobulins, IgM or IgG, for activation which first

arose in jawed vertebrate [4]. The classical pathway is activated by binding of its only recognition molecule, C1q, that consists of a hexamer of heterotrimers, each of which has an N-terminal collagen-like domain and a C-terminal globular region. C1q is a pattern recognition molecule that binds to the Fc-tail of immunoglobulins (IgG and IgM), acute phase proteins (C-reactive protein, pentraxins,...), DNA, bacteria, viruses and apoptotic cells or necrotic cell debris [23]. The C1 complex consists of one C1q and two C1r and C1s molecules and is a Ca^{2+}-dependent complex that otherwise dissociates. In order to prevent spontaneous auto-activation of the two pro-enzymes, C1r and C1s, the C1 complex is inhibited and controlled by C1-Inhibitor (C1-INH).

Binding to IgM does not occur when freely circulating but requires binding to antigen and subsequent reorientation of the Fc regions to enable C1q binding. Contrary, Fc regions of IgG are available in freely circulating IgG molecules but activation of C1 occurs only when at least two of the six monomers of C1q bind to Fc-regions. This requires at least two IgGs binding to a surface in close proximity (or 1 IgM). Therefore, IgM molecules which consist of 5 closely spaced Fc tails are much stronger activators of the classical pathway ($\approx$ 100x more efficient than IgG), while the Fc tails are more randomly spaced in IgG binding to a surface and it is less probable to find 2 or more IgG molecules in the correct position to bind and activate the C1 complex. Also, not all IgG subclasses bind equally well, the order from high to lowest binding is IgG3, IgG1, and IgG2 [24]. IgG4 does not activate the complement system.

Once bound to its substrate, a conformational change in C1q probably displaces C1-INH and results in the auto-activation of the serine protease C1r which is associated with the collagen-like tail of C1q. Subsequently, activated C1r cleaves C1s which in turn cleaves C4 to C4a and C4b, the later of which becomes covalently linked to the target surface. Next, C2 binds to C4b, is cleaved by C1s and the larger fragment, C2a, stays attached to C4b. The resulting complex, C4b2a, is called the classical pathway C3 convertase and as the name implies, cleaves C3 molecules to C3a and C3b.

1.1.2 The lectin pathway

The lectin pathway is the latest discovered pathway and its exact activation mechanisms are still controversial. There are several recognition molecules of the lectin pathway which recognise sugars or N-acetyl groups on bacteria, fungi, parasites, viruses or altered self-cells. Thereby, the lectin pathway is activated by pathogen associated molecular patterns (PAMPs) or altered self-structures and thereby becomes an effective initiator of the innate immune system. Currently, in human there are five known recognition molecules which are Mannose-Binding-Lectin (MBL), Collectin-11 (CL-11 or also CL-K1), Ficolin-1, -2 and -3 [25–27]. All have similar structures: an N-terminal collagen-like domain and a C-terminal globular domain that oligomerise to a higher structure [4]. Every recognition molecule has distinct, but overlapping carbohydrate specificities which are likely to broaden the spectrum of microbial structures that initiate a direct response of this arm of the innate immune system [28].

MBL has no binding activity on self cells because its normal binding ligands, i.e. sugar residues like N-acetyl-*D*-glucosamine (GlcNac), fucose and mannose, are usually hidden by sialic acids on self-cell membranes. Sialic acids recruit Factor H (FH) from circulation and bind it for protection from complement attacks. However, in altered e.g. apoptotic cells, these structures become naked and exposed to MBL and probably other recognition sub-components [29]. Ficolins bind to lipoteichoic acid (LTA), lipopolysaccharides (LPS), acetylated groups, GlcNac, N-acetyl-*D*-galactosamine (GalNac), fucose and capsular polysaccharides of bacteria such as *Salmonella typhimurium*, *Staphylococus aureus,* and *Streptococus pneumoniae* or certain parasites, e.g. *Trypanosoma cruzi* [30, 31]. CL-11 also recognizes a variety of carbohydrate structures such as fucose, mannose and GlcNac and it was shown to bind to nucleic acids and some microorganisms including *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* [27, 32]. MBL and Cl-11 require Ca^{2+} for binding whereas Ficolins do not always [33, 34].

Activation of the lectin pathway roughly resembles the classical pathway but instead of C1s, so-called MBL-associated serine proteases (MASPs) cleave C4 and C2. The MASP

[4] MBL and Ficolins resemble C1q and oligomerize "twice", e.g. three monomers form a trimer and assembled trimers oligomerise further whereas Cl-11 has a simpler, trimeric structure.

family consists of three serine proteases, MASP-1, MASP-2 and MASP-3 and the non-enzymatic proteins, MAp19 and MAp44. [5] Each carbohydrate recognition molecule of the lectin pathway associates with either MASP-1 or MASP-2 which are mainly found as homodimers in an anti-parallel orientation in circulation [28]. It is exactly this anti-parallel orientation that keeps the serine protease domain of the MASPs separated so that they cannot interact and activate each other within one complex. In fact, activation of the lectin pathway greatly depends on binding of many lectin pathway initiation complexes in close proximity on activator surfaces. This is necessary to allow transactivation i.e. a MASPs dimer in one complex cleaves another MASP dimer in a neighbouring complex which results in a chain reaction of activation events.

MASP-1 and -2 can autoactivate or activate each other but only the latter has a key function in the activation of the complement cascade and is the effector enzyme of the lectin pathway because of its ability to also cleave both C2 and C4 [35]. Recent studies show that MASP-2 can also directly cleave C3 which is also supported by the fact that C4 deficient mice are not protected from MASP-2 dependent ischaemia-reperfusion injury [35–37]. In the absence of MASP-2, the lectin pathway activation complex is unable to initiate the cascade and this results in complete inhibition of this pathway [35].

The role of the other two MASPs is still controversial and requires clarification. It is clear that MASP-1 cannot initiate the cascade by itself because, although it can cleave C2, it cannot cleave C4 [35]. It is believed to have role as an amplifier of complement activation by cleavage of C2 and by activation of MASP-2, which is also supported by its much higher serum concentration compared to MASP-2. In 2010, it was reported that *MASP-1/3* knockout mice have no detectable alternative pathway activity *in vitro* due to presence of zymogen (= pro-)FD and that MASP-1 can cleave pro-FD and thereby enable alternative pathway activation [38]. Nevertheless, the cleavage experiments were done in artificial *in vitro* conditions with purified components and cannot be of any physiological relevance because of the absence of plasma serine protease inhibitors, such as α_2-macroglobulin or C1-INH. It is now known that C1-INH inhibits MASP-1 and -2 *in vivo* but not MASP-3 which results in a

[5] MASP-1, MASP-3 and MAp44 are encoded by the same gene, *MASP-1/3* and are alternative splice products as well as MASP-2 and MAp19 which are both encoded by the *MASP-2* gene [28].

longer half-life of activated MASP-3. Therefore, MASP-3 could be responsible for activation of FD once it is secreted and thereby enable alternative pathway activation. Another role of MASP-3 arises from a link between mutations in the *MASP-1/3* gene and the rare autosomal recessive 3MC syndrome (3MC = Mingarelli, Malpuech, Michels and Carnevale), which is characterized by various developmental disorders [39, 40]. It was found that MASP-3 has an unexpected but important role in early developmental processes. Mutations in *CL-11* have also been linked to 3MC-syndrome, suggesting a possible interaction of CL-11 and MASP-3 in this physiological context [40]. A regulatory role has also been suggested for MASP-3 (together with MAp44 and MAp19), because of their potential ability to compete with MASP-1 and -2 for interaction with the recognition proteins [41, 42]. In summary, all roles of MASP-3 require clarification which is hopefully done in the near future.

1.1.3 The alternative pathway

The alternative pathway of the complement system functions slightly different than the other two activation mechanisms and is in evolutionary terms a very old immune mechanism because already present in insects and echinoderms [43].

In contrast to the classical and lectin pathway, the alternative pathway has no distinct initiation event but is rather governed by a balanced rate of two distinct reactions: C3b amplification and breakdown. The alternative pathway does not require specific antibodies to recognise potential pathogens but performs a continuous surveillance function and is under strict control of plasma and membrane bound regulators (further described in Sec.1.3). Activation of the alternative pathway occurs on surfaces where the effect of these regulators is dampened, e.g. on bacteria, fungi, certain viruses, virus infected cells, some tumour cell lines and erythrocytes from other species [44].

Today, it is generally accepted that the alternative pathway is activated either by one of the other two complement pathways (positive feedback amplification) or by so-called "activated C3" (i.e. C3(H_2O)) or C3b which is constantly generated in serum at very low concentrations by mechanisms responsible for the "tickover". The tickover hypothesis was first postulated after *in vitro* replication of a Factor I (FI) deficient phenotype (summarized

in [43]). Before, a patient was presented in a Boston hospital who had recurrent bacterial infections and an inactive complement system: FB was circulating as Ba and Bb, C3 was cleaved to C3b in serum and deposited on red blood cells. Coincidentally, at the same time, complement FI was described for the first time and it was proposed that this patient might be FI deficient which turned out to be correct. Treatment with FI transfusions restored the patient's complement functions for up to 2 weeks [45]. Lachmann and Nicol then depleted serum of FI in the cold without activating complement [46]. [6] It was noted that as soon as serum was heated up again, C3 and FB were immediately converted to C3b and Bb. There was no lag phase or an activating factor which was required for initiation of the alternative pathway. From this experiment, it was concluded that the alternative pathway is controlled solely by the relative rates of amplification and breakdown loops. In analogy to an idling car engine, the term tick-over was introduced to describe the permanent active state of the system [46].

For the alternative pathway convertase (i.e. C3bBb), a C3b molecule is necessary which raises the question how the initial convertase is formed in the absence of the classical or lectin pathway. Native C3 cannot react with FB but the mere absence of the regulator FI was shown to be sufficient to induce alternative pathway activation which led to the conclusion that there must be some so-called "activated C3" that has C3b-like properties and is capable of binding FB. A very elegant explanation was provided by Pangburn and Muller-Eberhard, who proposed that nucleophilic modification and scission of a thioester in C3 results in a molecule with C3b-like properties [48]. C3 belongs to the family of α_2-macroglobulins which are characterized by an internal thioester. The thioester in C3 is formed between the side chains of cysteine-988 and glutamine-991 (Fig. 1.2b) and is orientated inwards between the thioester domain (TED) and the MG8 domain [49]. In native C3 (Fig. 1.2a), the thioester is hidden in a hydrophobic pocket between the TED and MG8 which is stabilized by the anaphylatoxin domain. The interactions between TED and MG8 in native C3 prevent the chemical transition of the thioester into a acyl-imidazole intermediate which is a highly

[6] The feedback cycle of the alternative pathway is extremely temperature sensitive and is not active in the cold but can only be activated at elevated temperatures (above 30°C). This is in contrast to the classical pathway where it is quite usual to do complement fixation reactions in the cold overnight.

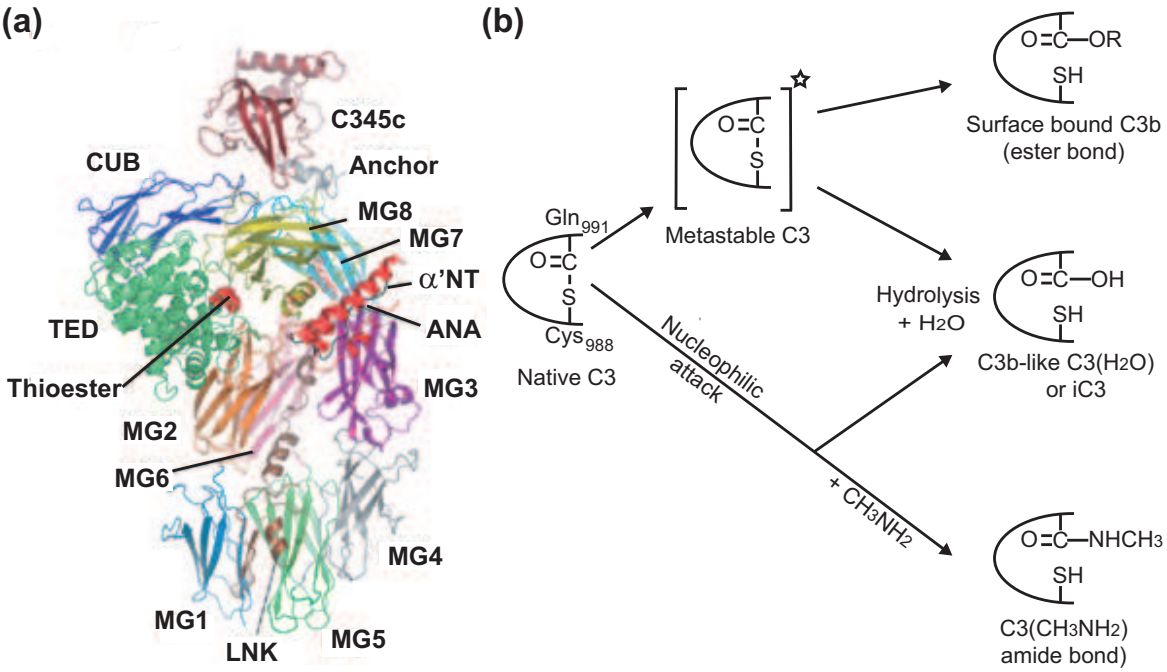

Fig. 1.2 Complement C3 structure and its thioester. (a) Ribbon structure of native C3 showing all its 13 domains. The thioester (red) is hidden inside the molecule in a hydrophobic pocket. (b) Proposed chemical reaction for activation of native C3. Once C3a is cleaved off, C3 is in a metastable state that is quickly either inactivated by hydrolysis or attaches to a nearby surface. If native C3 is altered by nucleophilic attack or hydrolysis, the molecule becomes C3b-like in that the scission of the thioester enables FB or FH to react with the it. Figures adapted from [47, 44].

reactive species [50]. Removal of the anaphylatoxin domain (i.e. C3a) causes the MG7 and MG8 domains to swivel, the new α'nT to relocate, and the CUB domain and TED to swing out [51]. Consequently, the thioester is exposed to the environment and is converted into the highly reactive acyl-imidazole intermediate which may react with any accessible nucleophile and leads to C3b opsonisation of target and nearby surfaces. Hydrolysis of native C3 with water or nucleophilic attacks by molecules such as ammonia, methylamine, ethylamine or free amino acids in plasma can break the thioester and result in a molecule called C3(H_20) or C3(CH_3)NH_2 which can be bound by both, FB and FH [44] (Fig. 1.2b). These new C3 molecules are sometimes also called iC3 (for inactive) or C3(H_2O) and are C3b-like proteins that still have C3a attached but a cleaved thioester bond. Nevertheless, the exact nature of "activated" C3 is not absolutely clear and even though Pangburn's hydrolysis hypothesis is

widely accepted, it has never been proven experimentally, because of its minute amounts and rapid FI degradation. It is therefore also possible that other mechanisms play a role in maintaining the tickover. First, it has to be pointed out that the lectin and classical pathway are the most important mechanisms in maintaining the alternative pathway which merely acts as an amplification mechanism once complement is activated; therefore it is possible that C3 convertases formed in minute amounts by the other pathways account for the tickover. Alternatively, it is also reported that some enzymes (Factors Xa, XIa, thrombin, and plasmin) of the coagulation cascade have the ability to cleave C3 into C3a and C3b [52]. Elastase, derived from polymorphonuclear leucocytes, can cleave C3 as well [53]. Therefore, it is still not absolutely clear what maintains tickover concentrations of activated C3 in serum or at what relative rate but it is accepted that it is mainly the location of the thioester which renders C3 “activated” or “inactivated”.

Summarised, the tickover provides the required minimal amounts of activated C3 that can be bound by FB which is further cleaved by FD to form the initial C3 convertase complex, $C3(H_2O)Bb$, that can convert native C3. Once C3a is cleaved and removed, the remaining C3b molecule undergoes a structural rearrangement that enables the serine protease FB to bind to C3b. FD then can join the complex and cleave FB into Bb and Ba. FD does not cleave FB in serum unless FB is bound to either C3b or $C3(H_2O)$ [54]. The newly generated C3b will bind covalently to the surface of pathogens (or any other nearby surface), where it can be bound by another molecule of FB. This binding is stabilized by Properdin that increases the half-life of the alternative pathway C3 convertase by antagonizing the functional activity of FH.

Apart from the missing recognition molecule and the described mechanism, the alternative pathway has several other unique properties. As mentioned before, it can only work at temperatures over 30°C and also only at high serum concentrations (more than 20% serum in physiological buffers). This is probably not very relevant for *in vivo* situations but enables measurements of the classical (and lectin) pathway only by simply using higher dilutions for *in vitro* assays. The limiting factor for alternative pathway activation at higher dilutions is probably Properdin because it was shown that addition of recombinant Properdin (although

this is much more active than plasma purified Properdin) can restore alternative pathway activation at low serum concentrations [55]. Further, the alternative pathway can also be inactivated by raising the temperature to 50°C for 30 minutes, because of the heat-lability of FB. In contrast to the classical pathway and partially the lectin pathway, the alternative pathway only requires Mg^{2+} but no Ca^{2+}, which enables *in vitro* assays for measurement of the alternative pathway only, i.e. by the use of Mg^{2+}-EGTA buffers. A "usual" alternative pathway buffer thus consists of barbital buffered saline with 10 mM EGTA (preferentially chelates Ca^{2+}) and 2 mM Mg^{2+}. Concentrations higher than that will spontaneously activate the alternative pathway [56] and can lead to artefacts of *in vitro* results. Substitution of nickel with magnesium ions *in vitro* produces a more stable C3bBb convertase [57].

1.1.4 The terminal pathway

The terminal pathway starts with the formation of a C5 convertase which consists of a C3 convertase with an additional C3b molecule integrated into the complex, i.e. C4b2aC3b (classical and lectin pathway) or C3bBbC3b (alternative pathway). The resulting C5 convertase cleaves C5 into C5a and C5b. The anaphylatoxin, C5a, is a potent chemoattractant and triggers inflammation and activation of leukocytes, including polymorphonuclear neutrophils (PMNs), which is exacerbating local injury. One molecule of C5b binds one molecule of C6, and the C5b,6 complex then binds one molecule of C7. A conformational change in the complex results in exposure of a hydrophilic site in C7 which subsequently inserts into a nearby membrane or lipid bilayer. When this insertion occurs on a cell that does not otherwise have any deposited complement products, it is called the "innocent bystander phenomenon". C8 which is a complex of two proteins, C8β and C8α-γ, binds to C5b,6,7 via C8β, and thus enables a hydrophobic domain in C8α-γ to insert into the lipid bilayer. The formed C5b,6,7,8 complex can already cause slow and inefficient lysis of the cell. C8α-γ induces the polymerization of 10 to 16 molecules of C9 into a pore-forming structure called the membrane attack complex (MAC) which has a hydrophobic external face, allowing it to associate with the lipid bilayer, and a hydrophilic internal channel. Disruption of the lipid

bilayer leads to loss of cellular homoeostasis, disruption of the proton gradient across the membrane and finally to osmotic lysis of the affected cell or bacterium.

Nucleated cells are difficult to be killed by MAC because they resist lysis because of ion pumps that counter ion influx and they can also bud off or internalize and degrade the affected, MAC containing membrane. Nevertheless, MAC deposition does not only cause lysis but also triggers increase in intracellular Ca^{2+} concentrations which activates the NLRP3 inflammasome [58]; danger signals are subsequently translated to inflammatory responses by generation of IL-1β and IL-8.

1.2 The function and downstream effector functions of complement activation

As described before, the term complement refers to a group of proteins in the plasma that serve as the source for a large variety of biological activities. The complement system forms a first line of defence against infections by triggering inflammatory responses to alert the immune system to impending danger [59]. It has an essential role in tagging microbes and infected or damaged cells to promote their killing by lysis or phagocytic clearance. Other tasks extend to the removal of apoptotic cells or immune complexes and to the modulation of adaptive immune responses.

In general, effector functions of complement begin with activation of C3 which is considered to be the most important of the phlogistic actions of complement. All activation pathways converge at the cleavage of C3 into C3a and b. Before this cleavage (or hydrolysis), C3 is relatively inert and does not bind to any receptors [50].

Anaphylatoxins are the smaller, bioactive fragments released from C3 and C5 during complement activation, i.e. C3a and C5a.[7] They are humoral mediators of complement that have pro-inflammatory and immunoregulatory function, e.g. ability to contract smooth muscle tissue, enhance vascular permeability (widening of gaps between endothelial junctions to enable immune cells to enter the side of infection), chemotaxis (predominantly C5a mediated chemotaxis of polymorphonuclear neutrophils), inflammation (promote release of TNF-α, IL-6, IFN-γ, IL-1β,...), degranulation, release of histamine and recruitment of various leukocytes. Anaphylatoxins exert their effector function by binding to their receptors (C3aR and C5aR) which are found on many different cells, e.g. dendritic cells, activated mast cells, macrophages and polymorphonuclear neutrophils [61]. Activation of neutrophils by C5aR signalling leads to their secretion of elastase which can cleave more C3 but also C5, generating a positive feedback loop [43]. Expression of C3aR and C5aR on both

[7] The term anaphylatoxin was introduced by Friedberger and describes "an activity found in complement-activated serum that produced rapid anaphylactoid-like death when injected into laboratory animals" [60]. These anaphylactic shock-like reactions are mainly caused by C5a.

renal and circulating leukocytes was also shown to contribute to the pathogenesis of renal ischemia-reperfusion injury [62].

One of the most important functions of complement is enhancement of phagocytosis which is mediated by adherence of complement activation products (opsonins) to cells carrying C3 receptors and activation of neutrophils by C5a. The importance of phagocytosis in *in vivo* host defence against bacterial, viral and fungal infection has been clearly indicated by the finding that abnormalities in the complement system or of phagocytic cells lead to a marked increase of infections. In fact, a defect in phagocytosis is often how individuals with complement deficiencies were and still are found in the first place. Individuals with deficiencies in the early complement components or C3 suffer from a broad range of bacterial infections while patients deficient in C5 or later components suffer from a more restricted spectrum of infections [63]. The opsonic activity of particle bound complement activation fragments results from the presence of complement receptors on the membrane of professional phagocytes [64]. C3b is one of the major opsonins of complement and forms a covalent bond with a nearby surface. [8] The presence of C3b but also iC3b on a bacterium, virus, erythrocyte membrane or other particle identifies this particle for adherence to certain cells and can also activate the later components of complement leading to cytolysis. Receptor sites for C3 activation products have been found on a large number of different cell types, including neutrophils, eosinophils, lymphocytes, macrophages or erythrocytes. The biological consequences of this adherence to cells will depend on the cell type to which the particle adheres to. All complement receptors will be explained below and it should be pointed out that not all are used for phagocytosis.

Complement receptor 1 (CR1 or CD35), in human, is mainly expressed on erythrocytes but is also found on phagocytes, and on kidney glomerular podocytes [65]. The primary function of CR1 on erythrocytes is the clearance of soluble immune complexes by binding to the opsonising C3b. This way, immune complexes are transported by erythrocytes to liver (and spleen) where both the immune complex and CR1 are removed from the erythrocyte surface by macrophages and the erythrocyte then continues to circulate [65]. The binding

[8] Both, C3 activation fragments and IgG are the classical opsonins but complement opsonisation allows fast elimination of a pathogen before an adaptive immune response has been mounted.

and removal of immune complexes is a dynamic event: CR1 on erythrocytes binds C3b on an immune complex but also functions as a co-factor for FI. The resulting iC3b is only bound weakly to CR1, which causes release of the complex and binding via a new C3b molecule. This on-off cycle provides an efficient and dynamic system of transport [66]. In human, CR1 is also the only physiological co-factor for the cleavage of iC3b by FI [67]. This reaction is very important because iC3b is by no means an inactive protein as its name implies (iC3b stands for inactivated C3b) even though it can no longer take part in the feedback cycle. In fact, iC3b is an important effector molecule of the complement system that, by reaction with complement receptor 3 on neutrophils, is absolutely essential for the production of much of complement mediated inflammation.

In rabbits, CR1 is present on platelets and mice lack CR1 but express complement receptor 1 related protein y (Crry), a functional analog of human DAF and MCP which functions as co-factor for FI cleavage of C3b and iC3b [68]. Different distribution of CR1 in species obviously plays a role in the pathogenesis and localization of complement-mediated pathology which requires careful consideration in animal models [43] .

Complement receptor 2 (CR2 or CD21) is mainly expressed on B lymphocytes where it preferentially binds to C3dg but also to iC3b and forms a co-receptor complex with CD19 and CD81 [31]. Other CR2 expressing cells are follicular dendritic cells in lymphoid organs and epithelial cells [69]. C3dg binding to CR2 has an adjuvant effect by lowering the threshold for B-cell activation by 1,000 to 10,000 times [70]. The reaction of CR2 with C3dg does not promote inflammation or phagocytosis of the particle. If erythrocytes are coated with C3dg, as compared with iC3b coating, they have a supra-normal lifespan which has been found in studies of patients with cold autoantibody disease [71]. Interestingly, CR2 is also the cell surface receptor of Epstein-Barr virus in human [69].

Complement receptor 3 (CR3, also Mac-1 or CD11b/CD18) and 4 (CR4 or CD11c/CD18) are both specific receptors for iC3b although this is not their only ligand [67]. CR3 is present on monocytes and its expression is up-regulated in macrophage differentiation [65]. Once activated, neutrophils also express large numbers of CR3. CR4 on the other side is also expressed poorly on monocytes but strongly on differentiated macrophages and dendritic

cells. Both, CR3 and 4, induce phagocytosis mainly in conjunction with other stimuli, such as pro-inflammatory cytokines, which activate phagocytic cells [72]. This is why iC3b is considered to be one of the most important inflammators of complement which is supported by studies that show that the development of dense deposit disease is entirely dependent on the generation of iC3b [73].

Another complement receptor, CRIg, is also able to promote phagocytosis of iC3b-coated particles by macrophages [74]. Collectin receptor (C1q receptor), a further phagocytosis receptor, is widely distributed on leukocytes, platelets and endothelium and acts as a receptor for a group of structurally-related complement or lectin opsonins (= the collectins), which include C1q, MBL, Lung Surfactant Protein A (SP-A) and Conglutinin [75, 76].

Another function of complement is formation of a MAC which forms 10 nm wide pores in target membranes [77]. Many pathogens, usually gram-positive bacteria, are able to resist MAC-induced lesions and are resistant to lysis [78]. On the other hand, gram-negative bacteria, because of their thinner cell wall, are very sensitive to lysis. In particular, defence against pathogenic bacteria of the *Neisseria* genus, critically relies on rapid bacteriolysis because of the ability of these bacteria to replicate in neutrophils and to resist intracellular killing.

1.3 Regulation of the complement system

The complement system is a powerful effector mechanism and it is of uttermost importance that it is tightly regulated in order to impede uncontrolled activation but at the same time to enable adequate recognition of foreign particles. Regulation is therefore essential not only on host cells but also to limit the duration of complement activation on microbial cells or antigen-antibody complexes. The importance of complement restriction is highlighted by the large number of regulators identified to date, which exceeds that of the components of the complement cascade [79]. Many regulators belong to one family, termed Complement Control Proteins (CCPs) or Regulators of Complement Activity (RCA) and are encoded by homologous genes, located adjacent to each other in a locus on chromosome 1q32 [80, 81]. Complement regulators can be divided either into fluid-phase and membrane-bound regulators or into their stage of action. Here, regulators that have an effect until a C5 convertase is formed (so-called early regulators) and regulators of the terminal pathways of the complement system will be considered separately.

Although the early components of human and mouse complement cascades are very similar, it is important to note that complement receptors and regulators are not exclusively used interchangeably in these two species [82]. This difference is of particular importance when translating results from mouse to other models or human.

1.3.1 Regulators of the early steps of complement activation

Endothelial cells are in permanent contact with high levels of circulating complement proteins and are also constantly exposed to low levels of activated C3 generated by the tickover. Therefore, self surfaces are protected from complement attack by membrane-bound regulator proteins. Regulators of the early steps of complement activation either inhibit assembly of the recognition complexes, are co-factors for the FI mediated cleavage of C3b or have decay-accelerating function to dissociate C3 convertases. In general, membrane-bound regulators control all three activation pathways and inactivate both C3 and C4 while fluid-

phase regulators are more specific and regulate either the alternative, classical or the lectin pathway [79]. All regulators will briefly be introduced below.

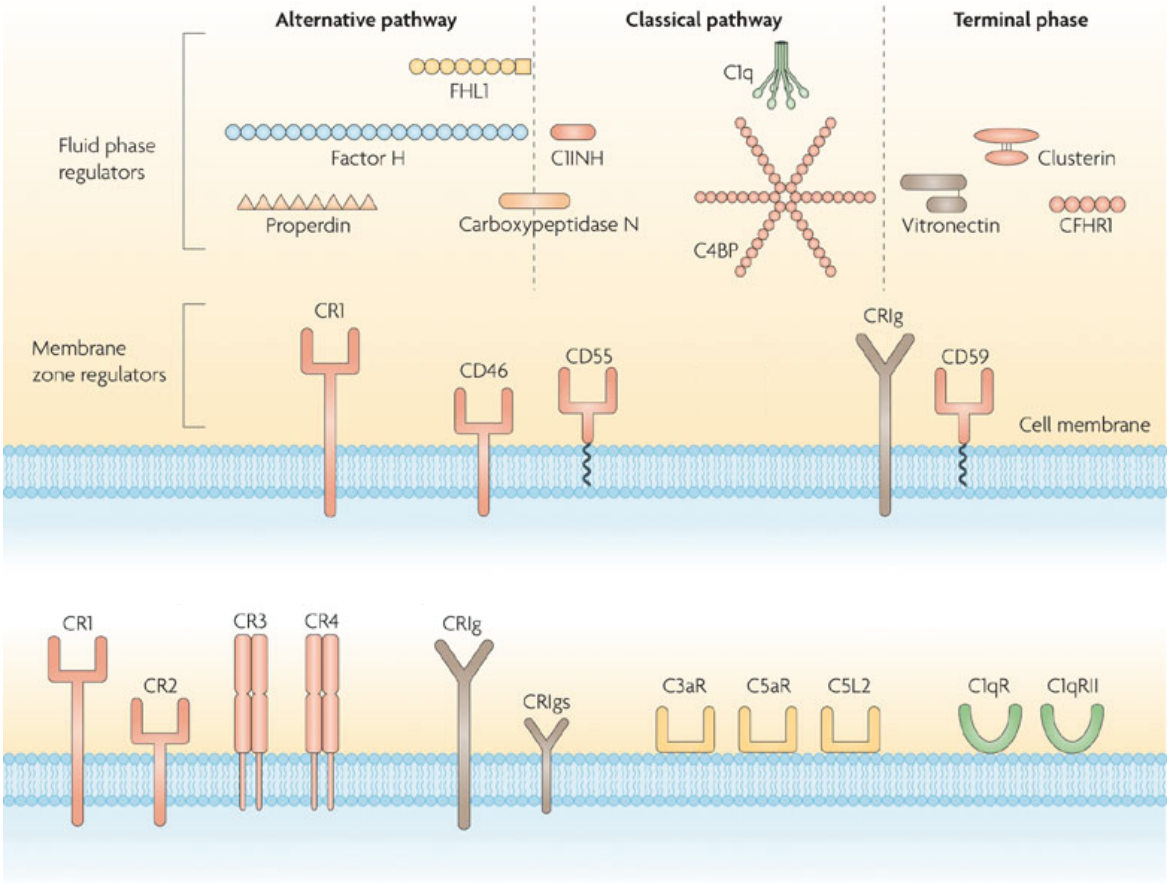

Fig. 1.3 Regulators and receptors of the complement system. Figure adapted from [79].

C1-INH is a serine protease inhibitor that irreversibly binds to and inhibits C1r and C1s in the C1 complex as well as MASP-1 and -2 in the lectin pathway recognition complex [83]. Thereby, it prevents cleavage of C4 and C2. Additionally, C1-INH can also inhibit serine proteases of the coagulation, contact and fibrinolytic system, i.e. Factor XI, XII, thrombin, plasmin, tissue plasminogen activator and kallikrein [84–86]. C1-INH is the most important physiological inhibitor of kallikrein, Factor XIa and XIIa. It is an acute-phase protein whose concentration doubles during inflammation.

Activation of the lectin pathway is further regulated by MAp19 and MAp44, two alternative splice products of the MASP-2 and MASP-1 gene, respectively. They compete with the MASPs for binding to pattern recognition molecules such as MBL and ficolins [41].

Carboxypeptidase N is responsible for rapid inactivation of the anaphylatoxins C3a and C5a. It exerts its regulatory effector function by cleavage of their N-terminal arginine rendering C3a and C5a to bind to their receptors [87].

C4b-binding protein (C4bp) is a fluid-phase negative regulator of the classical and lectin pathway C3 convertase. It binds to C4b in the classical or lectin pathway C3 convertase, displaces C2a (= decay accelerating factor) and acts as a co-factor for the FI-mediated cleavage of C4b into iC4b which has no further role in complement activation [83, 88, 89].

FH is one of the most important fluid phase regulators that is present at high serum concentrations (220-540 μg/ml). FH functions as FI-co-factor but also has decay-accelerating activity. It has an elongated structure presenting several binding sites to C3b and glycosaminoglycans along its length and consists of 20 CCP domains. It binds to C3b via CCP 1-4 at the N-terminus and thereby, accelerates the decay of the C3 convertase by removing C3b but also positions C3b so that the FI cleavage site becomes accessible [90]. Via CCP 6-8 and 19-20, FH binds to sialic acids, heparan sulphates and glycosaminoglycans on host cells and thereby inhibits alternative pathway activation on self-surfaces [91]. It is therefore important for the discrimination of self from non-self cells and many pathogens recruit FH or FH-like proteins for protection of complement recognition [79]. The human *CFH* gene family consists of FH, Factor H-like protein 1 (FHL-1, a truncated version of Factor H derived by alternative splicing) and five complement Factor H-related proteins 1-5 (CFHR-1-5) that are believed to compete with FH for binding to surfaces and thereby regulate FH [92].

CR1 is found on human erythrocytes and is the only co-factor *in vivo* for cleavage of iC3b by FI [67]. CR1 promotes phagocytosis of C3b- or C4b coated particles and clearance of immune complexes from the circulation (= immune adherence).

Almost every cell in the human body expresses the membrane bound complement regulators membrane cofactor protein (MCP or CD46) or decay-accelerating factor (DAF or CD55), usually both are exposed. Exceptions are for example erythrocytes that lack expression of MCP and Natural Killer cells that have no, or barely detectable DAF [93]. In human, MCP is a widely distributed complement regulator while in mice, expression

is largely restricted to the testis [94]. [9] MCP acts as another co-factor for FI [83]. DAF is anchored by a glycosyl phosphatidylinositol residue and found on almost every cell. It binds to C3b and C4b in a convertase and accelerates their dissociation (decay-accelerating factor) [83].

Properdin is the only known positive regulator of the complement system. It binds to the alternative pathway convertase and stabilizes and extends the half-life of this otherwise rapidly dissociating complex [95] [10]. It is essential for alternative pathway activation and Properdin depleted serum lacks the ability to activate the alternative pathway [96]. Recombinant and purified Properdin has the property to aggregate to higher order oligomers which are formed during the purification process or during subsequent freeze–thaw cycles [97]. Even though it is an artificial artefact, the functional activity of Properdin increases with the size of the polymers formed [98] and this property of recombinant Properdin was already successfully exploited in a mouse model where it enhanced opsonisation and lysis of *Neisseria meningitidis* and *Streptococcus pneumoniae* [33].

FI is the only regulator that irreversibly inhibits C3 by cleavage of the α-chain. A detailed description of this regulator can be found in chapter 3 (section 3.1.1).

1.3.2 Regulators of the late steps of complement activation

Regulators of the terminal pathway of complement inhibit formation of the MAC complex and include protectin, vitronectin and clusterin.

Protectin (CD59) is attached to the membrane of erythrocytes, epithelial or endothelial cells via glycosyl phosphatidylinositol residues and inhibits lysis by binding to C5b-8 complexes. Thereby, it prevents C9 binding, polymerisation and MAC formation [83].

Vitronectin (S-protein) and Clusterin, on the other side, are fluid-phase regulators, that inhibit MAC formation on the surface of host cells [86]. These proteins bind to C5b-7 and prevent integration of C8 or C9 into the complex [99].

[9] In mice, the widely expressed protein Crry appears to perform human MCP's regulatory activity.

[10] Nephritic factors (NEFs) are also stabilizers of the alternative pathway C3 convertase but are pathological

The most important *in vivo* inhibitor of lysis is C8 itself, because once it is bound, the membrane binding sites of the C5b67 complex are blocked [100]. The C5b67 complex thus only has a limited time in which it can insert into a membrane to start MAC pore formation.

With the exception of C8, all late steps fluid phase inhibitors have functions outside the complement system and it is likely that their C5b-7 binding properties are the products of their non-specific affinity for hydrophobic molecules [66].

1.4 The amplification loop of the complement pathways

It better reflects the alternative pathway if it is not seen a mere cascade of vertical reactions but as a horizontal system that consists of a balance between two competing cycles that both act on C3b (Fig. 1.4). These are the C3b feedback and breakdown mechanism which lie at the heart of complement activation. Once C3(H_2O) (= iC3) or C3b is formed and deposited on a surface, two different reactions can occur independent of how C3b was generated in the first place: it can either be bound by FB (feedback) or FH (breakdown).

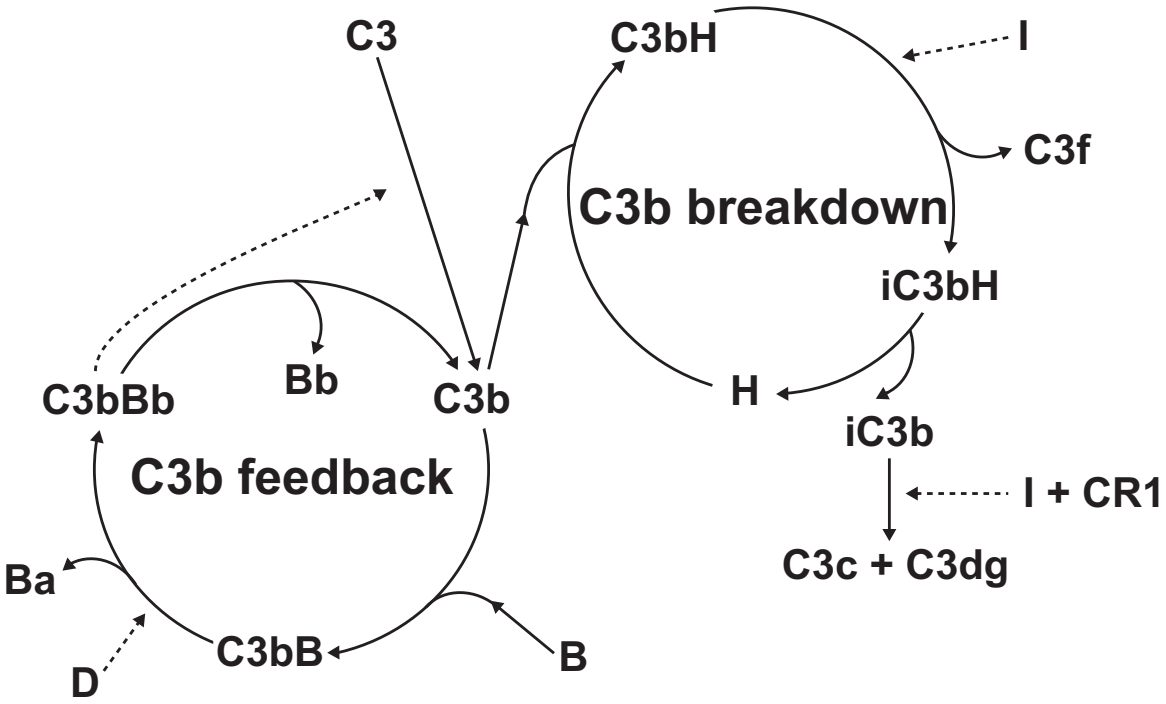

Fig. 1.4 The feedback loop of the alternative pathway of vertebrate complement. Dashed lines indicate enzymatic reaction while solid lines indicate conversions of proteins. D, B, H and I stand for FD, B, H and I, respectively. Figure adapted from [43].

Binding to C3b (or C3(H_2O)) either leads to amplification of the C3 convertase (presence of FB, FD and Properdin) and initiation of the assembly of the MAC or to inactivation of C3b (presence of FH and FI) [101]. In the early days of the discovery of the alternative pathway, it was unclear how conventional alternative pathway activators (i.e. zymosan, endotoxins, inulin,...) act, until Fearon and Austen proposed the concept of a so-called "protected surface" on which FH binding (and therefore also FI cleavage of C3b) is impaired [102]. Whether

amplification or inactivation occurs, depends solely on the nature of the surface to which C3b is attached. For example, FH binding to C3b on LPS of *Escherichia coli 04* was shown to be far weaker than that of FB [103] and removal of membrane-bound sialic acid from sheep erythrocytes (otherwise protected from alternative pathway attack) by neuraminidase treatment made them to an activator and led to haemolysis [104].

The fact that the alternative pathway is regulated at several steps makes it an attractive target for therapeutic intervention. The balance of the feedback loop which determines the amount of generated C3b can be tipped at both ends that will now be described.

More C3b can be generated either by amplification of the feedback loop or by inhibition of the C3b breakdown mechanism. The first can be amplified by increase of C3b input i.e. more C3 convertases from classical/lectin pathway or other C3 splitting enzymes (elastase, plasmin,...), by acceleration of the feedback reactions i.e. gain of function mutants of FB, increase of FD or Mg^{2+}, by addition of cobra venom factor (mechanism explained later) or by stabilisation of the alternative pathway C3 convertase i.e. by Properdin or nephritic factors. Nephritic factors (NEFs or C3-NEFs) are pathological stabilizers of the alternative pathway C3 convertase and can substitute for Properdin. First described by Spitzer [105], they were later defined as autoantibodies to C3 that stabilise the alternative pathway C3 convertase by protecting the convertase from C3b inactivation by FI [43]. That way, they induce a state of ongoing, uncontrolled complement activation and can result in kidney disease but also in drusen development in the macula [106]. NEFs are strongly associated with membranoproliferative glomerulonephritis type II (MPGN 2 or dense deposit disease), a rare kidney disease that is characterized by onset of hematuria and/or proteinuria, acute nephritic or nephrotic syndrome and deposition of electron dense material in the glomerular basement membrane. The disease results in end-stage renal failure within 10 years in 50% of affected individuals [107]. Some patients with NEFs however have partial lipodystrophy in the absence of kidney diseases mentioned above [108]. Cobra venom factor (CVF) is a complement-activating protein found in cobra venom. It is a structural and functional analog of C3 and, although structurally resembling C3c, has C3b-like properties and can bind FB of all mammalian species and form a C3 convertase after cleavage of FB by FD [109, 110].

The new convertase, CVFBb, is resistant to FI cleavage because FH binding is prevented. This property can be used to completely de-complement an individual because all available native C3 will be consumed temporarily by the reaction and thus allows assessing the role of complement involvement in a certain disease model. In serum depleted of C3, CVF is the only activator of the alternative pathway [46].

The C3b breakdown can be prevented by either absence or low concentrations of FH and FI or MCP or by a susceptible C3 genotype i.e. C3F (SNP: R102G) has a lower affinity to FH than C3S and is therefore slower cleaved by FI [111]. Additionally, C3 breakdown is also impeded on protected surfaces.

Negative regulation of the feedback loop is achieved by promotion of the C3b breakdown. This is done by either raising the concentrations of FH or I, by inhibition of FD, B or Properdin. This thesis is focused on negative regulation of the alternative feedback loop and possible therapeutic interventions are discussed in Sec. 1.4.2.

1.4.1 Immunopathology of an uncontrolled amplification loop

In health, the complement system is a homoeostatic state and the tickover confers the capacity of rapid activation and amplification. Even though this is an essential prerequisite in the fight against infection, it can cause great collateral damage if this mechanism gets out of control and attacks self tissue. During the past decades, it has become apparent that genetic variations in complement proteins that affect their function are associated with various chronic and infectious diseases [59, 112]. Recently, the term "hyperinflammatory complotype" (for complement phenotype) was introduced to "represent the pattern of genetic variants in complement genes inherited by an individual which alters risk for both inflammatory disorders and infectious diseases involving complement" [59]. Initially, the term complotype was used to refer to only alternative pathway associated polymorphisms but it is now extended to other pathways as well. In order to understand the hyperinflammatory complotype's disease pathology it is important to have a clear understanding of the feedback mechanism which plays a key role and which is extensively described in the sections above. It is predictable

that changes in the central components can have an effect on plasma homoeostasis and affect an individual's risk of acute or chronic inflammation and disease [59].

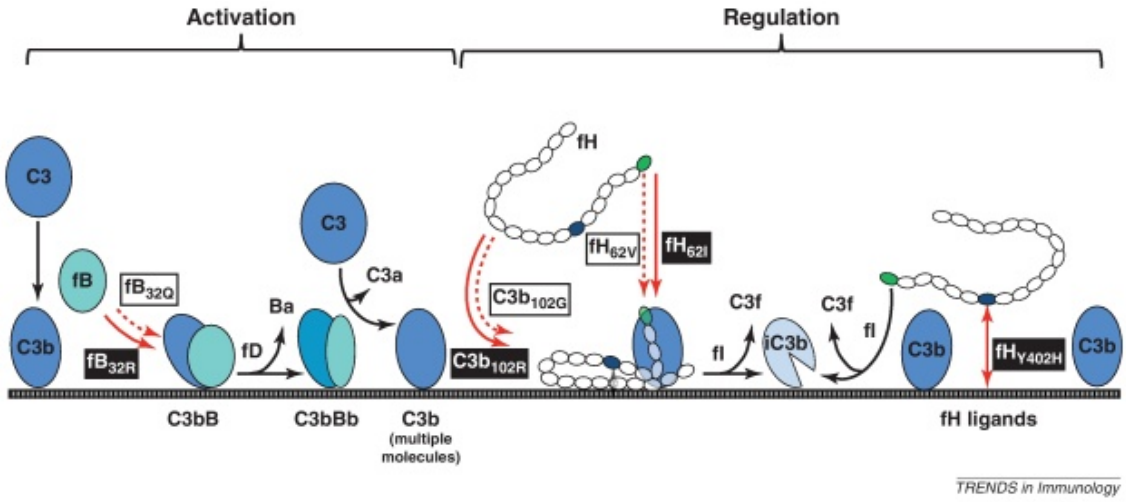

Fig. 1.5 Functional effects of complement protein polymorphisms. Four relevant polymorphisms are shown, all of which have an effect on alternative pathway activation and solid red arrows indicate increased while dashed red arrows show reduced binding/action. fB_{R32Q}: fB_{32R} binds better to the pro-convertase and results in more alternative pathway activation than fB_{32Q}. $C3_{R102G}$: $C3_{102G}$ binds FH less strongly and drives more alternative pathway activation than $C3_{102R}$. fH_{V62I}: fH_{62V} binds less strongly to C3b and results in less regulation and more alternative pathway activation than fH_{62I}. fH_{Y402H}: fH_{402H} shows reduced binding to heparan sulfate and dermatan sulfate glycosaminoglycans within Bruch's membrane and results in more alternative pathway activation. Figure adapted from [59].

The best described polymorphisms are associated with AMD, DDD and aHUS. They are introduced below and their mechanism of action is shown in Fig. 1.5.

The first, AMD, is the most common disease influenced by variations of alternative pathway genes (AMD is introduced in the introduction to Chap. 5). Increased AMD risk is associated with several common *FH* haplotypes: *FH* haplotype 1 which includes the fH_{Y402H} SNP [113–115], *FH* haplotype 2 which includes the fH_{V62I} SNP [116] and *FH* haplotype 3, which includes the deletion of *CFHR1/CFHR3*, protective [117]. Inheriting two copies of the *FH* haplotype 1 was shown by cited studies to increases the risk of AMD sevenfold. Other AMD associated genetic variations were found in *CFB* and *C3*. $C3_{R102G}$ was the first polymorphism to be described in 1967 by Alper et al. [118]. Back then, C3F (= $C3_{102G}$) was already known to have increased functional activity but it was only shown in 2011 that

this is due to decreased affinity of FH to the C3F convertase [111]. More details about the polymorphisms mentioned above can be found in Tab. 1.1. Quite recently, rare *CFI* mutations that predispose to low FI serum levels have been associated with a significantly increased risk for advanced AMD [119].

SNP name	**Coding variant**	**Minor Allele**	**Odds Ratio (OR)**
rs1061170	fH_{Y402H}	H (risk)	1.99-2.5 [117, 120]
rs800292	fH_{V62I}	I (protective)	0.54 [116]
Δ*CFHR1/CFHR3*	deletion of CFHR 1 and 3	[Δ (protective)]	0.48 [117]
rs641153	fB_{R32Q}	R (risk)	0.32 [121]
rs2230199	$C3_{R102G}$ (also S/F)	G (risk)	2.6 [122]

Table 1.1 Polymorphisms associated with age-related macular degeneration

Dense deposit disease is clinically linked to AMD in that these patients also develop retinal changes as in AMD but at much earlier age (if they survive) than "AMD-only" patients [123]. In fact, polymorphisms in H1 and H2 haplotypes also confer an increased or decreased risk for DDD, respectively, as reported for AMD [124] as well as polymorphisms in C3. Combinations of several risk haplotypes increase DDD risk dramatically [125].

Both, AMD and dense deposit disease are associated with changes in fluid phase alternative pathway regulation and can therefore be seen as systemic, rather than eye- or kidney-specific disease. The reason why these two organs are particularly affected probably lies in the exact distribution of complement regulator proteins and organ architecture, i.e. in both organs, there is a separation of erythrocytes and plasma and a basement membrane that is in direct contact with plasma, making protection from complement attack much more dependent on FH co-factor and FI activity. It is interesting to note that most polymorphisms predisposing to AMD and DDD are found in regions of FH that are responsible for C3 binding.

Atypical haemolytic uremic syndrome, aHUS, is also associated with mutations in alternative pathway genes, particularly in the the FH gene (half of all aHUS cases), but as opposed to AMD and dense deposit disease, these mutations were usually found in the C-terminus of FH, a region responsible for sequestering FH to surfaces [126]. Other predisposing polymorphisms were found in the *C3* or *CFB* gene [127, 128]. In summary, risk

of aHUS is highly increased in the presence of risk polymorphisms that promote inappropriate alternative pathway activation on cell surfaces [129].

Although the focus is set on the three disease settings mentioned above, genome-wide association studies have identified further polymorphisms that lie in e.g. the *CR1* gene, clusterin (both further predisposing to Alzheimer's disease (AD) in individuals that already have one or two copies of the AD predisposing APOE-ε4 allele) [130, 131], C5 (associated with rheumatoid arthritis) [132]. It should also be pointed out again that amplification of complement and its effects via the feedback cycle is inherent of all complement pathways and that its manipulation has the potential to improve other pathophysiological processes as well.

Most of the mentioned polymorphisms have in common that the disease allele predisposes to an increase in the alternative pathway feedback activity that amplifies all complement pathways irrespective of the triggering stimulus [43]. A hyperinflammatory complotype thus protects against infection, particularly in early childhood but comes at the expense that, later in life, when an individual already has formed IgG antibodies against commonly invading pathogens, it predisposes to inflammatory diseases [59, 112]. Since, the list of diseases affected by complotype grows steadily as genetic studies implicate known or novel complement polymorphisms [59] and in most parts of the world, there is a tendency to an increased life-span, there is considerable interest and need in finding methods for reversing this hyperactivity of the C3b feedback cycle [112].

1.4.2 Possible interventions into the amplification loop

As introduced in Sec. 1.4, there are several targets for systemic intervention in the feedback mechanism and the most obvious targets for downregulation of the feedback loop are , FH and FI. Because AMD is the most common of complotype-associated disease, focus will be set on this complement related disorder.

A first therapy for the treatment of geographic atrophy has already been developed and is currently in a phase III clinical study (MAHALO study by Genentech/Roche, see also

Sec. 5.1.1.4 [11]. Here, a humanised monoclonal inhibitory antibody to FD(lampalizumab) administered by intravitreal injection is used to stop the rate of progression of a late form of AMD, geographic atrophy. FD is present in very low serum concentrations and is an essential factor for the alternative pathway. Nevertheless, FD is a less good therapeutic target because due to its small size (27 kDa), it is rapidly cleared out by the kidneys and quickly re-synthesized. Thus, the α-FD treatment works for local inhibition of FD in a small compartment such as the retina but not systemically. The route of administration, i.e. intravitreal injection, is not without associated risk which is another drawback of this treatment. Also, inhibition of FD does not accelerate breakdown of the hyper-inflammatory iC3b.

Regarding the other two target proteins, it has been known since the 70ies that the feedback loop can be down-regulated by increasing the plasma concentrations of FH and I [134, 135]. FI poses the better target for several reasons. First of all, it is present in much lower concentrations than FH, i.e. $\approx 35\,\mu$g/ml (FI) and $\approx$ 200-500μg/ml (FH), respectively. Lower quantities are easier to handle and also reduce the cost of therapy. Further, there are variants of FH, called complement FH-related proteins 1-5 (CFHR1-5), that compete with FH for binding to surfaces. CFHR1, 2 and 5 contain a shared dimerization motif that enables formation of three homodimers and three heterodimers which have significantly increased avidity and out-compete FH at physiolocigally relevant concentrations [136].[12] Increasing FH concentration probably does not overcome the dominant pathogenic effects of these competing antagonists [112]. The last and most important reason is that FI is the only regulator that not only promotes cleavage of C3b to iC3b but also accelerates the breakdown of iC3b which is the main inflammatory molecule (see Sec.1.2). It should be emphasised that increase of FH will also result in less iC3b because it assists the degradation of newly generated C3b in the C3b amplification cycle which can thus no longer form a C3 convertase

[11] Eculizumab was also tested as a therapy for AMD however no effect on disease progression was reported [133]

[12] The presence of a *CFHR1/CFHR3* deletion was shown to be protective in age-related macular degeneration [117], IgA nephtopathy [137] but shown to confer higher susceptibility to systemic lupus erythematosus [138] which is probably due to the lower antagonistic effect on FH. Contrary, *CFHR1/CFHR3* deletion, actually lack of *CFHR1*, is strongly associated with auto-α-FH antibodies which confer increased risk to aHUS [139].

itself, i.e. the cyclic nature of the C3b amplification mechanism is disrupted and shifted towards more C3b breakdown. Under physiological conditions, the affinity of FH to iC3b is too low to form a complex and thus, CR1 is the only co-factor for the cleavage of iC3b. Since iC3b reacting with the complement receptor CR3 is a major mechanism by which complement activation gives rise to inflammation, the breakdown of iC3b to C3dg is essential for reducing complement induced inflammation [43]. It is known from mouse studies that the development of dense deposit disease is entirely dependent on the capacity to generate iC3b [73]. In these studies, it was shown that while dense deposit disease does not occur in a *FI* knockout or *FI/FH* double-knockout mouse, it does develop if mice are only*FH* deficient or if double-knockouts are reconstituted with FI. There is therefore an absolute need in disease development for the presence of FI and complete FI deficiency abolishes risk for dense deposit disease because of the absence of the inflammatory mediator iC3b. Nevertheless, FI deficiency, apart from being very rare, behaves similarly to C3 deficiency and can give rise to bacterial immunodeficiency, as demonstrated in the initial FI -/- patient. In case of a non-deficient individual, it seems most logical to re-balance the feedback loop of the alternative pathway by raising plasma concentrations of FI which will promote C3b and iC3b breakdown and thus remove major disease factors in complement associated diseases that have an underlying defect in alternative pathway regulation.

In a separate study, we have already studied the effects of FI supplementation in the presence of zymosan or LPS as alternative pathway activator on iC3b formation and measured its subsequent breakdown to C3dg in three different complotypes [112, 140]. Chosen complotypes were divided into susceptible- or protective-homozygous and heterozygous with respect to three common polymorphisms associated with AMD, i.e. C3S/F$_{R102G}$, fH$_{Y402H}$ and fH$_{V62I}$. The results are highly promising because they do show not only that the susceptible complotypes have delayed and much less iC3b to C3dg conversion than both, the protected and heterozygous group but also that by increasing the total FI concentration, the most at risk genotype can be "converted" to a least at risk one and can overcome disadvantageous genetic variants in the *FH* and *C3* genes. Supplementation with $22\,\mu$g/ml FI (less than normal FI concentration) converts the "at risk"- to a protected genotype [140]. The presence

of just three loci therefore has striking influence on the regulation of alternative pathway complement activation by FI in that the hyperinflammatory complotype is more resistant to down-regulation by increased FI concentration [112]. By increasing plasma concentrations of FI we propose to reverse the effects of a disadvantageous complotype and slow down disease progress.

Together with new arising genomic data of steadily increasing associations of complement and/or alternative pathway disorders with a number of diseases [59, 113, 116, 119], it is certainly now the time to introduce a therapy against uncontrolled pathway associated disorder using FI or FH.

1.5 Thesis aims and outline

The overall aim of this project is the development of a treatment for prevention or even reversion of (early) dry AMD and this dissertation forms an initial part of this bigger project. Studies of AMD are hindered by the lack of optimal animal models that replicate the human disease, in particular because of the absence of a macula in mice. Since AMD now has turned out to have an underlying systemic rather than an eye-specific defect, which is shown by the increasing list of AMD-associated polymorphisms in complement proteins and regulators, it is clear that the key to a therapy lies in the control of systemic alternative pathway regulation. In order to extend studies of uncontrolled feedback cycle activation, the role of FI is studied in an already established model of renal ischemia which is known to be exacerbated by the alternative pathway. In terms of a future therapy for AMD, down-regulation of the feedback cycle by increase of plasma levels of FI can be achieved by either supplementation of plasma-purified or recombinant FI or by *in vivo* over-expression via a gene therapy. The latter one, gene therapy, offers a new way of treating various diseases and overcomes many of the obstacles of traditional therapeutic strategies (although, naturally, it comes with its own problems). This thesis describes the use of two methods of FI increase, a recombinant expression and by gene therapy and explores its therapeutic potential in renal ischemia. Another one of the main problems in AMD is the absence of a technique by which the potential and success of a therapy or follow-up can be quickly assessed. In order to enable this, a new *in vivo* biomarker needs to be introduced that fulfils these tasks. Therefore, another part of this project aims to the development of such a new biomarker.

The aims of this thesis are:

- Assessment of the therapeutic potential of FI in a mouse model of renal ischemia
- Generation of a vector construct that enables *in vivo* over-expression of FI and assessment of the extent of down-regulation of the feedback cycle.
- Generation of a biomarker that enables the detection of *in vivo* deposition of complement activation products for diagnosis of early dry age-related macular degeneration.

The structure of this work is as follows: After the general introduction to the complement system and the role of the alternative pathway reactions, in the next chapter, Chapter 2, materials and methods used in the following chapters are described to enable a detailed understanding of obtained results and to allow their repetition. Chapter 3, 4 and 5 present the results obtained in the course of this work. Chapter 3 starts with a detailed introduction of the most important protein of this project, FI (3.1.1), and then reviews relevant literature of renal ischemia reperfusion injury including the role of the complement system in this disease (3.1.2). In order to study the therapeutic role of FI in renal ischemia, a recombinant FI protein was produced (3.2.1) and then downregulation of the alternative pathway feedback loop in a mouse model of renal ischemia reperfusion injury was studied (3.2.2). In Chapter 4, increase of plasma FI levels was achieved by a different technique, namely by a gene therapy. In collaboration with a leading group in gene therapy, a FI vector construct was generated that enables over-expression of FI in murine hepatocytes. First, an introduction in gene therapy is given (4.1.1), before the used adeno-associated virus based expression system is explained in detail (4.1.2). After the vector was constructed, plasma levels of FI after its injection into mice, were determined (4.2.1.2) and the extent of down-regulation of the alternative pathway was assessed (4.2.1.3). Chapter 5 describes the generation of an *in vivo* biomarker for detection of complement activation products for the diagnosis and follow up of early dry age-related macular degeneration. First, the disease pathology (5.1.1.2) is introduced as well as the role of complement in AMD (5.1.1.4). Next, experimental results of the generation of this biomarker will be shown and discussed (5.2). Finally, Chapter 6 shortly summarises the obtained results and discusses them in the broader context. This dissertation ends with an outlook for future work of this project. In Appendices A and B, Primer sequences (A.1) and abbreviations (B.1) are listed. Already published Papers of work I collaborated in are listed in Appendix C.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Antibodies

All antibodies used will be described below, including their source, exact binding characteristics and application in this thesis.

α-His

The antibody is a monoclonal α-polyHistidine antibody produced in mouse and is available from Sigma Aldrich (# A7058). It recognises N- or C-terminal tags of 6 histidines in recombinant proteins. This antibody was used to detect the light chain of FI and the ScFv. Normal working concentration was 1:5000.

α-mouse Factor I

This polyclonal antibody is commercially available from Santa Cruz Biotechnology, Inc (# sc-69465). The antibody was raised in goats against a peptide in the heavy chain of mouse FI and recognizes the heavy chain in reducing and non-reducing western blots or ELISAs. The antibody also recognises the pro-enzyme. Nevertheless, the antibody is not precipitating

because the peptide used for immunisation of is too short. Normal working concentration was 1:500.

α-mouse Factor I antiserum

This antibody is not commercially available and was ordered from Absea Biotechnology Ltd. in order to get a precipitating antibody to mFI which would allow easy determination of FI levels in an Ouchterlony double immunodiffusion assay. A rabbit was immunised with a peptide fragment (203aa-510aa) of recombinant mouse FI that was produced in bacteria. Once tested, the antibody turned out to be non-precipitating and therefore, an inhibition ELISA was developed to measure the levels of FI in serum. To get a multivalent antibody to mouse FI that precipitates FI, FI purified from mammalian cell culture was sent to Absea Biotechnology Ltd. but the immunisation period (8 weeks) for this second α-mouse FI antiserum was not completed in time but will be used in future experiments.

Clone 9 antibody

Clone 9 is a rat α-human C3g antibody that recognises a neo-epitope in C3g that only becomes accessible if C3 is cleaved to iC3b or C3dg by FI [141]. Under native conditions, clone 9 only reacts with iC3b or C3dg of human origin, whereas under denaturing conditions it detects the α, α' chain and the 68 kDa fragment of human C3 because all these three fragments include its epitope in C3g. Normal working concentration for detection in a western blot is $0.5\,\mu$g/ml and as capture antibody for coating the concentration is $1.35\,\mu$g/ml. The names clone 9 and α-hC3g anbtibody will be used interchangeably.

Clone 4 antibody

Clone 4 a rat monoclonal α-C3c antibody that recognizes a conformational epitope in C3c and reacts with C3, C3b, iC3b and C3c [141]. It therefore does not bind to Cdg or C3g because of the absence of the epitope in C3c. Normal working concentration is $5\,\mu$g/ml.

α-human C3c

This antibody is commercially available from Dako and is used to detect C3b and iC3b deposition. It is used at a concentration of 1:5000 and detected with an α-rabbit secondary antibody.

SO16

SO16 is an IgM antibody directed against sheep erythrocytes. It is used 1:100 to coat/opsonise sheep RBCs with antibodies which promotes their lysis [142].

Secondary antibodies

Used secondary antibodies are α-rabbit-AP or -HRP, Extravidin-AP or -HRP, α-goat-AP or -HRP. They are generally used at a concentration of 1:5000.

2.1.2 Primer sequences

Primer sequences can be found in Appendix A.1.

2.1.3 Buffers

Buffer recipes can be found in App. A.2.

2.1.4 Kits

Kits used are listed below

- QIAprep Spin Miniprep Kit (Qiagen)
- RNeasy Mini Kit (Qiagen)
- QIAquick Gel Extraction Kit (Qiagen)
- Rat Immunoglobulin Isotyping ELISA Kit (BD Pharmingen)

- ProtoScript II First Strand cDNA Synthesis Kit (NEB)
- EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Scientific)
- Infinity Urea Harnstoff (Microgenics, Thermo Fisher Scientific)

2.2 Methods

All procedures were performed at room temperature (22-25°C) unless specified otherwise.

2.2.1 Molecular biology methods

Plasmids

Plasmids used are listed below

- pCDNA3.1 (Invitrogen), for eukaryotic expression
- pRSET A (ThermoFisher), bacterial expression
- pET29b (Novagen, courtesy of Andrew Grant, Cambridge University, Department of Veterinary Medicine), bacterial expression
- Plasmids used for gene therapy are explained in detail in Sec. 4.1.3.

Bacterial strains

- DH5α
- BL21(DE3)
- BL21(DE3)LysS
- Rosetta(DE3)LysS
- SHuffle® T7 Competent E. coli (NEB)

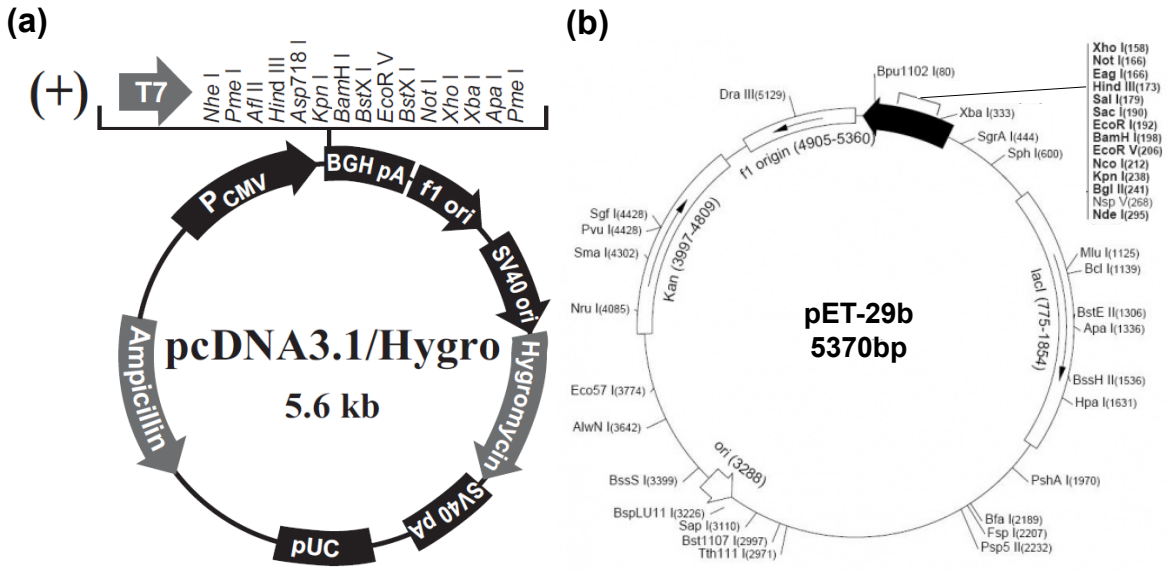

Fig. 2.1 Vector maps of (a) pcDNA3.1/Hygro + and (b) pET29b.

RNA extraction and cDNA synthesis

In order to generate a cDNA pool which would later serve as a template to amplify the respective genes, RNA first had to be extracted from hybridoma cells or organs expressing the target gene.

For the expression of recombinant mouse complement FI, liver and kidneys were excised from a freshly killed C57/Bl6 mouse and the organs were cut to pieces of 5 mm. Next, the cells were separated from tissue using a cell strainer. Cells were washed once with PBS and RNA was extracted according to protocol using the RNeasy Mini Kit. All steps were performed on ice. In brief, the cells were lysed with 600 μl buffer RLT and mixed with 600 μl 70% Ethanol. Next, the lysate was transferred into an RNeasy Mini spin column and centrifuged at 8.000 xg for 15 seconds. The flow-trough was discarded and 700 μl of buffer RW1 were added. After centrifugation, the pellet was washed twice with 500 μl buffer RPE and finally the membrane was dried by spinning for 1 minute. The RNA was eluted in 30 μl provided RNase-free water. RNA was used directly for cDNA synthesis and then stored at -80°C.

In case of expression of the ScFv biomarker, Hybridoma cells stably expressing clone 9 antibodies were grown in RPMI medium supplemented with 10% FCS. The cells were grown to 80% confluency ($\approx$ 1x10^7 cells), detached and centrifuged to remove all cellular debris and medium. Next, they were resuspended in buffer RLT and processed as described above.

The cDNA of mouse complement FI and the immunoglobulin genes of the α-C3g antibody were reverse transcribed from extracted RNA as follows: 100 ng RNA were mixed with 2 μl oligo-dt primer 1:10 (Promega), 1 μl 10 mM dNTPs. Heating up to 90°C followed by incubation of ice for 5 minutes ensures that the RNA is disentangled so that the primers can anneal. Next, 4 μl 5x ProtoScript II reaction buffer, 2 μl 0.1 M DTT and 1 μl ProtoScript II Reverse Transcriptase were added and the reaction was incubated at 42°C for 40 minutes. Finally, the enzyme was inactivated by incubation at 85°C for 5 minutes and the cDNA pool was used on the same day for PCR and then stored at -80°C.

PCR amplification

All proteins expressed were initially amplified from a cDNA pool using a polymerase chain reaction (PCR) :

- Mouse complement FI from mouse liver and kidney cDNA
- ScFv biomarker from cDNA of hybridoma cells expressing the parental antibody

The genes were amplified in a PCR reaction by mixing 1 μl cDNA pool with 1.25 μl of a 1:10 dilution of forward and reverse primer (see Tab. 2.1 for PCR program and Appendix. A.1 for primer sequences), 2.5 μl Advantage Buffer (10x), 0.75 μl 10 mM dNTPs and 0.25 μl Advantage 2 Polymerase Mix (Clontech). The reaction was filled up to 25 μl with sterile H_2O and the PCR reaction was started. The primers were designed to incorporate restriction sites, with the forward primer also including a Kozac sequence if the recombinant protein was intended to be used in a mammalian expression system.

After amplification, the reaction was loaded onto an agarose gel and the correct amplified bands were excised and purified using the QIAquick Gel Extraction kit. In brief, the excised gel was weighted and dissolved in 300 μl buffer QC per 100 mg gel. Once completely

Step	Temperature (C)	Time (sec)	No. of cycles
Inital denaturation	95	180	1
Denaturation	95	10	25
Annealing and extension	68	60/kb	25
Final extension	68	500	1

Table 2.1 Usual PCR program

dissolved, 100 μl isopropanol per 100 mg gel were added, mixed and the sample was added to a provided QIAquick column. After centrifugation, the membrane of the column was washed with 750 μl buffer PE followed by an additional centrifugation step to remove all ethanol. For elution, 30 μl H_2O were added onto the membrane and after 1 minute incubation, the DNA was eluted by centrifugation and is then ready for subsequent procedures.

Restriction digest, ligation and transformation

Purified PCR product and vector have to be digested with the same enzymes in order to create compatible sticky ends for correct cloning. In a typical restriction digest, 5 μl DNA were mixed with 1 μl of each restriction enzyme, 2 μl 10x buffer and 11 μl H_2O followed by incubation in a 37°C water bath for 30 minutes. Digested PCR products and vectors were then gel purified and either stored at 20°C or used for ligation.

For ligation, 3 μl insert and 1 μl vector backbone were mixed with 1 μl T4 ligase (NEB), 1 μl T4 ligase buffer (10x) and 4 μl H_2O. The reaction was either carried out for 15 minutes at RT or overnight at 4°C.

Transformation was performed by adding 2 μl ligation reaction to 50 μl competent cells. The mix was incubated on ice for 20 minutes and then heat shocked for 40 seconds at 40°C. The cells were put on ice for 5 minutes and then incubated at 37°C in SOC medium. After 1 hour, the bacteria were plated onto LB plates containing the respective antibiotic and then incubated at 37°C overnight.

Plasmid purification

Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). 3 ml overnight cultures of single colonies were harvested and pellets were resuspended in 250 μl buffer P1. Next, 250 μl Lysis buffer P2 were added and the reaction was incubated until the suspension was completely blue colour and cleared up, indicating complete lysis which was then stopped by addition of 350 μl neutralization buffer N3. The suspension was mixed by inversion until all traces of blue colour have gone and the suspension was colorless, indicating that the SDS has been effectively precipitated. DNA and cell debris were separated by a long centrifugation step and the clear supernatant containing the plasmids was transferred into a QIAprep spin column. The pellet was washed with 750 μl buffer PE and then eluted with 30 μl H_2O. 2 μl of purified plasmid were then digested with the respective restriction enzymes to confirm presence of the construct. The constructs were also sent for sequencing to ensure that no mutation was introduced.

Preparation of competent cells

Chemically competent cells were prepared according to the rubidium chloride method which renders the cells highly competent and can yield transformation efficiencies of about 10^8-10^9 CFU/μg DNA. A frozen culture of bacteria was streaked overnight and on the next day, a single colony was inoculated (= pre-culture). Next day, 250 ml pre-warmed LB medium were inoculated with the pre-culture supplemented with 20 mM $MgSO_4$ and the cells were grown until the OD600 reached about 0.5. Next, the cells were chilled on ice for 15 minutes and then harvested by centrifugation. The pellet was gently resuspended in 0.4x original culture volume ice-cold transformation buffer 1. After a 15 minute incubation step on ice, the cells were pelleted again and resuspended in 0.04x original culture transformation buffer 2. The cells were incubated 15-60 minutes on ice and then shock frozen in pre-chilled tubes. The competent cells were stored at -80°C until use.

2.2.2 Expression of recombinant proteins

Eukaryotic expression of recombinant proteins

Once a plasmid with the right sequence was identified, it was transfected into a eukaryotic cell line. CHO-K1 or HEK293T cells were seeded in a 6-well plate in DMEM medium supplemented with 10% foetal calf serum and penicilin/streptomycin. DMEM medium for CHO-K1 cells was further supplemented with 0.1 mM proline, alternatively, RPMI or F12 medium were used. As soon as the cell confluency reached 90%, medium was exchanged with 2ml antibiotic free medium since antibiotics interfere with the used transfection method. 250 μl Opti-MEM® I Reduced Serum Media were mixed with 10 μl Lipofectamine 2000 and incubated 5 minutes at room temperature. Meanwhile, 1 μg DNA miniprep was mixed with 250 μl Opti-MEM and added to the Lipofectamine mix. After incubation for 20 minutes, the suspension was dropped onto the cells and distributed by gently rocking the plate. For a transient transfection, cells were incubated for 48 hours, washed with PBS and incubated for another 48 hours in serum-free medium (SFM). The conditioned supernatant was then harvested and tested for recombinant protein expression. For a stable transfection, medium was exchanged to selection medium containing 150 μg/ml Hygromycin B after 24 hours. Cells were incubated for 14 days during which time, cells without the construct will die. Once positive cells were selected, they were washed with PBS and transferred to serum free medium for 48 hours. Protein expression was confirmed by a western blot and cells expressing the recombinant protein were propagated and secreted protein was purified.

Bacterial expression of recombinant proteins

For inducible expression of recombinant proteins in bacteria the used e.coli strain has to be suitable for protein expression, namely, the desired gene must be under the control of a T7 promotor and the bacteria must have a T7 RNA Polymerase. Therefore, the ScFv-constructs were transformed into BL21(DE3)LysS and Rosetta(DE3)LysS by methods described above.

An overnight culture was diluted 1/50 and inoculated at 30°C until an OD600 of 0.7-0.9 was reached. Cells were grown in 2YT medium (Invitrogen). Protein expression was induced

by addition of 0.5mM IPTG and cells were grown for 3-4 hours at an optimized temperature. The cells were harvested by centrifugation and either analysed by SDS PAGE or sonicated to extract protein. For initial screening of expression, the gel was stained with Coomassie Blue. Additionally, the gel was blotted onto a PVDF membrane and ScFv was detected with an α-His-HRP conjugated antibody (1:5000).

Cellular fractionation and purification of recombinant protein from bacteria

In order to find the cellular localization of expressed recombinant proteins, the different cellular compartments were fractionated. Protein expression was performed as described above and after 3 hours growth, the periplasmic fraction was prepared by resuspension of the harvested cells in ice cold periplasmic extraction buffer. The cell suspension was incubated for 30 minutes on ice with continuous stirring. Vigorous vortexing or shaking was omitted since this led to contamination of the periplasmic fraction with cytoplasmic proteins. Next, the cell suspension was centrifuged for 10 minutes at 13,000xg at 4°C and the supernatant was taken off as periplasmic fraction. For separation of the spheroblast fraction, the pellet was respuspended in ice cold spheroblast extraction buffer and again incubated stirring on ice, followed by centrifugation. To break the cells, the pellet was resuspended in bacterial resuspension buffer and sonicated on ice. Soluble and insoluble proteins were separated by ultracentrifuge at 150.000 xg , 1 hour, 4°C. The soluble cytoplasmic fraction (= supernatant) was taken off and the pellet was washed in inner membrane (IM) solubilisation buffer. After a second ultracentrifuge step, the pellet which now consists of outer membrane and all insoluble proteins, was solubilised overnight in pellet solubilisation buffer at 4°C rotating. Next day, all insoluble particles were spun down and the supernatant kept as inclusion body fraction.

Finally, all fractions were run on SDS-PAGE or blotted with an α-His antibody and fractions containing recombinant protein were determined.

2.2.3 Protein purification and chromatography

As soon as expression of the recombinant protein was confirmed, protein expression was scaled up and purified by either nickel sepharose affinity chromatography (IMAC) or ion exchange chromatography (IEX). For eukaryotic expression, serum-free conditioned supernatant was concentrated more than 10x using an Amicon stirred ultrafiltration cell (Millipore) or by ammonium sulphate precipitation. In case of bacterial expression, the cellular fraction containing the recombinant protein was prepared. All steps involving chromatography were performed at 4°C unless specified otherwise.

Ammonium sulphate precipitation

The required amount of ammonium sulphate was calculated using an online ammonium sulfate calculator from EnCor Biotechnology Inc. and slowly added to the cell culture supernatant and dissolved by stirring. 2 mM PMSF and 2 mM EDTA were added as proteinase inhibitors to protect the recombinant protein from proteolytic degradation. Once all salt was dissolved, the solution was stirred for 2 hours at 4°C and all precipitated protein was harvested by centrifugation at 11.900 xg, 30 minutes. The supernatant was decanted carefully and the pellet was resuspended in the respective buffer, followed by dialysis overnight.

Immobilized metal affinity chromatography (IMAC)

Before every use, the IMAC resin, Ni Sepharose 6 Fast Flow (GE Healthcare) was cleaned with 0.1 M NaOH and poured bubble-free into a column. The column was cleaned with several column volumes (CV) of distilled, filtered water and charged with 0.1 M $NiCl_2$. The column was equilibrated with wash buffer. Concentrated supernatant was then adjusted to 20 mM phosphate, pH 7.8, 0.5 M NaCl and 20 mM imidazole and microfiltered. The protein was subsequently loaded and the column was washed with at least 10 CV wash buffer. Next, bound protein was eluted with an imidazole gradient or with 250 mM imidazole in binding buffer. The protein peak was detected by measuring absorbance at 280 nm and fractions containing recombinant protein were analysed in a SDS PAGE. Dialysis against the respective

buffer was performed overnight at 4°C. Protein was stored in aliquots at -80°C and repeated freeze/thawing was avoided.

Ion exchange chromatography (IEX)

Lysate or supernatant containing the recombinant protein was dialysed into the respective buffer overnight at 4°C. Next day, either anion or cation exchange chromatography were performed (Source S or Source Q, GE Healthcare) using FPLC. The column was first equilibrated with Buffer A (same as dialysis buffer) for at least 10 CV and protein was loaded onto the column. The column was operated at 1 ml/min. Unbound protein was washed off with another 5 CVs of buffer A. Then, a salt gradient was generated by introducing buffer B (high salt buffer) over (usually) 20 CVs. Elutions were first analysed by nanodrop measurement and fractions containing protein/peaks were analysed by SDS PAGE. To keep purified proteins free of endotoxins which are negatively charged, anion exchange chromatography was employed.

IgG purification

A 5 ml ProSepA column (Milipore) was prepared and equilibrated with PBS. 5 ml α-serum were filtered and diluted 1:1 with PBS. The solution was loaded onto the column which was washed with 10 CV PBS. Bound IgG molecules were eluted with 0.1 M glycine pH 2.8 and immediately neutralized with 2 M Tris pH 8.5.

Silver staining of proteins

Sample was separated by SDS PAGE and the gel was fixed with destain buffer. To remove all acetic acid, the gel was washed several times with H_2O. The gel was rocked for 15 minutes in 0.1% $AgNO_3$ and then quickly rinsed in water before developer was added. Reaction was stopped with destain buffer.

In order to intensify the bands, the gel was first washed with H_2O and silver was reduced (soluble) with Farmer's reducer. After several washes, the gel can be stained again as before.

Biotinylation of proteins

Biotinylation was done using the EZ-Link™ Sulfo-NHS-LC-Biotin kit. In brief, buffers had to be exchanged to either HEPES or PBS buffer to ensure that no free primary amine was present in the buffer which would interfere with the reaction. 10 mM biotin reagent was prepared and mixed as a 20-fold molar excess with protein to be labelled. The reaction was allowed to incubate for 1 hour at room temperature. Labelled protein can then be either used straight away or is dialysed into the respective storage buffer.

2.2.4 Immunological methods

Isotyping of immunoglobulins

The isotypes of the parental α-human C3g antibody were determined using a rat immunoglobulin isotyping ELISA kit (BD Pharmingen) according to the recommended protocol of the manufacturer. Maxisorb ELISA plates were coated with the respective dilution of provided mouse anti-rat purified mAbs and incubated 1 hour at 37°C. After washes with PBS-T (0.05%), blocking buffer was added for 30 minutes. The wells were washed again and microcentrifuged supernatant of α-human C3g Hybridomas was incubated for 1 hour. Unbound contents were removed by extensive washing and HRP-labeled mouse α-rat Ig mAB solution was added. After incubation for 1 hour, the plate was washed 6x with PBS-T by soaking the wells for 30 seconds between each wash. Substrate solution was added and the results were read visually after 5 minutes.

Preparation of Fab fragments

Fab fragments of clone 9 were prepared by limited papain digest. 10 μl of clone 9 (3.68 mg/ml) were mixed with 18.4 μl PBS and 0.34 μl 1 M $CaCl_2$ for 10 minutes at 37°C. Papain (10 μg) was activated with 5 mM L-cysteine. Next, 0.4 μl papain and 1.2 μl PBS were added to the IgGs and incubated for 3 hours at 37°C. To inhibit papain, 15 mM iodoacetamide was added to the reaction and incubated for 5 minutes. Next, 500 μl PBS was added and the whole solution was dialysed against PBS. The Fabs were biotinylated and stored at 4°C.

Enzyme-linked immunosorbent assay (ELISA)

iC3b deposition assay – "clone 9 assay" Maxisorb Nunc Plates were coated overnight at 4°C with clone 9 at a concentration of 1.25 μg/well in coating buffer. Next day, the plates were washed 5x with washing buffer (=PBS-0.05% Tween 20) and blocked with 4% Marvel milk powder in PBS-T for 2 hours at room temperature. The plates were washed 5x and diluted serum samples (1:2000 in PBS/gelatin) were added to each well (preparation of serum samples is described in Sec. 2.2.7). After 1 hour incubation, the plate was washed and incubated for 1 hour with biotinylated clone 4 at a concentration of 5 μg/ml in PBS/gelatin. The plates were washed 5x and incubated with 1:1000 Extravidin-HRP in PBS/gelatin. Finally, after extensive washing, 100 μl TMB substrate (Invitrogen) were added and plates were incubated on a plate shaker for 30 minutes. The reaction was stopped with 50 μl H_2SO_4 and the plates were read on a Microplate reader (450 nm).

C3b deposition assay Maxisorb Nunc Plates were coated overnight with mannan (Sigma, mannan from *Saccharomyces cerevisiae* or lipopolysaccharide (Sigma, L3755) 1 μg/well at 4°C in coating buffer. On the next day, the plates were washed in TBS-0.05% Tween-20 and blocked with 1%BSA in TBS-T at room temperature. After 2 hours, the plates were washed and incubated with serial dilutions of recombinant proteins and/or sera. After 1 hour incubation at 37°C, plates were washed with TBS-T and incubated for 1 hour with polyclonal rabbit α-human C3c complement (Dako), used 1:5000 in TBS-T. After extensive washing, α-rabbit IgG (whole molecule)-alkaline phosphatase 1:5000 in TBS-T was added for 1 hour. The plates were washed 4x with TBS-T and the assay was developed using Fast p-Nitrophenyl Phosphate Tablets (Sigma) and measured (405 nm). Alternatively, the assay development was stopped by addition of 3 M NaOH and read afterwards.

Inhibition ELISA In an inhibition ELISA, microtiter plates are coated with antigen and, after blocking, the concentration of antiserum is determined that gives a strong signal when detected with the secondary antibody. This concentration is then used in the inhibition assay and mixed with serial dilutions of a sample with an unknown antigen concentration. At the

same time, a dilution series of a known antigen concentration is prepared. During this pre-incubation step, immune complexes form and when the mix is loaded on the coated microtiter plate, only samples in which the concentration of antigen does not exceed the amount of specific antibody give a positive signal. If there is excess antigen, no free antibodies are available that can bind to the immobilised antigen on the microtiter plate. Therefore, a positive result in an inhibition assay shows the dilution of sample in which the amount of free antigen is limiting and by comparison with the standard dilutions of known antigen concentrations, the unknown concentration can be determined.

For determination of the concentration of mouse FI in a serum sample, Maxisorb Nunc Plates were coated overnight at 4°C with purified recombinant mouse FI (1 μg/well) in coating buffer. Next day, plates were blocked with 1% BSA in TBS-T for 2 hours at room temperature. During this incubation, dilutions of mouse serum (5% downwards) or purified mouse FI (2 μg/ml downwards) were prepared in 1%BSA/150 mM NaCl. Next the dilutions were mixed 1:1 with the determined concentrations of purified and biotinylated α-mFI IgGs, i.e. 75 μg/ml. The samples were incubated at room temperature for 1 hour on a plate shaker and then loaded onto the coated plate. After one hour incubation, the plate was extensively washed and bound biotinylated α-FI antibody was detected with Extravidin-alkaline phosphatase at a concentration of 1:5000. The plates were washed 4x with TBS-T and the assay was developed using Fast p-Nitrophenyl Phosphate Tablets (Sigma) and measured (405 nm).

Western blot

Western blot analysis was performed to detect specific proteins or to confirm their presence in a sample. First, gel electrophoresis was performed to separate the proteins according on their size. The protein samples were boiled up in 4x loading buffer to denature them; β-Mercaptoethanol was added if reduced protein was required for analysis. SDS gel electrophoresis was performed on a 4-12% bis-tris protein gel (Invitrogen) for 50 minutes at 200 V. After separating the proteins, a wet transfer was performed to transfer the separated proteins onto a PVDF membrane where target proteins can detected with specific antibodies.

The blot was assembled by stacking of in transfer buffer-equilibrated blotting paper, gel and membrane (first activated with MeOH) into a transfer cassette. The transfer was performed for 1 hour at 350 mA, 60 W.

Next, the membrane was blocked for 30 minutes on a rotator in blocking buffer. Primary and secondary antibody were both incubated for 1 hour rolling, in between which the membrane was washed 3x 5 minutes in wash buffer. The last washes were done in TBS-T, pH 8, because both used substrates gave stronger signals when the last washes have been in slightly alkaline pH. Depending on the conjugate of the secondary antibody, different substrates were used: proteins were detected chromogenically by addition of 3 ml of TMB or AP substrate (Life Technologies) or, in case of peroxidase conjugated antibodies were also detected chemiluminescently after addition of ECL reagent.

Hemolytic assays

Fluid phase haemolytic assay Red blood cells were separated from plasma by centrifuging whole blood (3000xg, 5 minutes); whole blood and RBCs were always kept on ice or at 4°C. Rabbit erythrocytes were ordered from Harlan and kept at 4°C until use. First, they were washed 3x with sterile saline (= 150 mM NaCl) at 3.000 xg. After the cells have been washed sufficiently, they were equilibrated with classical or alternative pathway complement fixation buffer. Next, the OD451 was adjusted to 0.7 (before 1:15 dilution of RBCs in H_2O, followed by measurement of the OD of the lysed supernatant).

If mannan coated RBCs were needed, the cells were were diluted 1:1 with classical pathway complement fixation buffer and incubated with 100 μg/ml mannan for 5 minutes at RT. To stop the reaction, 0,1% gelatin was added. The cells were washed again and kept in 0,1% gelatin until further use.

The erythrocytes were mixed 1:1 with the diluted serum and loaded onto a round-bottomed microtiter plate. The plate was incubated into a humidified chamber at 37°C until the endpoint of the assay. To measure hemolysis, the erythrocytes were centrifuged down and the supernatant was transferred into a new plate which was subsequently measured at 490 nm.

Plate haemolytic assay Rabbit erythrocytes were prepared as described above but diluted to 10%. 2% Agarose was prepared and then kept in a 56°C water bath, while the erythrocytes and 2x complement buffer were kept in a 37°C waterbath. 4x4 cm glass plates were cleaned with 70% alcohol and prewarmed. Quickly, 0.5 ml RBCs were mixed with 4.5 ml 2x buffer and 5 ml 2% agarose and distributed onto two glass plates. The agarose-erythrocyte-mix was left to settle and then, holes were punctured into which 10 μl serum were loaded. After incubation overnight at 4°C in a humidified chamber during which the serum diffuses into the agarose, the plates were put in a 37°C incubator. Once, hemolysis was completed, the diameter of the haemolytic rings was measured.

C6 hemolytic plate assay To quantify serum levels of complement C6, a hemolytic assay using C6-deficient rat serum was designed. The protocol was adapted from [142, 143].

Sheep erythrocytes were washed with 150 mM NaCl and VBS and then, antibody sensitised sheep erythrocytes (EA) were prepared. In brief, RBCs were incubated on a rotator for 15 minutes at 4°C with SO16, with a before determined concentration, i.e. 1:100. The cells were washed and EA1423 cells were prepared. For this, guinea pig R3 reagent had to be prepared first (= guinea pig serum depleted of C3): 400 μl of 50% zymosan in VBS were spun down, 1 ml of guinea pig serum was added and the mix was incubated for 45 minutes on a rotator at 37°C. Next, zymosan was centrifuged down and the supernatant (= guinea pig R3), was tested to see how much is needed to generate cell intermediates EAC142. Serial dilutions of R3 reagent in EAs were prepared in duplicates and then guinea pig C-EDTA (guinea pig serum + 10 mM EDTA) was added to one set of tubes. After 30 minutes incubation at 37°C, lysis was measured. The dilution of R3 reagent for EAC1423 preparation is the one that gives no lysis on its own but possesses about 8 minimal hemolytic doses in C-EDTA at about 1/8 of that dilution. In this experiment, the dilution of required R3 was determined to be 1/50. To generate EAC1423 cells, EAs were diluted to 5% and pre-warmed to 37°C. Next, guinea pig R3 was added to 1/50 and gently stirred for 5 minutes. The cells were washed 3x with VBS and 400 μl of 10 μg/ml human C3 were added for 15 minutes at 37°C. The resulting cells are EAC1423. In order to prevent Factor I inactivation of C3, 0.5 ml of 5 mg/ml Antrypol

was added and incubated for 5 minutes. 5 ml of 1% EAC1423 cells were spinned down and mixed with 0.5 ml C6-deficient rat serum. Next, 4.5 ml 1.2% Agarose in VBS/2 mM EDTA was added and poured on a plate. Holes were punched and 10 μl serum were loaded. The plate was put in a humidified chamber at 4°C overnight and then incubated at 37°C until hemolytic rings appear.

2.2.5 Refolding of recombinant proteins

Several refolding techniques have been employed and will be described below. Additives that either prevent aggregation, stabilize intermediate products or promote folding were added to the buffers. They include arginine, sucrose, Mg^{2+} or Ca^{2+}, urea, guanidine-hydrochloride, detergents, salts,... GSH and GSSG were added as redox coupling reagent to support formation of the correct disulfide bonds.

Refolding by dialysis

Refolding by dialysis was performed by sequential reduction of denaturant, i.e. from 8 M Urea to 4, 2, 0.5 M. In order to enhance correct refolding additives were added. After every dialysis step, ScFv was 0.2 μm filtered to ensure removal of aggregated protein that could prevent further refolding.

Refolding by dilution

For refolding by dilution, the purified ScFv was diluted to $\approx 10\,\mu g/ml$ to prevent aggregation during refolding. Several buffers were tried, whose composition can be found in Sec. A.3. ScFv was added dropwise to the buffer that was being stirred . Usually, ScFv was refolded overnight at 4°C.

Refolding by immobilisation of recombinant protein on IMAC

On column refolding is performed by prior immobilisation of purified ScFv on an IMAC column. Any reducing reagent has to be removed or diluted before because otherwise, nickel

gets reduced and cannot bind His-tags any more. Urea on the other hand has no effect on protein binding to the column. On column refolding has the advantage that theoretically, the aggregation problem should be kept at a minimum because of immobilisation of ScFv. Once bound, denaturant can be removed by washing the column with reducing concentrations of urea or guanidine hydrochloride. When the protein is refolded, it can be eluted with imidazole.

2.2.6 Preparation of serum

Serum preparation was performed as described in [144]. Blood was taken under sterile conditions and left to clot at room temperature. The initial centrifugation is usually carried out in a bench centrifuge at about 3.000 xg for about 5–10 minutes at room temperature. The serum is taken and a second, high speed, centrifugation at; 20.000 xg for 2-5 minutes is carried out. This step is essential to remove all fragments of RBCs that can later distort the results. The serum removed from this second centrifugation can then be chilled, aliquoted and frozen for future experiment. It should be noted that serum used for functional assays should never be stored at temperatures above -80°C. Repeated freeze/thawing should be avoided by preparation of aliquots.

2.2.7 *In vitro* assays

Furin digest of pro-Factor I

Both, Hek and CHO cells, were secreting only partially processed mFI and the majority was the inactive pro-enzyme of FI. In order to get active FI, the purified pro-enzyme was digested with the protease furin (NEB). In brief, purified mFI was dialysed against TBS and then adjusted to 10 mM $CaCl_2$. 1 unit of furin was added per 25 μg mFI. The reaction was carried out overnight at 30°C. On the next day, FI was used immediately or aliquoted and stored at -80°C.

C3b cleavage assay

In a C3b cleavage assay, C3b was first prepared by limited tryptic digest as described in [145]. In brief, 2 μl human C3 were incubated with 0.8 μl trypsin (10 μg/ml) for exactly 60 seconds at 20°C. The reaction was stopped by addition of 2.4 μl soy-bean trypsin inhibitor (10 μg/ml). This limited digest results in generation of C3b of C3. Next, C3b was mixed with various amounts of murine or human FI and 2 μl human FH and the reaction volume was completed to 10 μl with TBS. Alternatively, murine serum was added as a source of FI. The reaction was carried out for 30-60 minutes at 37°C and then stopped by addition of reducing 4x loading buffer and boiling. The samples were either analysed by western blot or stored at 4°C.

Time course

In order to test the ability of recombinant mouse FI to cleave C3b and iC3b, a time course assay was developed. For this, serum was thawed rapidly at 37°C, vortexed briefly, and then placed on ice. The serum was then mixed with recombinant human or mouse FI and then, zymosan was added to a final concentration of 5%. Alternative pathway buffer was used as buffer in this assay. Once prepared, the mixture was rotated in a 37°C incubator and at selected time points 40 μl of each sample were removed into 10 μl of 50 mM EDTA. The sampling times were: 0, 30, 60, 120, 180, 240, 480, 600 and 1440 minutes. At each time point the samples were microfuged to remove the zymosan and frozen at -80°C until tested by ELISA (see Sec. 2.2.4). Alternatively, the assay was also done with LPS (1 mg/ml) as complement activating reagent which was the advantage that it is soluble.

2.2.8 Mouse model of renal ischemia

All experimental work for this experiment was performed in the lab of Professor Steven Sacks (King's College London, Guy's hospital, MRC center for Transplantation). Animal handing and surgery was kindly performed by Dr Conrad Farrar.

In vivo dose escalation

Three male C57/Bl6 mice were injected s.c. with different doses of recombinant mouse FI in order to determine the optimal dose range for therapeutic intervention. Injected doses were 100 μg, 200 μg and 400 μg FI. Mice were tail bled after several time points (6, 12, 24, 36 and 48 hours) and the serum was analysed for reduced alternative pathway activity as well as serum elevation of FI.

Experimental set up

12 male C57/Bl6 mice (8 weeks) were divided into three groups. Therapeutic and control protein was injected s.c under light anaesthesia between 6 and 8 hours before reperfusion of the kidneys. Renal ischemia reperfusion injury was induced by occluding both renal vessels for a specified time. In brief, mice were anesthetized by inhalation of isoflurane. During surgery, mice were placed on a heated pad to keep the body temperature constant. Using a midline abdominal incision, renal arteries and veins were isolated and bilaterally occluded for 40 minutes using microaneurysm clamps. After occlusion, 0.5 mL saline was placed in the abdominal cavity and the abdomen was closed. Mice were under anaesthesia during the whole surgery. After removal of the clamps, the kidneys were observed for an additional minute to see the colour change indicative of blood reflow. Next day, after 24 hours, tail blood samples were taken for BUN analysis. After 48 hours, all mice were culled. Blood samples were taken again and kidneys were harvested for histopathology.

Assessment of renal function

Serum was prepared as described in 2.2.6 and blood urea nitrogen (BUN) levels were measured using a standard urease kit (BUN Infinity) which is based on the measurement of coupled enzyme reactions of urease and glutamate dehydrogenase.

Assessment of renal histopathology

Kidneys were fixed in a solution of 4% formalin in PBS for 24 hours and embedded in paraffin by Guy' Hospital Service. Paraffin sections of 2 μm were cut and stained with periodic acid-schiff (PAS). Sections were fixed for one hour at 60°C and de-waxed in xylene. Then, they were re-hydrated slowly to prevent damage to the kidney sections by placing in different concentrations of ethanol (100, 90, 70% EtOH and water). Plates were then stained in 1% periodic acid for 10 minutes, washed and placed in Schiff's reagent for 10 minutes. After washes, plates were placed in haematoxylin for 5 minutes and washed again before being de-hydrated again (70, 90 and 100% EtOH). Last, plates were transferred into xylene.

The severity of tubular injury at the area of corticomedullary junction was graded in a blinded fashion by two people using a 6-point scale as follows: 0 = normal kidney, 1 = less than 10% necrosis, 2 = 10-25% necrosis, 3 = 25-50% necrosis, 4 = 50-75% necrosis and 5 = more than 75% necrosis.

2.2.9 The adeno-associated Virus (rAAV) gene delivery system

Project was carried out in collaboration with Prof. Ian Alexander's lab at the Children's Medical Research Institute at Westmead (Australia). Workflow is only described briefly because it was performed by Dr. Szun Tay.

Preparations of rAAV2/8 virions

The cDNA of murine complement FI was cloned into pAM2AA using XbaI and HindIII restrictions site and transformed into SURE2 cells to prevent unwanted recombination events. HEK293 cells were transfected with all three plasmids using $CaPO_4$. Two days after transfection, cells were harvested and cells were lysed by repeated freeze-thawing in a dry ice/100% ethanol bath and then stored at -80°C. AAV virions were purified on a cesium chloride gradient and virus particles were quantified by real-time PCR.

In vivo experiments

AAV_mFI was first tested by injection into mice. 12 mice were divided into 4 groups, which received different concentrations of AAV_mFI or the control virus preparation:

- low mFI = $5x10^9$ virions
- med mFI = $5x10^{10}$ virions (=100 % liver transduction in previous experiments)
- high mFI = $5x10^{11}$ virions
- GFP = $5x10^{11}$ virions

The mice were injected intravenously into the tail vein. Blood was taken after 4 weeks and after 8 weeks, the mice were culled by cardiac puncture and their serum was analysed.

Chapter 3

Therapeutic application of complement Factor I

3.1 Introduction

In this chapter, the therapeutic potential of complement FI in a renal ischemia reperfusion injury model is assessed. First, FI will be introduced and its actions within the complement system will be described. Then, the used model will be explained as well as the role of complement in renal ischemia reperfusion injury.

3.1.1 Complement Factor I

Complement FI is a serine protease that acts as a negative regulator of complement activation. It cleaves the α'-chain of C3b to iC3b which can be further degraded to C3dg and C3c; all these steps only occur in the presence of the respective co-factor proteins. This proteolytic cleavage of complement C3b is a key step in the self protection of the complement system.

FI was first characterized by Lachmann and Müller-Eberhard as a plasma protein that enables bovine conglutinin to react with surface bound C3 [146]. It was initially anticipated that bovine conglutinin reacts with C3b, but then found that there is another factor required, a so-called conglutinogen-activating factor or KAF. This factor (i.e. FI) acts on C3b, cleaves

it at two sides and thereby exposes a hidden carbohydrate. Only then, conglutinin can bind C3 (actually iC3b) and cross-link the cells; a reaction visible by eye.

In the 1970ies, it was found that FI could also cleave C4b [147]. Therefore, the name was changed to C3 inactivator, C4b inactivator or C3b/C4b inactivator (C3b/C4bINA) and finally again to complement FI. At that time, FI was purified out of human serum but as the methods for protein purification improved, it was seen that more highly purified FI does not cleave C3b or C4b anymore. It was therefore concluded that FI needs a co-factor for cleavage of its substrates [148, 149]. These co-factors are FH (FH) [150], membrane co-factor protein (MCP) [151], complement receptor 1 (CR1) [152] and C4b-binding protein (C4bp) [153, 154].

Even though, most of the available information about FI refers to the human protein, it was already early found that there was also an activity in mouse serum that resembled that of human FI and which inactivated the haemolytic activity of the red blood cell intermediate EAC4b prepared with human C4 [155]. After its purification out of mouse serum, it was soon found to be homologous to human FI by functional and structural criteria [156].

3.1.1.1 Structure and expression of complement Factor I

Complement FI is a serum glycoprotein with an average concentration of about $35\,\mu g/ml$ [80]. It is an acute-phase protein and its expression is increased by IL-6 during inflammation [157, 158]. FI is mainly expressed in the liver by hepatocytes [158], although it has also been found to be expressed in monocytes [159], fibroblasts [160], keratinocytes [161] and human umbilical vein endothelial cells [162]. It is also known that several carcinomas express FI to protect against the complement system, i.e. breast cancer [163] or cutaneous squamous cell carcinoma [164].

FI is a heterodimer, consisting of a heavy and a light chain which are covalently linked by a disulphide bond [165]. The FI gene encompasses 63 kb and its coding region is 1.9 kb. FI is expressed as a single chain of 66 kDa, the pro-peptide of 603 amino acids [166]. Post-translationally, the protein undergoes several modifications, including N-glycosylation in the endoplasmatic reticulum and Golgi. Each chain can be glycosylated at three asparagin

residues (Fig. 3.1); these heavy weight glycan sugar molecules make 20-25% of the apparent protein molecular weight [166]. The pro-enzyme has a molecular weight of around 88 kDa. Additionally, FI is proteolytically cleaved in the trans-Golgi network by furin which cuts out four positively charged amino acids, namely RRKR [167]. These four amino acids are found at the interface between heavy and light chain and form the linker peptide. After their removal, the pro-enzyme is processed into the enzymatically active, cleaved heterodimeric form. Furin or PACE (paired basic amino acid cleaving enzyme) is an ubiquitous endoprotease that cleaves a variety of mammalian pro-peptides, including growth factors, pro-albumin, von Willebrand factor, serum proteins or viral-envelope glycoproteins [168, 169]. Although pro-FI contains 28 paired basic amino acid residues throughout its sequence, furin recognizes and cleaves only at the Arg-Arg-Lys-Arg site [167]. FI has an 18 residue N-terminal leader sequence that is cleaved off before secretion and the mature protein is released into the blood stream [170].

FI has been recombinantly expressed by several groups [167, 171, 172] but only partial cleavage of the pro-enzyme was reported while no pro-form of the enzyme has ever been detected in serum. The observed low rate of processing of recombinant pro-FI in mammalian cells may be due to several factors such as the high level expression of the gene, saturation of the pro-peptide cleaving capacity of the cells or the rate of transit through the cellular compartments being too rapid for complete cleavage to occur [167]. The main FI expressing cells are hepatocytes and it is quite possible that cell types used for tissue culture have a lower processing rate of the pro-enzyme. The limiting factor is probably the amount of furin predent in the expressing cell. Wong et al. have demonstrated that co-transfection of Factor I and PACE (= furin) cDNA in COS-1 cells results in the secretion of more than 90% processed recombinant FI [167].

FI consists of several domains (Fig. 3.1); some of them share sequence similarity with domains found in both, complement and non-complement proteins [80]. The heavy chain consists of an N-terminal FI membrane attack complex domain (FIMAC), a scavenger receptor cysteine-rich domain (SRCR, also known as CD5 domain), a low-density lipoprotein receptor 1 and 2 domain and a small region of unknown homology, sometimes called the

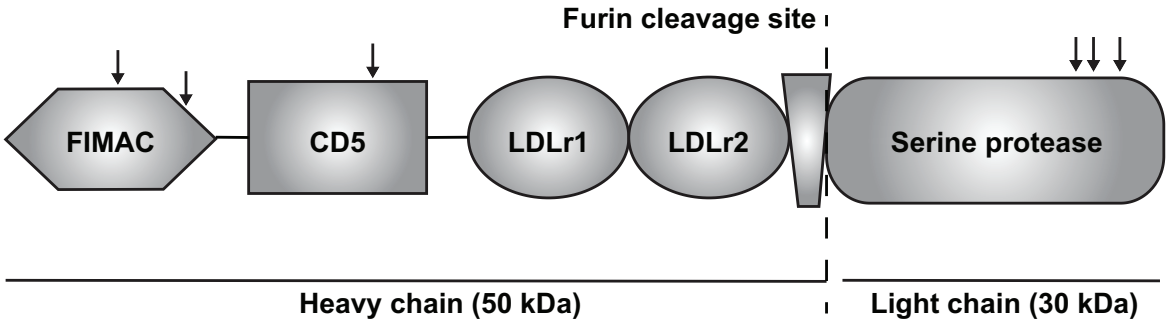

Fig. 3.1 Schematic structure of Factor I. Complement FI consists of a heavy and light chain which are linked by a disulfide bridge. FI is expressed as a single polypeptide chain and post-translationally cleaved by furin (dotted line) that separates FI into the two chains. Each chain can be glycosylated at three sites (arrows). Figure is taken from Nilsson et al. [80].

D-region [80]. The light chain of FI is the enzymatically active domain which consists of a chymotrypsin-like serin protease (SP) domain [173] containing the residues that form the His-Asp-Ser catalytic triad.

Until recently, the role of the heavy chain in complex formation was not quite clear. Since the FIMAC domain consists of a Kazal-type protease inhibitor domain that has been reported to prevent the action of thrombin/trypsin-like proteases [176], it was proposed that FI-co-factor-substrate complexes might cause separation of the SP and Kazal domain, thus allowing FI to become fully functionally active [173]. A few years later though, FI was shown to be active against synthetic substrates in the absence of co-factors [177]. The same group also demonstrated that intact FI cleaves C3b(NH_3) rapidly and specifically in the presence of FH, but this cleavage was slow and less specific when only the SP domain and no FH was present [178]. In these experiments, the SP domain alone cleaved C3(NH_3) at more than the two usual sites and presence of FH did not stimulate cleavage like in the intact protein but inhibited this unspecific cleavage. This led Tsiftsoglou et al. to suggest that the heavy chain of FI binds to the substrate and orients the SP domain of intact FI towards the two cleavage sites in C3b which are cleaved to form iC3b.

In 2011 the crystal structure of FI was published and shed more light on the the arrangement of the heavy and light chain in the C3b-FH-FI complex [175]. It was suggested that the substrate (i.e. the C3b-FH complex) induces structural remodelling in the active site of FI.

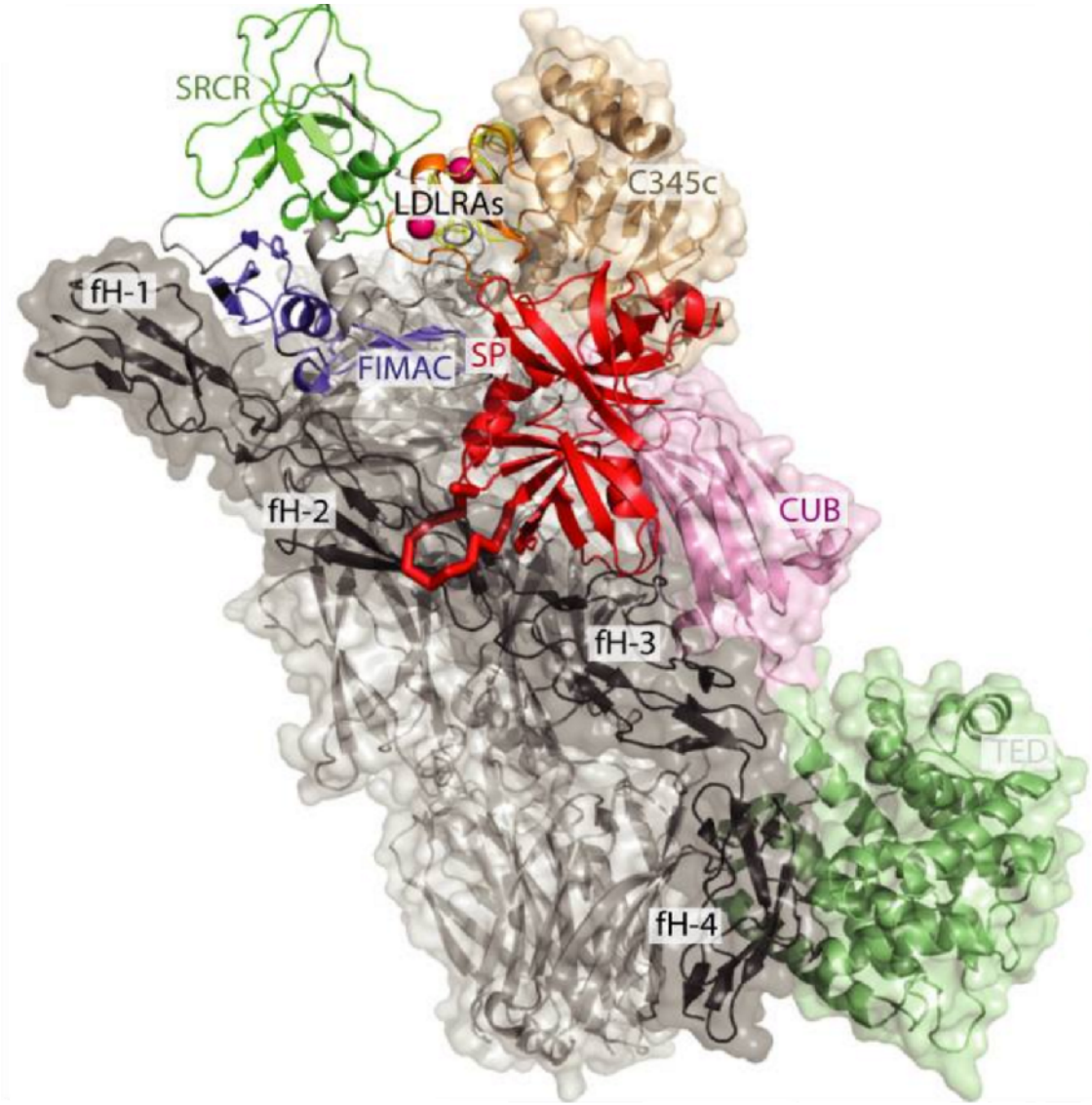

Fig. 3.2 Model of a complex of FH(1-4), C3b and FI. C3b and FH(1 - 4) are depicted in semitransparent surfaces and FI in full colour. Only the first four CCPs of FH are shown, which are required for C3b binding (in grey). The domains of C3b shown are the C345C domain (light bronze), the CUB domain (pink), the thioester containing domain (TED, light green), and the linker domain, C3 α' N-terminal domain, and macroglobulin domains of C3b (light gray). The figure shows binding of FI to the FH-C3b complex with its FIMAC and SP domain. The SRCR domain of FI does not take part in binding and mutations in that domain were also found to not impair FI binding [174]. Figure taken from Roversi et al. [175].

This is further supported by the finding that the protease inhibitor diisopropyl fluorophosphate is only able to react with the active site serine if FI is pre-incubated with C3b [179]. Superimposition of FI on the crystal structure of C3b-FH(1-4 complex shows that apart from the SRCR domain, the heavy chain is closely packed against C3b and co-factor [175] (Fig. 3.3). This binding abrogates the allosteric inhibitory effects of the heavy chain and induces remodelling of the SP domain, which then becomes active and cleaves C3b. The importance of the FIMAC and FI domain accessibility in proper FI function was elegantly demonstrated in a series of mutagenesis series [174].

3.1.1.2 Cleavage of complement C3 and C4 by Factor I

Complement FI cleaves three peptide bonds in the α-chain of C3b and two bonds in the α-chain of C4b. Notably, these cleavages are restricted to arginyl bonds in its natural substrates, C3b and C4b [178]. These bonds are, in C3b LPSR-SS and SLLR-SE; in iC3b, RLGR-EG; and in C4b, HRGR-TL and STGR-NG [80]; cleavage occurs after arginine (R) in each case.

Degradation of C3b The breakdown of C3 during complement activation is depicted in Fig. 3.3 and will be described further in the section below.

C3 (185 kDa) consists of two chains, the α and the β chain while in C3b, the N-terminal peptide, C3a (9 kDa), is cleaved off by a C3 convertase. Cleavage of C3b is achieved by two FI-mediated cuts in the α'-chain of C3b. A small fragment called C3f (3 kDa) is released, dividing the α'-chain into a 68 and a 43 kDa fragment [101, 180]. This proteolysed C3 is now called iC3b and it can no longer take part in a C3 or C5 convertase. This first cleavage to iC3b occurs quite quickly and requires FH as a co-factor while the second cleavage of iC3b is much slower. Only in the presence of the co-factor complement receptor 1 (CR1), iC3b can be further cleaved by FI. Co-factor activity of FH for the cleavage of iC3b in vitro is minimal. It requires buffers at low ionic strength and is observed only after extensive incubation [181], so the physiological relevance is questionable. FI cleaves iC3b again in the 68 kDa fragment which releases a large part of the molecule, called C3c. Solely C3dg remains bound to the surface because of the strong covalent nature of the thioester binding.

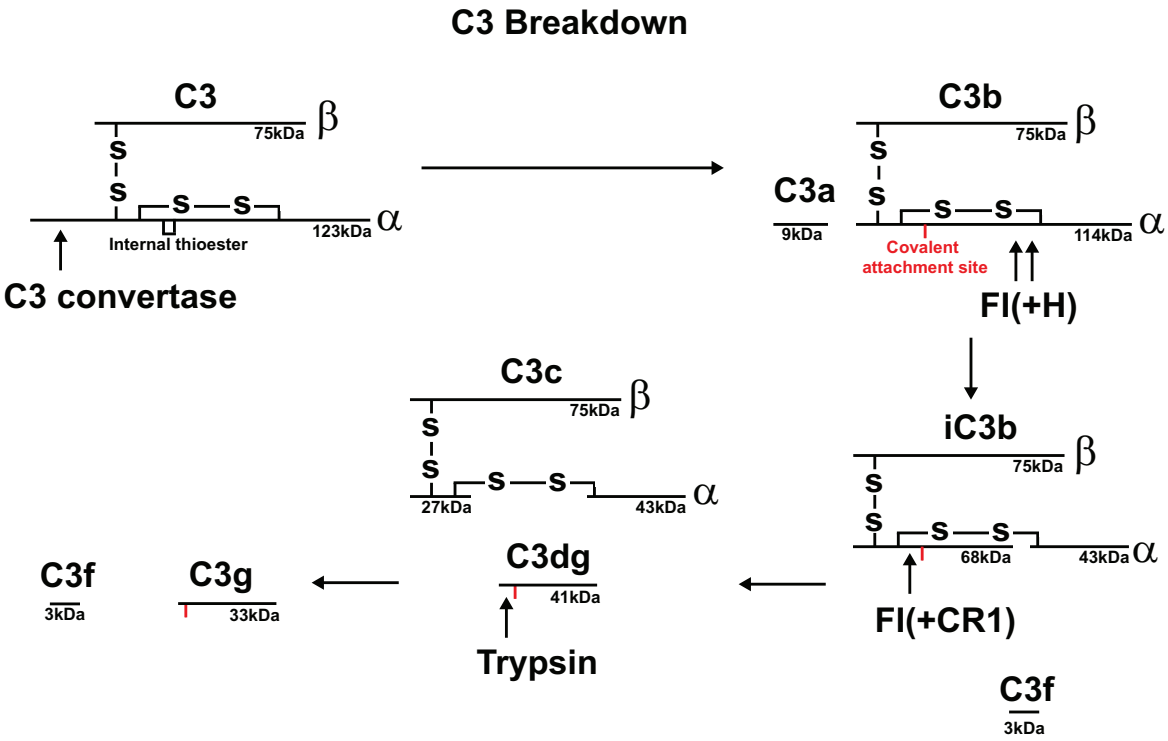

Fig. 3.3 Proteolysis of C3 during complement activation. C3 consists of the α and β chain. The β-chain is not modified while the α-chain is cleaved several times: 1.) C3a is cleaved off by a C3 convertase and the remainder protein is now called C3b; 2.) FI cleavage at two sites, releasing the C3f peptide (FH as co-factor), it is now called iC3b or C3bi; 3.) with CR1 as co-factor, FI cleaves again in the 68 kDa fragment of iC3b. While C3c diffuses off. C3dg stays attached to the cell surface and can be cleaved further by trypsin-like proteases. Figure is taken from [43].

Lachmann et al. showed that C3dg can be further degraded at the amino terminus by serine proteases, e.g. trypsin to C3d, but this reaction does not occur in plasma. [182].

Degradation of C4b C4 (200 kDa) contains three polypeptide chains: the α, β and γ-chain. During activation, C4 is cleaved into a small fragment of 8.8 kDa, C4a, and the major fragment, C4b that stays attached to C2a and forms a classical or lectin pathway C3 convertase. Like C3b, C4b now exposes several binding sites for proteins that can either promote the complement cascade or inactivate it. Binding of C4bp or MCP to C4b allows the cleavage of C4b by FI into iC4b and then C4c and C4d [183].

In recent years, the immunohistochemical detection of C4d in renal allograft biopsies has gained clinical interest. The accumulation of C4d along peritubular capillaries is used as a marker for an antibody-mediated allo-response because it has been shown to correlate with the levels of donor-specific antigens. [184, 185]. C4d deposition is associated with poor graft survival.

3.1.2 The complement system in renal ischemia reperfusion injury

A very brief introduction into the kidney structure and function will be given below, before the topic will be switched to renal ischemia reperfusion injury.

The kidneys are two bean-shaped organs that perform several essential homoeostatic functions. They are located in the abdominal cavity and consist of an outer cortex and the inner medulla. The nephron is the smallest structural and functional unit of the kidney (Fig. 3.4); there are $\approx$ 1 million functional nephrons in a human kidney . It is composed of a renal corpuscle, consisting of the glomerulus surrounded by the Bowman's capsule, and the renal tubules that are formed by the proximal tubule, the loop of Henle and the distal tubule. The glomerulus is a network of small blood vessels where $\approx 180\,\mathrm{L}$ plasma are filtered out of the blood every day. The resulting glomerular filtrate is further processed along the renal tubules and forwarded into the collecting duct. The end product is passed into the urinary bladder via the ureter from where water-soluble waste products are secreted as urine.

The kidneys fulfil several functions, the most important of which is removal of waste products from the plasma. Additionally, they regulate the acid-base balance, electrolyte concentrations, extracellular fluid volume and the blood pressure. They accomplish this by above mentioned filtration of plasma. In the tubules, glucose, electrolytes and water are either added to or removed from this filtered fluid.

Generally, humans can live normally with only one kidney if the other kidney is is severely diminished in its function or had to be removed because of disease.

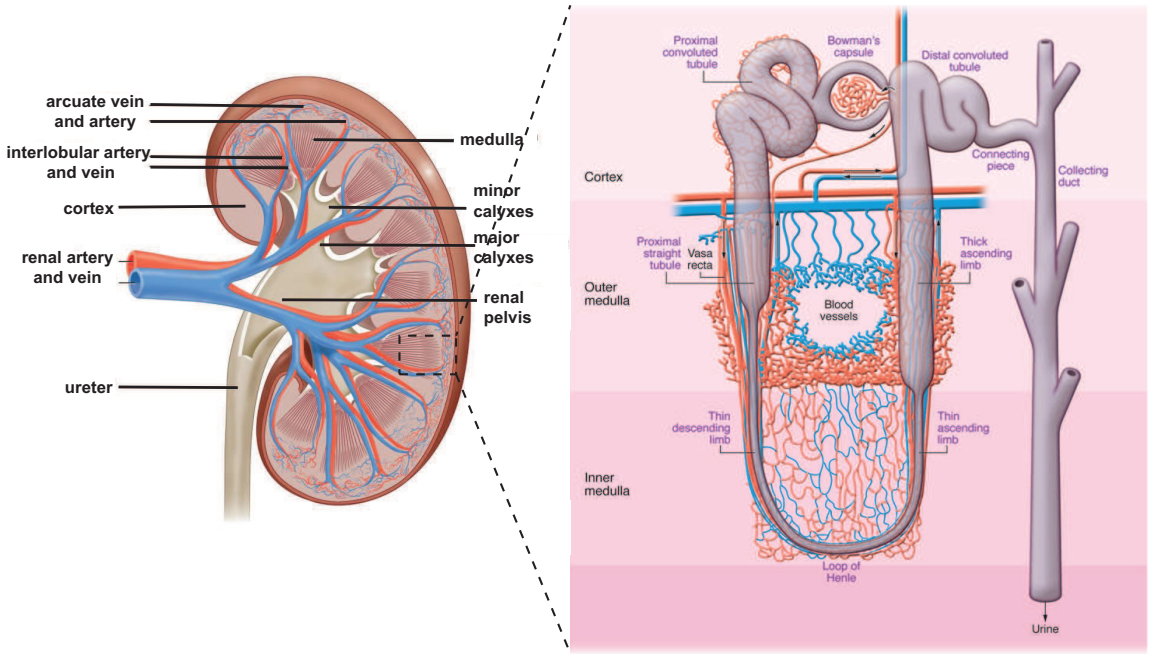

Fig. 3.4 Schematic structure of a kidney and nephron. Nephrons stretch from the outer cortex through the renal medulla. Once plasma is filtered out of the glomerulus into the Bowman's capsule, it enters the proximal tubule. Next, the filtrate is passed through the descending and ascending loop of Henle, before it enters the distal tubules, which end in a collecting duct. Figure adapted from Bonventre at al. [186].

Ischemia reperfusion injury

Ischemia occurs if an organ or tissue is temporarily cut off its blood supply and reperfusion injury happens when the blood supply is restored again. During the ischemic period the absence of oxygen and nutrients that are necessary to maintain a normal metabolism and energy homoeostasis, creates an environment that leads to inflammatory events in the reperfusion period [187]. In fact, it is depletion of ATP which is rapidly degraded when deprived of oxygen in early ischemia and induces critical alterations in the cytoskeleton [188]. The hypoxic and anoxic cells in ischemic tissues become necrotic and release endogenous ligands that lead to significant changes in epithelial and endothelial cell morphology and protein/receptor surface expression profiles which stimulate innate immune responses [36, 189]. Once perfusion is restored, incoming innate immune cells or serum proteins (such as complement factors) are activated by endogenous ligands from necrotic and apoptotic cells and exacerbate the inflammatory tissue and organ injury [190]. Ischemia reperfusion injury (IRI) can affect

almost every organ, such as heart [35], brain or kidneys, but also muscle tissue [191] and intestines [192]. IRI is a common source of morbidity and mortality in e.g. myocardial infarction, stroke, gut ischemia, and cardiopulmonary bypass [193]. Often, there are no specific therapies available. It has long been known that complement plays a role as a main pro-inflammatory mediator in IRI [62, 193–195] but which pathway of complement is activated varies in the different affected tissue types [187]. Likewise, the effect of complement mediated damage varies depending on the organ and the animal model used [194]. Nevertheless, it is important to stress that the complement system also has a beneficial role after IRI in clearing up debris and dead cells, which otherwise might lead to inflammation and further immune responses. This opsonisation and efficient clearance is mainly mediated by the classical pathway and dampens inflammation, although also recognition molecules of the lectin pathway were shown to bind to apoptotic cells [196, 197].

Renal ischemia reperfusion injury

In this thesis, the focus will be set on renal ischemia reperfusion injury (IRI) which is an inflammatory process of the kidney that leads to acute renal injury. Renal IRI is an inevitable event during renal transplantation and the duration of ischemia greatly effects short- and long-term transplant function [198–200]. In fact, IRI is one of the major causes of morbidity and mortality after organ transplantation [201] but can also occur as a consequence of impaired kidney perfusion e.g. during major surgery or sepsis. Even though, complement is an important player, it is not the only one and neutrophils, oxygen radicals and other factors produced by an activated endothelium, also contribute to the process of injury [202–205]. Nevertheless, since complement activation is an early event in reperfusion injury, it might have a direct influence on all other above mentioned factors [206].

In renal IRI, the area most affected are the tubules, more exactly the S3 fragment of the proximal tubules [207] (see Fig. 3.4, segment of proximal tubules stretching between the end of the cortex and the outer medulla). Within a short time during an ischemic event (several minutes), the proximal tubular brush border begins to shed [208] and the polarity of adhesion molecules, complement receptors and other membrane molecules (e.g. integrins

or Na^+-K^+-ATPase) is lost [209]. The cytoskeleton gets disrupted and tight junctions open up, leading to reflux of the glomerular filtrate from the lumen to the interstitium. In later, advanced ischemia, there is detachment of viable and necrotic tubular epithelial cells which together with remnants of brush boarder vesicles and cellular debris form tubular casts which can be seen in PAS staining for example [186]. All these events are depicted in Fig. 3.5. Danger-associated molecular patterns (DAMPs)[1] that are released during ischemia, promote an inflammatory environment and activate innate and adaptive immune responses once reperfusion is restored [210]. One reason for the susceptibility of the S3 segment to IRI is that even under normal circumstances, this region is characterised by a slight hypoxia caused by a low partial pressure of oxygen which is not only a consequence of the high metabolic requirement of the proximal tubular epithelial cells but also of the counter-current arrangement of vessels, especially the vasa recta, that drains the outer and inner medulla (Fig. 3.4) [188, 211]. The nephron fragments at the cortico-medullary junction are therefore more sensitive and predisposed to the relative hypoxia and its downstream effects. C3 was shown to be deposited primarily on the basolateral side of the tubular basement membrane at the cortico-medullary junction and this deposition correlated with tubular injury [193]. Damage of the tubules by complement deposition induces release of pro-fibrotic growth factors, cytokines, and matrix proteins [212]. Additionally, it is also known that the distribution of complement receptors varies in that segment. In human, it was found that, although present in the kidney, both, DAF (CD55) and MCP (CD46) are not expressed in the apical portion of proximal and distal tubules, and only MCP could be detected on the basolateral side [213]. In mice also, several complement inhibitors are present within the kidney but only Crry, homologue to human MCP and FI co-factor, is present on tubular epithelial cells [214]. As in humans, the regulator protein was only found at the basolateral but not at the brush boarder (apical side) of tubules. In hypoxic cells this polarized expression of Crry is lost, permitting complement activation on the basolateral membrane [215], a finding that could be repeated in vitro by the same lab. It is highly likely that a similar re-polarisation of MCP occurs in human tubular cells which, together with

[1] DAMPs are host-derived molecules that initiate an immune response in a noninfectious inflammatory response

increased access of serum to the unprotected apical side, leaves the tubules largely exposed to complement attacks. This hypothesis is further supported by observations that the detection of complement breakdown products in the urine correlates with the occurrence of tubular C3 deposition in non-selective proteinuria [216] as well as the appearance of casts and tubular cells in the urine [186].

Many studies have been conducted so far to elucidate the role of complement in renal ischemia reperfusion injury [36, 62, 193, 195, 217] and there is a clear interest in treatment aimed at blocking, attenuating or reducing the complement system and its activation [187]. Nevertheless, there is some degree of controversy to what extent each pathway plays a role in complement activation in IRI [218]. Deficiencies in the complement system showed protection in renal IRI in mice: C3-, C5, and C6-deficient mice had less tubular injury [193] than C4-deficient mice [219]. It was therefore speculated that both the alternative and the terminal pathway of complement cause tubular injury, a notion that had been reported before [212, 220]. Additionally, C3a and C5a were shown to exacerbate renal tubular injury by C3a- and C5a-receptor signalling that leads to generation of pro-inflammatory mediators and recruitment of leucocytes [62]. C5a-receptor expression was shown to be upregulated on tubular epithelial cells after IRI [221]. Mice deficient in the alternative pathway (FB deficient or after administration of α-FB antibodies) show some degree of protection, highlighting the importance of an intact alternative pathway in renal IRI [217, 222, 223]. Also, MBL is thought to play a role in renal IRI because MBL deficient mice are protected [224], but since C4 deficient mice are not protected, other effector functions of MBL besides activation of the lectin pathway of complement might exist. Further, it was shown that sublytic serum concentrations of complement can activate the alternative pathway on human and mouse tubular epithelial cells *in vitro* [193, 212].[2] Conversely, it was also reported that complement activation by human proximal tubular epithelial cells *in vitro* was dependent exclusively on the classical pathway [225]. In this study classical pathway activation was mediated by IgM binding to modified phosphomonoesters exposed on human hypoxic proximal tubular

[2] Although, it was later shown that complement is initially activated by the lectin pathway and subsequently amplified by the alternative pathway [36]; see below.

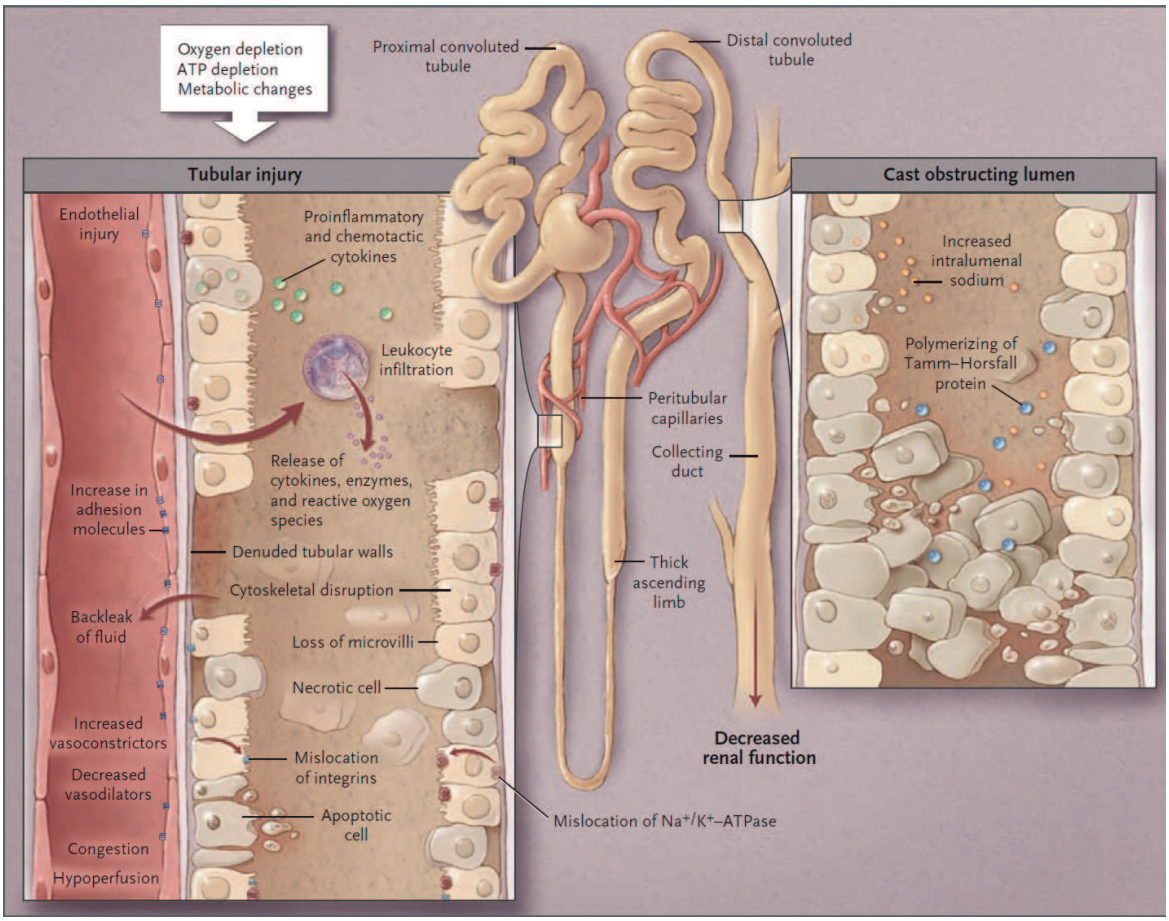

Fig. 3.5 Schematic depiction of renal tissue damage in ischemia reperfusion injury. Normally, tubular epithelial cells are protected by expression of complement control proteins at the basolateral side. In an ischemic event, complement, amongst others, up-regulates expression of endothelial cell adhesion molecules which allows immune cells and further complement components to leak out of the vessels into the surrounding interstitium that separates the endothelium of blood vessels and epithelium of tubules. The polarized expression of complement regulators as well as the luminal brush boarder is lost, leaving the tubular epithelial cells largely exposed to complement and immune cell attacks. Infiltration and action of neutrophils, macrophages, natural killer cells and MAC insertion of complement cause direct injury to tubular epithelial cells. The distal tubules are obstructed by cast which is formed out of dead tubular cells and brush-boarder membranes. Figure adapted from Abuelo [211].

epithelial cells (PTEC) and further supported by the use of C1q depleted serum. In each case, formation of terminal pathway components then leads to MAC-induced cytotoxicity [220].

Nevertheless, it is not quite clear what exactly triggers complement activation in the first place. Quite recently, however, the lectin pathway has gained importance in renal ischemia in mice and it was shown that MASP2 knockout mice (= lectin pathway deficient) were significantly protected in a renal transplant IRI model [36], which must be achieved by an C4-independent mechanism since C4 deficient mice were not protected [193, 219]. The lectin pathway has several recognition molecules (i.e. in human: mannan binding lectin [MBL], Collectin-11 [CL-11] and Ficolin [Fcn] 1, 2 and 3; in mice: MBL-A and -C, CL-11 and FcnA) [33], some of which were now found to function in the absence of Ca^{2+} and therefore have caused false assumptions regarding alternative pathway activation and absence of classical pathway and lectin pathway in certain experimental settings. The lack of knowledge that the lectin pathway can operate via a C4-independent bypass mechanism, earlier led to the wrong assumption that this pathway is not involved in renal IRI [218]. Cl-11 expression in the kidney is mainly associated with proximal and distal tubules and was found to bind preferentially to L-fucose and D-mannose [27, 32]. The first, L-fucose, was long known to be expressed in renal tubules [226] and it was shown that after ischemia reperfusion injury, there is an aberrant L-fucose pattern which co-localizes with CL-11 binding (submitted [227]). This study is further supported by results that show that efficient binding of CL-11 failed (and complement remained inactive) after L-fucose had been removed and that both, CL-11 and MASP-2 knockout mice were protected in renal IRI.

Although not much is known about complement activation in ischemic PETC in human, we know that complement is activated and exacerbates tissue damage. It is therefore essential to establish the validity of mouse studies in relation to man and to take differential complement pathway activation between species under consideration.

Summarised, renal ischemia reperfusion injury in mice is presumably mediated by lectin pathway activation in the renal tubules after DAMP (i.e. abberant L-fucose pattern) presentation. Once activated, the alternative pathway is also activated and amplifies complement activation. Due to a re-polarisation and subsequent loss of Crry, the main membrane-bound

complement regulator of renal tubules in mice, they remain unprotected and complement activation cannot be inhibited but proceeds to further inflammatory responses and MAC-induced cytotoxicity. Therefore, the most logical points of intervention in renal IRI are either inhibition of lectin pathway activation or of the feedback cycle of the alternative pathway. Since Masp2- and FB-deficient mice were both shown to be protected in renal IRI, this study aims at elucidating the role of the alternative pathway feedback inhibitor FI. By increasing serum concentrations of FI, the alternative pathway will be shifted towards its breakdown cycle while the feedback mechanism is impeded which was demonstrated *in vitro* [112, 135]. As stated before in Sec. 1.4.2, FI has several advantages that make it a perfect candidate for inhibition of the alternative pathway. With regards to species differences in complement activation, FI has another advantage in that it performs the same reaction in all relevant species. In other words, no matter how complement is activated, C3b and iC3b are cleaved and the terminal pathway is impeded. The aims of the studies described in this chapter were accomplished by expression of recombinant mouse complement FI. Its functional activity was confirmed first and then the therapeutic application of FI in a mouse model of renal ischemia reperfusion injury was examined.

3.2 Results and discussion

3.2.1 Generation of recombinant mouse complement Factor I

3.2.1.1 Expression, purification and in vitro processing of recombinant mouse complement Factor I

In the first year of my PhD, I used a FI construct prepared by another student (mFI cDNA with His6-tag in pSecTag). The expression cassette was cloned into pcDNA3.1 Hygro (+). FI protein expressed as pro-enzyme, was digested with furin and shown to be active in functional assays and to cleave both human and mouse C3b (results not shown). After a few months, the expression rate was increased tenfold but the purified enzyme was no longer functionally active when tested in an iC3b deposition assay on mannan (Fig. 3.6). Even though FI was used at a very high concentration ($80\,\mu$g/ml), it did not show any activity as opposed to human FI. Transfected cells with low passage numbers were thawed and tested but active protein could no longer be prepared. At that point, it was also found that there was a stretch of about 20 amino acids between the FI sequence and the His tag, also including a cysteine, derived from one of the cloning vectors used before. This cysteine could potentially cause problems during folding into the native structure.

Because there was no active mouse FI for *in vivo* studies, a pilot study was started to see whether human FI can be used in mice instead. Based on *in vitro* C3b cleavage results (see App. A.1), it was assumed that human FI could be used for mouse experiments as well, provided they did not last too long so that an immune response against the human protein could be mounted. Human FI was purchased from Comptech and injected into mice so that the overall plasma concentrations of FI are increased by 25 and 50%. Exact injection volumes are based on following equation:

$$FI_{tot} = C_{PL} \cdot V_{PL} + \frac{1}{2} \cdot C_{PL} \cdot (V_{ECF} - V_{PL}), \tag{3.1}$$

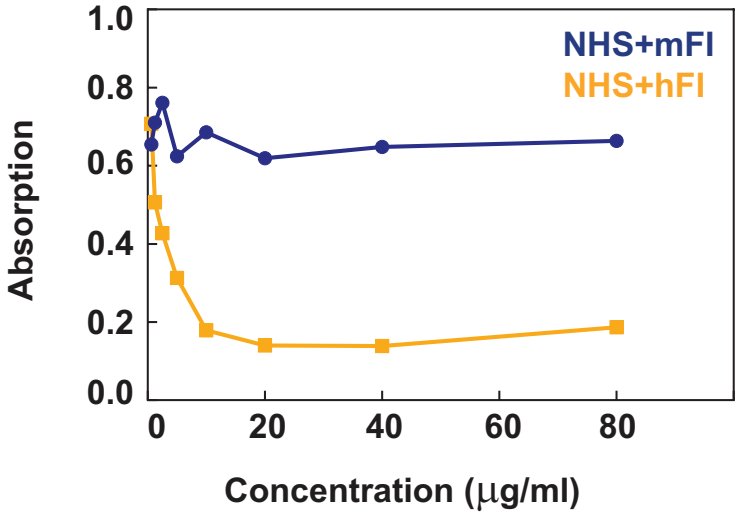

Fig. 3.6 iC3b deposition assay on mannan using inactive FI. Serial dilutions of either human or mouse FI starting from 80 μg/ml were added to 25% NHS and incubated for 1 hour at 37°C on a mannan coated plate. All dilutions were prepared in alternative pathway complement fixation buffer and bound iC3b was detected with an α-human C3c antibody. Absorption was measured at 415 nm. Experiment was performed three times in duplicates.

where FI_{tot} refers to the total amount of FI, C_{PL} to the plasma concentration of FI (35 μg/ml), V_{PL} to the plasma volume ($\approx$ 1.2 ml in 20 g mouse) and V_{ECF} to the extracellular fluid volume ($\approx$ 20% of body weight [ml/g]).

Two mice were injected s.c. with human FI, bled after 2 hours and culled after 3 days. Serum was diluted to 25% and tested for reduced alternative pathway activity in an iC3b deposition assay on mannan (Fig. 3.7). However, no effect of injected hFI could be observed and there was no down-regulation of the alternative pathway.

Since according to this assay, hFI seemed to have no effect which is opposed to the C3b *in vitro* cleavage assay (App. A.1), the assay conditions were repeated with normal mouse or human serum and human FI. 25% human and mouse serum were prepared in alternative pathway complement fixation buffer, Comptech human FI was added and serially diluted. The assay shows that while hFI is highly active in human serum, it shows absolutely no activity in mouse serum which also explains previous results but contradicts results from C3b cleavage using purified components (App. A.1).

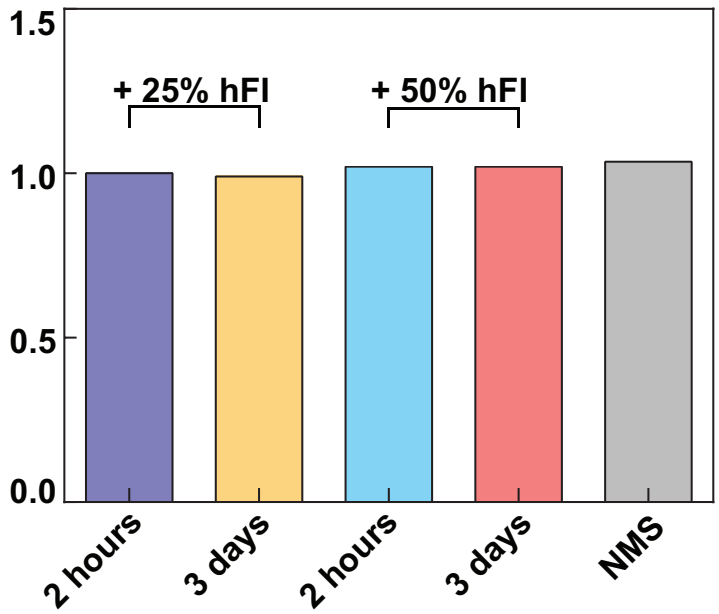

Fig. 3.7 iC3b deposition assay after injection of human FI into mice. Mouse serum (3 hours and 3 days post FI injection) was diluted to 25% in alternative pathway complement fixation buffer incubated for 1 hour at 37°C on a mannan coated plate. Bound iC3b was detected with an α-human C3c antibody. Absorption was measured at 415 nm. Experiment was performed once in duplicates

Because human FI could not be used in mice and to avoid further complications with the faulty FI construct from previous experiments, I prepared a new mouse FI-expression plasmid. The DNA sequence of murine complement FI was downloaded from the European Nucleotide Archive (ENA, accession number: AAI50752.1) and the protein sequence was taken from UniProt (accession number: Q61129). For easy purification of the recombinant protein, a His6-tag was added to the C-terminus. A Kosak sequence (GCC ACC) was added upstream of the start codon to increase the transcription rate of the gene. (Construct shown in Fig. 3.9). Primers for the new construct were designed to amplify only the cDNA of FI with its signal sequence (FI-F and FI-R). A short stretch of 4 amino acids, i.e. glycines and serines

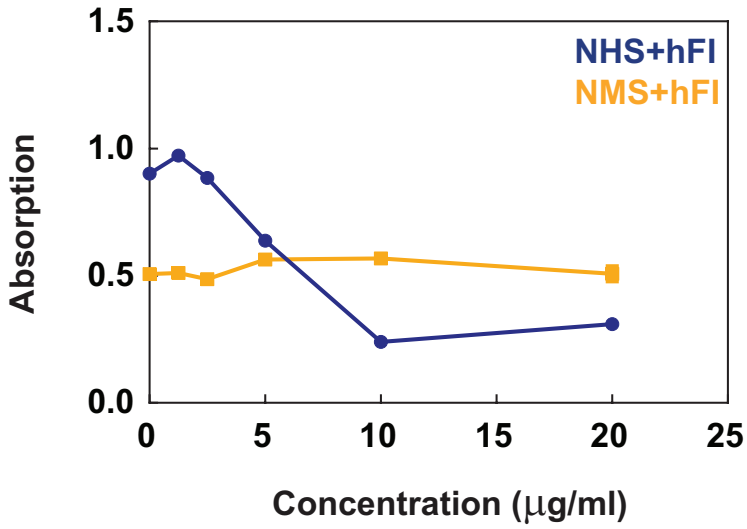

Fig. 3.8 25% human and mouse serum were prepared in alternative pathway complement fixation buffer and Comptech human FI was added in serial dilutions starting from 20 μg/ml. After loading on a mannan coated plate, it was incubated for 1 hour at 37°C. Bound iC3b was detected with an α-human C3c antibody. Absorption was measured at 415 nm. Experiment was performed twice in duplicates

(GSGS), was introduced between the end of the FI sequence and the His6 tail, followed by a Stop codon.

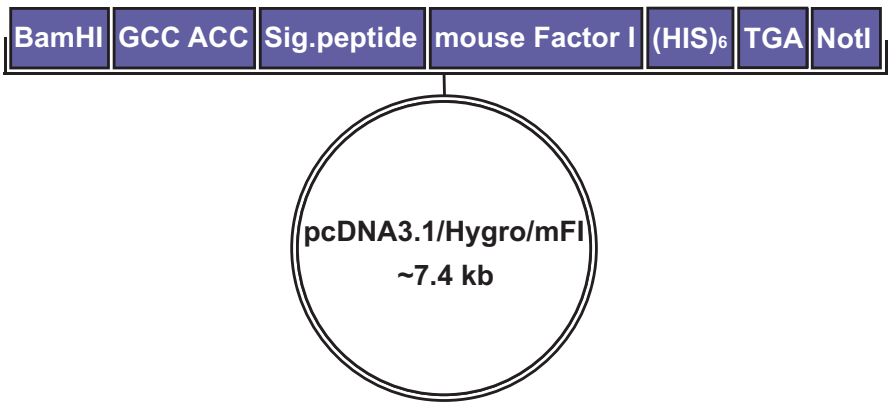

Fig. 3.9 Schematic diagram of Factor I expression construct

Mouse FI was amplified from a cDNA library and cloned into the eukaryotic expression vector pCDNA3.1/Hygro(+) (Fig. 3.10). The sequence was confirmed by sequencing and the construct was transfected into HEK293T cells. Hygromycin B was used for selection of

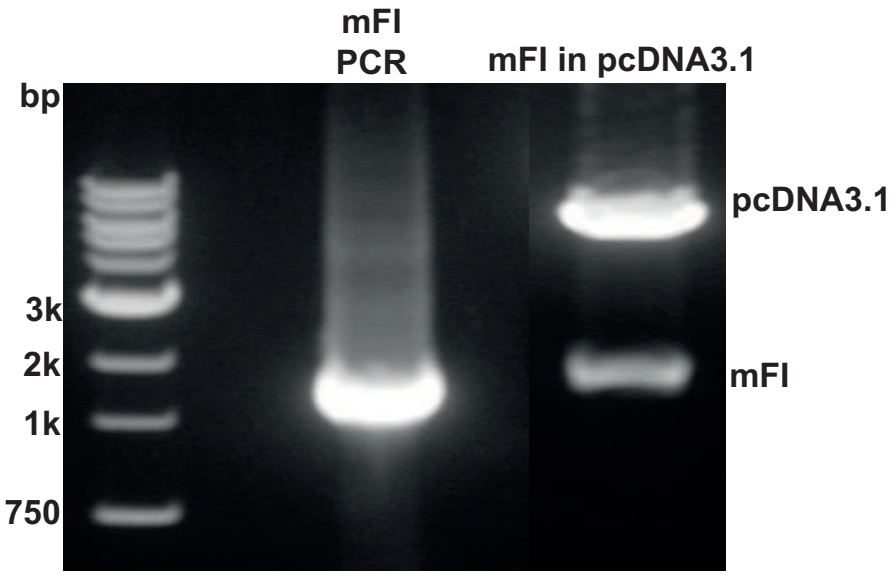

Fig. 3.10 Agarose gel of mouse FI PCR amplification (lane 1) and mini prep analysis (lane 2) of recombinant mouse FI in pcDNA3.1/Hygro(+).

stable transfected cells that secrete FI. After 14 days, the cells were screened for FI expression by dot blot analysis. Positive clones were amplified and frozen 10%-DMSO/FBS stocks were prepared (-80°C for short- and liquid nitrogen for long term storage).

In order to purify the tagged protein efficiently, the supernatant had to be concentrated. Otherwise, a great fraction of recombinant protein does not bind to the nickel column. Initially, an Amicon stirred cell was used for concentration but this was later found to cause irreversible precipitation of proteins in the supernatant and to fragment recombinant FI. Therefore, an ammonium sulphate (AS) cut was performed to precipitate all proteins in the supernatant. The right concentration of AS was first determined. Concentrations greater than 60% were found to precipitate mFI with 80% giving the maximum precipitation. Thus, an efficient AS concentration of 75% was used in order to ensure that all mFI is precipitated but also that precipitated protein can be redissolved after centrifugation. After AS precipitation, proteins were harvested by centrifugation and dialysed against 20 mM phosphate, pH 7.8, 0.5 M NaCl overnight (EDTA chelates Nickel and thereby strips it off the column). The IMAC column was prepared as described in Sec. 2.2.3 and recombinant his-tagged FI was purified accordingly.

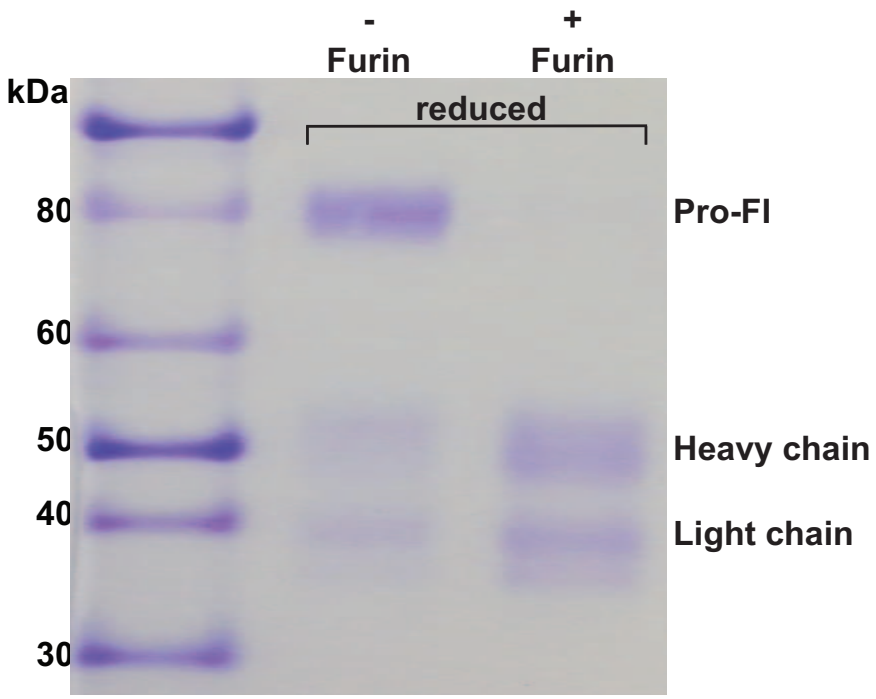

Fig. 3.11 SDS PAGE analysis of recombinant FI purified by IMAC. Lane 1: The protein is expressed mainly as pro-FI. Heavy and light chain of FI in the pro-enzyme cannot be separated by reduction. Lane 2: After overnight incubation with furin, the pro-enzyme is converted to the enzymatically active enzyme. The shown SDS PAGE is a representative of a usual FI purification. The ratio of pro-enzyme to processed enzyme in the purification was found to be variable to a certain degree.

FI could be purified using IMAC and it was found that the cells primarily secrete pro-mFI which appears as a 80 kDa band on a reducing SDS page, although there is also a small percentage of processed FI, visible as the 50 kDa (heavy chain) and 38 kDa band (light chain) (Fig. 3.11, lane 1). In order to get enzymatically active FI, the purified protein was digested with the endoprotease furin which cleaves at its recognition site (RRKR), separating heavy and light chain. After overnight incubation with the protease, all FI pro-enzyme was converted into the active heterodimeric form (heavy chain and light chain), (Fig. 3.11). Efficient cleavage of FI was further confirmed by western blotting: 2 different antibodies recognizing either pro-enzyme and heavy chain (α-mFI) or pro-enzyme and light chain (α-His) were used (Fig. 3.12a and b) (antibodies and their antigens are listed in Sec. 2.1.1). When loaded under reducing conditions, the 80 kDa pro-enzyme band only appears if there

is undigested FI present. Otherwise, the disulfide bond that links the two chains is reduced and FI appears as two distinct chains.

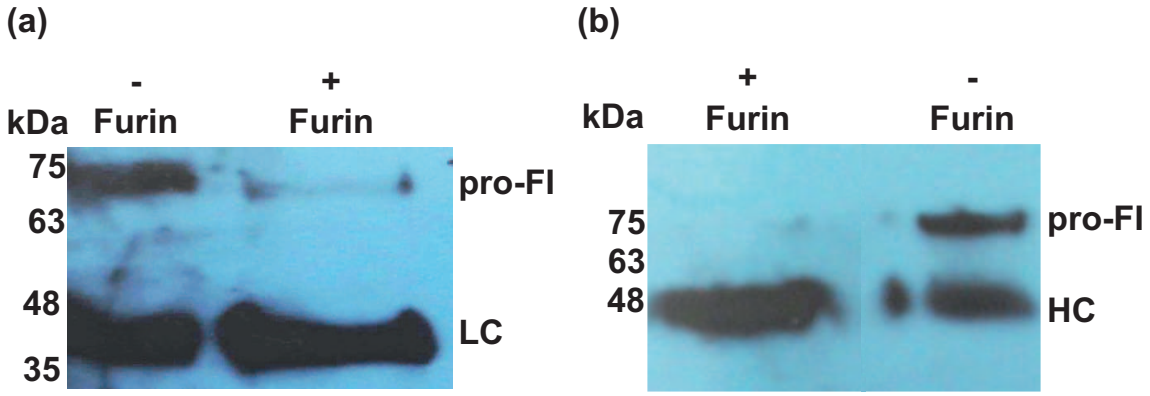

Fig. 3.12 Western blot analysis of reduced recombinant FI. 10 μl of 2 μg/ml of FI were separated by SDS PAGE and detected with two different antibodies. (a) FI is probed with an α-His antibody (1:4000) that recognizes the pro-enzyme and the light chain of FI. (b) FI is preobed with an α-FI antibody (1:500). The antibody recognizes an epitope in the heavy chain which is also accessible in the denatured pro-enzyme of FI. The Experiment was performed for every batch of recombinant FI.

3.2.1.2 In vitro testing of recombinant mouse Factor I's functional activity

In order to be able to proceed to *in vivo* studies, the functional activity of the expressed protein had to be confirmed. The ability of FI to cleave its natural substrates C3b and iC3b was tested by different methods, using purified components and whole serum. All these different assays were necessary to be absolutely sure of the protein's activity and to avoid misleading results as obtained with human FI cleaving purified mouse C3b but not in whole serum. It was however quickly found that mouse FI is active in human serum which enabled comparison with human FI in the performed assays.

C3b cleavage by Factor I Fig. 3.13. Since FI cleaves only C3b but not native C3, C3b was first prepared by limited digest with trypsin, during which the C3a anaphylatoxin was cleaved off (Fig. 3.13, lane 1 and 2). Incubation of hC3b with hFH as co-factor and h or mFI leads to cleavage of the C3α-chain. The first, rapid cleavage into iC3b appears as the 68 kDa band and the second, slower cleavage of iC3b into C3dg, as the 40 kDa band. Cleavage of

C3b by hFI served as a positive control (lane 3). It can be clearly seen that mFI is active and able to cleave the α-chain of hC3. It is also compatible with hC3b and FH which were used in the experiment and to perform both cleavages of the α-chain. It should be pointed out that cleavage, especially the second, slower cleavage, requires longer incubation periods, probably due to species differences.

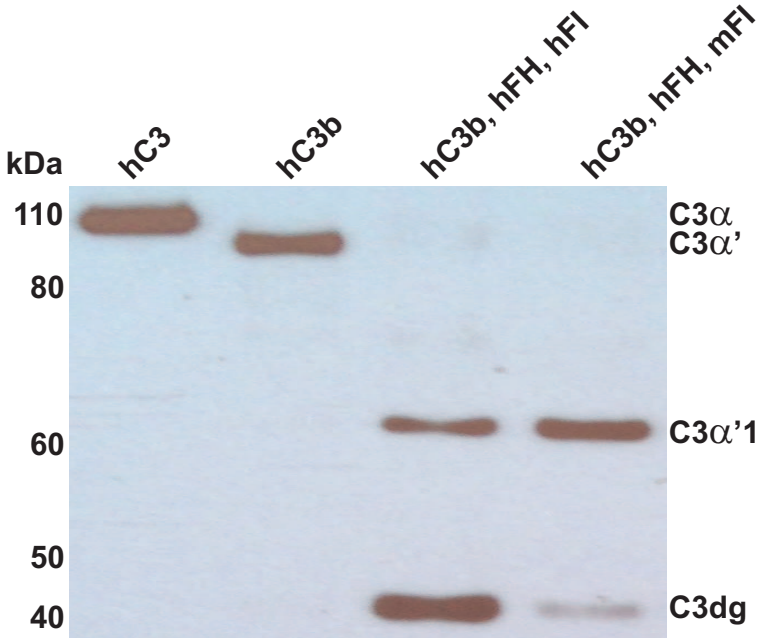

Fig. 3.13 Western blot of C3b cleavage by FI. hC3b was generated by limited trypsin digest (lane 1 and 2). hC3b was incubated for 30 minutes with hFH and h or mFI at 37°C. All samples were separated by SDS PAGE and detected with an α-hC3g antibody (clone 9). Experiment was performed three times.

iC3b deposition on mannan coated microtiter plates - Inhibition of the feedback cycle by Factor I Fig. 3.14. In this assay, the complement system is activated by mannan, which is bound by one of the lectin pathway's recognition molecules. The assay is done in alternative pathway buffer (Mg^{2+}-EGTA) which ensures absence of the classical pathway but does not rule out lectin pathway activation in a Ca^{2+}-independent way. Once complement is activated, the alternative pathway is recruited, FB binds to deposited C3b and is cleaved by FD. The resulting alternative pathway C3 convertase (C3bBb) continues to cleave more C3 into C3b, resulting in the positive feedback loop of the alternative pathway. FI interrupts

this feedback cycle by cleaving C3b into iC3b which can no longer form a C3 convertase. Therefore, elevated levels of FI will lead to less C3b (actually iC3b) deposition onto mannan coated microtiter plate. This assay measures the inhibiting effect of FI on the feedback loop of the alternative pathway. The more FI is in the system, the more C3b will be inactivated and can no longer act in an alternative pathway C3 convertase, cleaving more C3 to C3b. For this assay, a batch of recombinant FI was chosen that consisted of only pro-FI and no processed FI as assessed by western blot analysis. Serial dilutions of either pro-mFI or mFI were incubated with 25% normal mouse serum and added to the mannan coated plate. As expected, increasing the amount of FI inhibits the positive feedback loop and C3b/iC3b deposition in a dose-dependent manner, whereas the enzymatically inactive pro-FI shows no effect at all (Fig. 3.14). Around 50% less C3b deposition is accomplished on addition of 20 μg to 25% mouse serum, the remaining iC3b deposition results from C3 convertases formed by lectin pathway activation on mannan independent off the feedback cycle of the alternative pathway. Down-regulation by FI is titrateable and in this assay reaches its maximum at a concentration of 20 μg or higher.

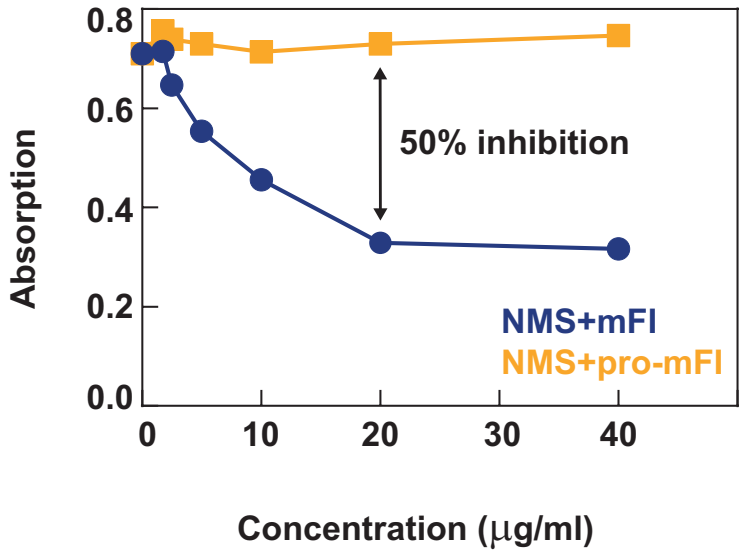

Fig. 3.14 iC3b deposition assay on mannan using recombinant Factor I. NMS was diluted to 25% in alternative pathway complement fixation buffer and serial dilutions of either pro-FI or FI (starting from for 40 μg/ml were added The mixture was incubated for 1 hour at 37°C on a mannan coated plate. Bound iC3b was detected with an α-human C3c antibody. Absorption was measured at 415 nm. Experiment was performed three times in duplicates.

Inhibition of rabbit erythrocyte lysis by Factor I Fig. 3.15. The ability of the complement system to lyse erythrocytes of another species can be used in functional hemolytic assays. Recombinant FI was also tested in its ability to inhibit the lysis of mannan coated rabbit erythrocytes (Fig. 3.15). The RBCs were coated with mannan because even though rabbit RBCs are easily lysed by human serum via the alternative pathway, mouse serum does so less efficiently. In order to "push" lysis via the alternative pathway, mannan was used to increase complement activation and opsonization of the cells. Elevated FI levels lead to increased inactivation of C3b and this effects that the terminal pathway of the complement system cannot take part any more and thus, rabbit RBCs are protected from lysis. It can be clearly seen that even slightly increased FI levels significantly reduce lysis. More exactly, while around 80% of the RBCs compared to the control were lysed, lysis was reduced by 50% in the presence of recombinant FI.

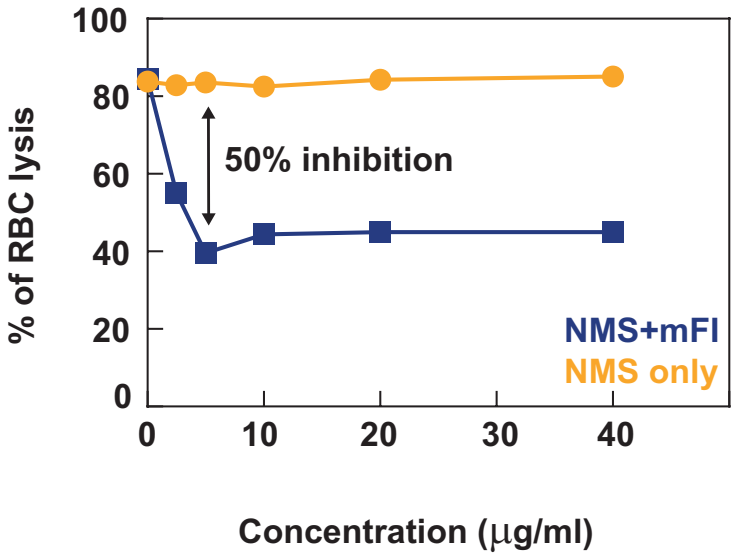

Fig. 3.15 Rabbit erythrocyte hemolysis assay using recombinant Factor I. Rabbit RBCs were coated with mannan and incubated with a mixture of 25% NMS and serial dilutions of pro-FI or FI starting from 40 μg/ml. The assay was incubated in a humidified box for 3 hours at 37°C. Hemolysis was measured by absorption at 450 nm and percentage of lysis is calculated. The experiment was performed twice in duplicates.

The effect of Factor I on iC3b degradation during complement activation Fig 3.16. Functional activity of recombinant FI was further tested in whole normal human serum

(NHS) for its ability to cleave C3b and iC3b. A capture ELISA was developed using clone 9 which does not bind to neither free C3 nor C3b but does react with a neo-epitope only available in iC3b and C3dg [141]. This antibody can therefore be used in time course assays, both to measure the formation of iC3b but also of its breakdown product C3dg. iC3b in solution is captured by clone 9 and after the serum is washed off, bound iC3b is detected by an antibody to C3 (clone 4). First, the protocol was developed and the right concentrations for coating with clone 9 and detection with clone 4 were determined. Both antibodies were purified from historical stocks of ascites supernatant using a ProSepA column. Because both antibodies were raised in rats, the detecting antibody had to be labelled so that it can be detected specifically and distinguished from the coating antibody. Purified clone 4 was thus labelled with biotin and detected with extravidin. Using this set-up, the initial stage of FI activity as well as the final stage can be measured and determined. In order to activate the complement system in the first instance, zymosan which is insoluble or endotoxin (results shown), which is soluble, were used as activating reagents.

In this experiment, human serum was used since the capture antibody (clone 9) recognizes only human iC3b but not its murine counterpart. The curve in the assays shows the amount of iC3b that has been captured by clone 9 at the respective time point. $50\,\mu$g/ml of either human FI (Comptech) or mouse FI were added to whole human serum and incubated at 37°C.

As early as 30 minutes after the start of the time course experiment, high levels of iC3b were generated. As demonstrated in the results above, mouse FI can cleave human C3b bound to human FH. With normal serum FI levels, complement activation continuous throughout the incubation period, leaving large amounts of inflammatory iC3b in the serum (Fig. 3.16, NHS only, blue line). If, however, FI concentrations are elevated by addition of purified or recombinant protein, generated iC3b gets further degraded into the non-inflammatory C3dg (Fig. 3.16) which is not recognized by clone 4.

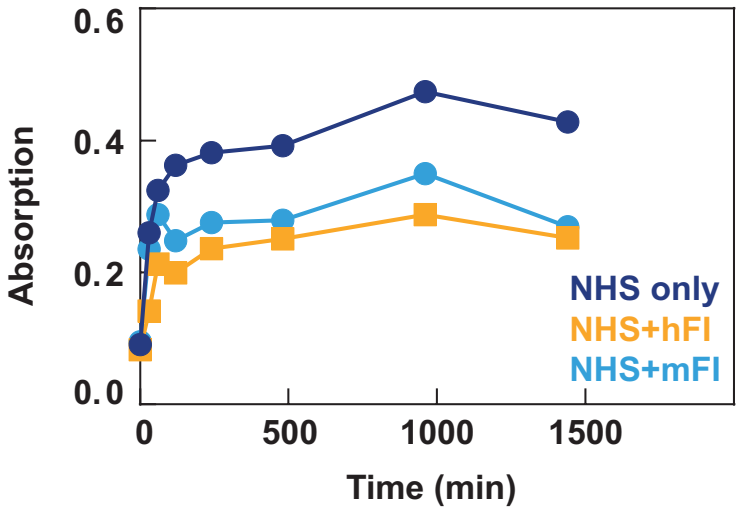

Fig. 3.16 Time course assay to measure iC3b formation and degradation. Whole NHS was incubated with LPS alone or with 50 μg human or mouse FI. Samples were taken at several time points, the reaction was stopped by addition of EDTA. Samples were serially dilutes and loaded onto a plate coated with α-human C3g. iC3b is captured and detected by an α-hC3 antibody. Absorption is measured at 450 nm. The experiment was performed three times in triplicates.

3.2.2 Application of recombinant murine Factor I in a mouse model of renal ischemia reperfusion injury

Once recombinant FI was shown to be functionally active, it was used in a mouse renal ischemia reperfusion injury (IRI) model. The therapeutic dose of FI was determined in a dose-escalation study and included three mice injected s.c. with 100, 200 or 300 μg FI. Subsequent analysis of their serum showed a dose dependent effect in a C3b cleavage assay (not shown). We decided to test two doses of recombinant FI in a pilot study to assess its therapeutic value in this model. All work requiring animal handling was performed by Dr. Conrad Farrar (King's college London, Prof. Steven Sacks lab) but I assisted during surgery and performed all other work. This pilot study included twelve mice (C57/Bl6, male, 9 weeks) that were divided into three groups:

- 300 μg mouse albumin (control group)
- 100 μg mouse FI (low dose)

- 300 μg mouse FI (high dose)

Mice were injected and operated as described in Sec. 2.2.8 and shown in Fig. 3.17a and b. The only difference from that protocol is that for mouse #4 of the 100 μg FI group, only 50 μg FI were injected.

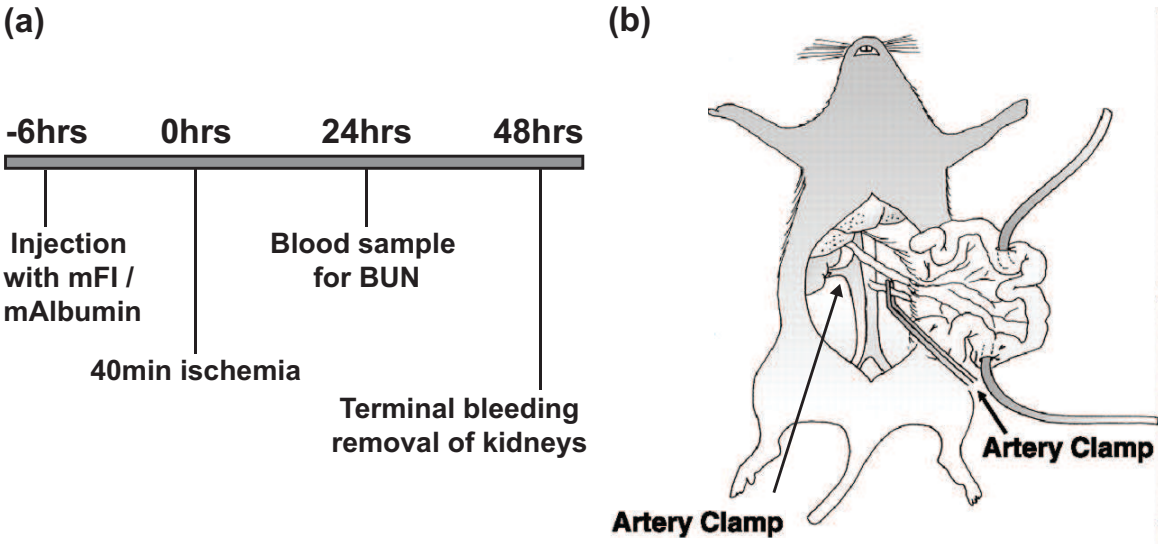

Fig. 3.17 Schematic diagram of timeplan and surgical details

Since this was only as pilot study, the number of mice was kept low. Unfortunately, time of ischemia (40 minutes) was a bit too harsh for the mice and around 60% of the mice had to be culled before the end of the experiment (Fig. 3.18c). It should therefore be noted that it was not possible to get blood and samples and kidneys from all mice at the 48 hours time point and additionally, blood was taken at 40 instead of 48 hours post reperfusion for some mice (Tab. 3.1).

3.2.2.1 Assessment of renal function and histology

To assess the therapeutic efficiency of recombinant mouse FI, renal function and kidney tubular damage were assessed and compared with control mice.

Analysis of blood urea nitrogen (BUN) Decline in real function was assessed by BUN measurements 24 and 48 hours after reperfusion (Fig. 3.18a and b) using the Infinity Urea

Harnstoff kit. BUN levels were found to be increased by almost 3 fold after 24 hours and 6 fold after 48 hours in untreated mice. FI treatment showed a protective effect: 24 hours post reperfusion, the BUN levels were raised from 9.4 mmol/L in an untreated mouse to 26.4 mmol/L (albumin group), 22.8 mmol/L (100 μg mFI) and 24.1 mmol/L (300 μg mFI). After 48 hours, this effect is becoming more profound: 54.7 mmol/L (albumin group), 39.0 mmol/L (100 μg mFI) and 40.3 mmol/L (300 μg mFI). This is a reduction of BUN of 9-13% after 24 hours and 27-29% after 48 hours, indicating less kidney damage after raising serum levels of FI. Again, it should be emphasised that this study is a pilot study including only a small number of mice. One mouse can therefore change the outcome especially if this mouse is an outlier.

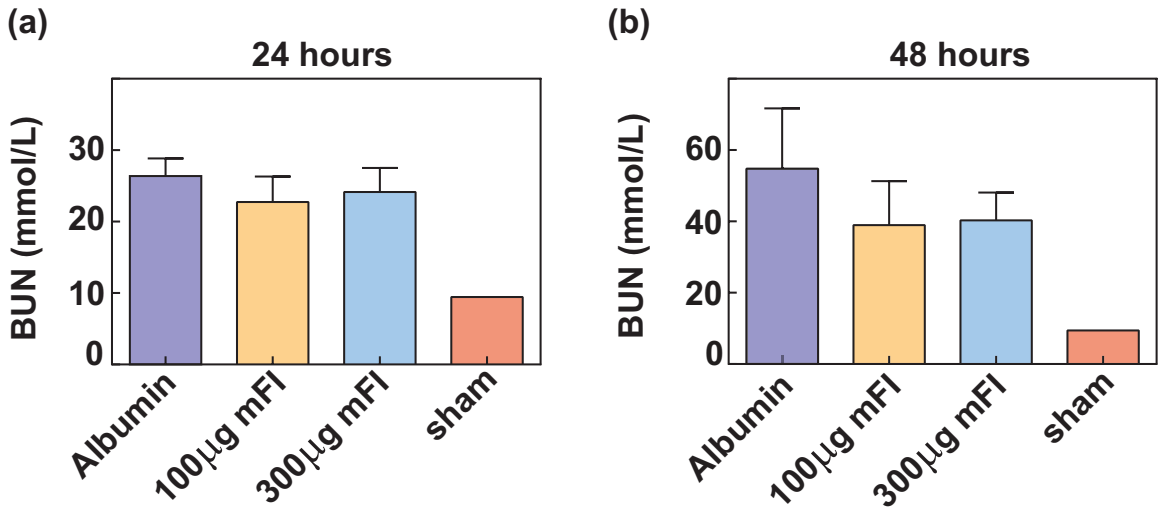

Fig. 3.18 Analysis of BUN levels. Mice were subjected to 40 minutes bilateral renal vessel occlusion and BUN was measured using the Infinity Urea Harnstoff kit after 24 (a) and 48 hours (b). Median of all samples per group is shown. Mice were either treated with a control protein (albumin) or with 2 different doses of FI. All BUN samples were measured twice.

Analysis of renal morphological changes The area most sensitive to IRI is the cortico-medullary junction where the proximal tubules lie. Renal sections were prepared, stained with PAS and tubular damage was scored as described in Sec. 2.2.8. PAS stains carbohydrates in the basement membranes of glomerulus and tubules and the brush boarder of the proximal tubules.

The severity of tubular damage in ischemic kidneys was proportional to functional impairment as determined by BUN (Fig. 3.18 and Tab. 3.1). Tubular injury leads to loss of brush boarder in tubular cells which can also be seen with PAS staining (Fig. 3.5 and Fig. 3.20b arrows) with subsequent necrosis resulting in accumulation of cellular debris in the tubules (Fig. 3.20 arrowheads). FI treated mice showed less tubular damage and necrosis, indicating a protective effect of FI (Fig. 3.20)

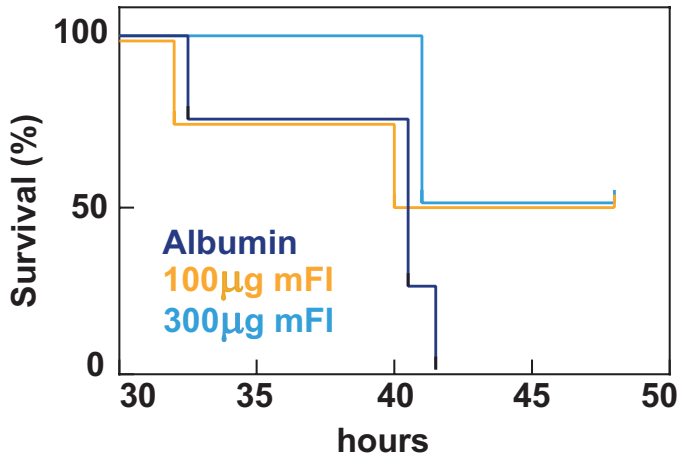

Fig. 3.19 Survival curve of mice. All mice from the albumin control group did not survive until the end of the experiment while 50% of mice from the treated groups survived.

Summary Below, a summary of all results is given (Tab. 3.1). Since many mice died or were culled before the end of the experiment, it is also appropriate to consider the ratio of premature deaths (Fig. 3.19). No mouse from the albumin control group survived more than 41 hours but 50% of mice from both FI treated groups survived. BUN levels correlated with determined percentages of tubular injury.

Altogether, there is a therapeutic, protective effect of FI but due to the small numbers of animals tested, it is not possible to apply a statistical analysis on the final results.

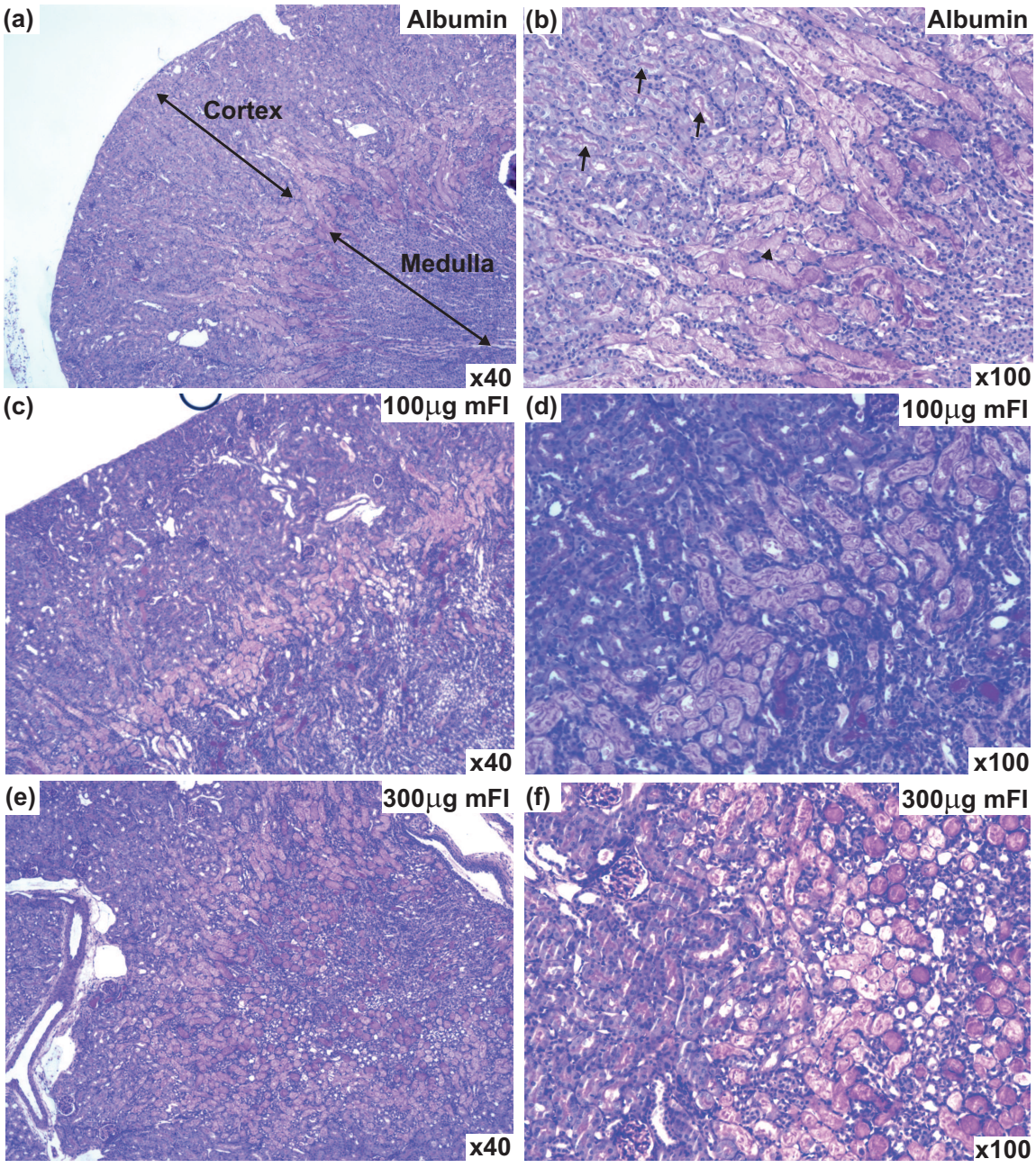

Fig. 3.20 Effect of ischemia reperfusion injury on renal tissue morphology in 3 groups. PAS staining: images are representative histology samples from each group. In both, albumin (a and b) and FI treated groups (c and d are 100 μg FI and e and g are 300 μg FI), there are visible signs of tubular damage in the cortico-medullary junction. Arrows show tubular injury and loss of brush boarders. Arrowheads show accumulating debris in tubules. Views are x40 and x100 as indicated in the bottom right corner.

300μg Albumin	**BUN (mmol/L) 24 hrs**	**BUN (mmol/L) 48hrs**	**tubular injury score**	**premature death**
#1	24.60	42.75	50%	after 40hrs
#2	29.22	66.73	50%	after 40hrs
#3	23.99	/	/	after 30hrs
#4	27.68	/	/	after 41hrs
100μg mFI	**BUN (mmol/L) 24 hrs**	**BUN (mmol/L) 48hrs**	**tubular injury score**	**premature death**
#1	18.45	25.22	10 – 25%	no
#2	27.06	49.20	25 – 50%	no
#3	22.14	42.44	50%	after 40hrs
#4 (50μg mFI)	23.37	/	/	after 32hrs
300μg mFI	**BUN (mmol/L) 24 hrs**	**BUN (mmol/L) 48hrs**	**tubular injury score**	**premature death**
#1	20.30	34.75	25 – 50%	no
#2	22.45	45.82	50%	no
#3	27.68	/	/	after 40hrs
#4	26.14	/	/	after 40 hrs

Table 3.1 Summary of renal ischemia reperfusion injury study

3.2.3 Discussion

Expression of a negative regulator of the complement system

The complement system is a delicate network of serum proteins and cell-bound receptors that relies on tight control in order to function without damaging the body's own tissue and cells. There are many regulators which have comparable roles in controlling complement activation. FI is a unique regulator which by cleaving the α-chain of C3b irreversibly inhibits complement once it is activated. In fact, it is FI which is the only protein that not only amplifies the C3 breakdown cycle of the alternative pathway but also inactivates the inflammatory iC3b.

In order to investigate the potential therapeutic effect of this regulator protein in an animal model, it is required to have access to a purified form either by plasma fractionation or by recombinant techniques. In contrast to the human protein, mouse FI is not commercially available and also has never been cloned before, although it was purified from mouse serum in a tedious way in 1980 [156] and its cDNA sequence was determined 15 years later [228].

Nevertheless, no further attempts to use this protein as a therapeutic have been undertaken. Human FI has been prepared recombinantly before. For mouse experiments, commercially available human FI could not be used because it was shown to be not active in whole mouse serum, although cleavage of mC3b by hFI was shown in *in vitro* experiments using purified components. Species specific limitations in the complement system are no surprise and is is also reported that mouse C4bp does not act as a co-factor for the cleavage of hC4b by hFI [156]. *In vitro* experiments using purified components can lead to artificial results and enable reactions that are not occurring under physiological conditions. Slightly changed buffer systems, prolonged incubations or increased amounts of protein can give false-positive results.

Therefore, a stable cell line was generated which secreted mouse complement FI. This recombinant protein was shown to be active in vitro and in vivo, cleaving both, C3b and iC3b. In the beginning, no problems regarding the functional activity of recombinant mFI occurred, but problems started to arise after some time. The early batches of transfected and recloned cells were initially active but became inactive as soon as a bigger batch was prepared for protein purification. Recombinant FI was still transcribed and translated but post-translational modifications were not performed accurately, in particular formation of the correct disulfide bridges. This faulty folding of protein can be detected by SDS gel analysis by comparing reduced and non reduced protein (and also by functional analysis). The exact reason for the sudden occurrence of missfolded recombinant protein was not investigated since cells transfected with the new construct, continuously secreted correctly folded FI.

Transformed cells were secreting mainly pro-FI, a fact that had already been discovered when human FI was initially expressed in COS cells [167] but also when expressed in HEK cells [172] or CHO cells (GlaxoSmithKline, Dr Ian Hiles, personal communication) but not when using a baculovirus expression system [171]. The pro-enzyme lacks the flexible structure of the processed heterodimeric protein and cannot exert its effector function. Altogether, because the pro-enzyme can easily be converted into the active enzyme by in vitro digestion with furin, more than 90% secretion of enzymatic-inactive pro-FI does not pose a severe problem.

Analysis of functional activity of recombinant mouse Factor I

FI's functional activity was tested in several *in vitro* assays to rule out the possibility of false-positive results but also to assess the protein's behaviour in different environments, i.e. whole serum, mouse vs human serum, purified proteins. It was shown that, as expected, the pro-enzyme does not show any functional activity (Fig. 3.14); a fact that is further supported by superimposition of the crystal structure of FI onto the C3b-FH complex which shows that reorientation of FI is required for cleavage, i.e. the heavy chain binds to the substrate and releases its allosteric inhibition [175].

Recombinant FI was tested for its ability to cleave its purified substrates, C3b and iC3b and also to reduce hemolysis of rabbit erythrocytes and to inhibit the amplification loop of the alternative pathway. In both, the C3b cleavage assay (Fig. 3.13) and time course assay (Fig. 3.16), it becomes apparent that mFI can cleave human C3b and iC3b in the presence of the necessary co-factors but is doing so less efficiently than hFI. Nevertheless, prolonged incubation resulted in complete cleavage of the C3α'1 chain, demonstrating that although there is some species-specific limitation that reduces mFI activity in human serum, C3b and iC3b cleavage still occurs. These species specific limitations were also shown previously by Kai et al. who performed a series of cleavage experiments using human and mouse FI and C4 in the cleavage of human C4b [156] but also by my own results showing that human FI is not active in whole mouse serum *in vitro* and *in vivo*. Shared activity between complement enzymes of different species is well known as opposed to molecules that are involved in "lock and key" interactions.

The iC3b deposition assay further shows that there is a maximum of reduction in complement deposition that can be reached by addition of FI (Fig. 3.14). Concentrations higher than 20 μg/mL do not lower iC3b deposition any further. This is either a limitation of the assay (i.e. background signal) or demonstrates initial lectin pathway activation independent of the alternative pathway. It has been shown before [34] that some recognition molecules of the lectin pathway can function in the absence of calcium. For this reason, LPS was used as complement activating reagent in chapter 4 (see Sec. 4.2.1.3).

The hemolysis assay convincingly shows how even a small increase in the physiological FI concentration results in a dramatic reduction of lysis of RBCs (see Fig. 3.15). In contrast to the iC3b deposition assay which measures only reduction of the alternative pathway feedback cycle, the hemolysis assay measures its downstream effects and these are more apparent even at minimal FI increase. As opposed to human serum which is quite readily activated by rabbit erythrocytes [102, 229], mouse serum does so less. For this reason, the rabbit erythrocytes were coated with mannan to boost complement activation.

In the iC3b capture time course assay, it should be noted that in the absence of exogenous FI but in the presence of an activating reagent, i.e. zymosan or LPS, iC3b deposition continuously increases over time. This cannot reflect the physiological situation because if this were the case, the complement system would be completely depleted every time, it encounters an activating substance. *In vivo*, the activating reagents will be removed by phagocytosis and in the assay, rather non-physiological conditions are generated due to the absence of erythrocytes and phagocytes. Minor amounts of soluble CR1 (sCR1) nevertheless, can act as co-factor of the second FI cleavage.

Summarised, purified recombinant FI was found to be functionally active and to behave as expected.

Therapeutic application of recombinant mouse Factor I in a renal ischemia mouse model

Next, the recombinant protein was tested in a mouse model of renal ischemia. Ischemia reperfusion injury can affect almost every organ and renal ischemia is a significant cause of morbidity and mortality in renal transplantation [201]. Renal IRI leads to acute tubular necrosis which can further result in renal failure. It has been found by many studies using knockout mice (MBL, FB, C3, C5, C6, C5a receptor, MASP2 and Cl-11 [36, 193, 217, 224]) that complement components have roles in IRI-mediated renal damage. We chose this model because the alternative pathway is known to be one of the major players in the complement mediated damage [217, 222, 223]. This is in contrast to murine models of IRI in heart, skeletal muscle, intestine and limb, which are all dependent on natural IgM and classical

pathway or lectin pathway activation. The experiment was designed as a pilot study to assess if recombinant FI, in this model, has a therapeutic potential. Our model consisted of bilateral occlusion of renal vessels for 40 minutes, followed by reperfusion for 48 hours. The climax of BUN rise is reached between 24 and 48 hours and FI improves kidney function in that it reduces mortality, tubular necrosis and BUN increase. Unfortunately, more than half of the animals died or had to be culled before the end of the experiment. Thus, it should be pointed out that all mice from the control group died while 50% of the FI treated mice survived. A high and a low dose of FI were used to see whether there is any dose-dependent effect but because of the small number of mice combined with varying time points of death it is not possible to deduce any reliable effects, even though one could argue that the 100 μg mFI-group is maybe slightly more protected. Although, it is theoretically also possible that this slight effect is caused by the smaller injection volume (100 vs 300 μl). Mice were injected 6 hours prior to surgery so it is possible that an increased plasma volume causes them to urinate more and thereby lowering the effective recombinant FI concentration.

Even though FI has a protective effect, it cannot prevent necrosis. Ischemic time has to be chosen carefully so that injury is still reversible by the therapeutic used. It is well known that susceptibility to renal IRI is dependent on the genetic background in mice and C57/Bl6 mice (used in this study) are particularly sensitive [230]. Moreover, Iwata et al. have shown that the time of ischemia greatly affects the mechanism of injury in IRI [231]. It is a general problem of animal studies that they do not necessarily reflect the situation in a clinical setting. In IRI the problem is that for most experiments, ischemic time is between 30 and 60 minutes which contrasts to a generally much longer ischemic period in clinical settings [231]. While short ischemic periods (under 60 minutes) mainly cause neutrophil mediated damage, longer periods (90 - 120 minutes) show apoptosis to play a crucial role in the development of muscle ischemia-reperfusion injury [221]. Since complement can activate and effects both short- and long ischemic periods, it can be used in either situation to improve the outcome. Additionally, in renal IRI, it was shown that blockage of P-selectin, which is in particular important in neutrophil recruitment, was equally effective in the absence of C3 after a 55 minutes ischemic period [195], implying that both, complement and P-selectin

mediated pathways are independent. It was found that both are separated spatially and by time and suggested that complement and P-selectin pose distinct targets in therapy. Also, C5a was shown to function independent of neutrophils [221].

Results between different research groups should be compared with care because of different ischemic and reperfusion times, mouse strains and surgical protocols used. Furthermore, the surgical procedure involved is a very delicate one that requires much practice and a skilful surgeon. Also, extrapolation to a clinical setting in human can and has proven itself difficult.

It is obvious that this pilot study needs repetition with a greater number of mice, an adequate ischemic time and an adjusted injection volume so that all mice receive exactly the same volume. Deposition of complement should also be quantified by immunohistochemistry. Nevertheless, raising serum concentrations of complement FI is shown to be a promising candidate whose therapeutic potential should be exploited further.

Chapter 4

Elevation of plasma complement Factor I levels using gene therapy

4.1 Introduction

In this chapter, *in vivo* over-expression of mouse FI will be described. This is achieved by a gene therapy using a virus that specifically transfects murine hepatocytes. First, a short introduction into gene therapy will be given before the viral expression system used, based on adeno-associated virus, will be explained.

As a proof of concept study, mice were injected with a viral construct harbouring the FI sequence and transgenic FI expression was confirmed and determined.

4.1.1 Gene therapy

Gene therapy is the delivery of genetic material into a patient with therapeutic intent. It includes gene replacement as well as addition and is divided into *in vivo* and *ex vivo* gene therapies.

In *in vivo* gene therapy, the vector is injected into the patient and enters its target cells where the transgene is subsequently expressed. *Ex vivo* gene therapy requires isolation of the target cell type which is then manipulated outside the body. *Ex vivo* gene therapy was

developed first and has the advantage that the patient is not exposed to the viral vector which can potentially elicit an immune response and also, the target cells can be selected before transduction. However, it is not always possible to extract the target cell or to proliferate it *in vitro* [232, 233].

In human, gene therapy includes only manipulation of somatic cells while germ-line cell alterations are strictly prohibited for ethical and safety reasons [234].

4.1.1.1 Gene therapy in the past and present

The idea that viruses can be used to transduce genes goes back more than 70 years. In 1966, Edward Tatum predicated:

> "Finally, it can be anticipated that viruses will be effectively used for man's benefit, in theoretical studies, in somatic-cell genetics and possibly in gene therapy. . . . We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis and introduction of new genes into defective cells of particular organs." [235]

The first authorized gene therapy was initiated in 1990. Two children suffering from adenosine deaminase deficiency - severe combined immunodeficiency (ADA- SCID) were treated with a retroviral vector replacing the missing gene [236]. Although the efficacy of this trial is still in question, the trial succeeded in proving safety of this new field [232, 237]. In 1999, the first death directly associated with gene therapy was reported: 18-year old Jesse Gelsinger developed an adverse reaction to the adenovirus used and died within 4 days after treatment [238]. Following investigations that reported a number of clinical trials in which trial design or ethical standards were not satisfactory, the whole field of gene therapy experienced a negative public backlash [239].

Nevertheless, only a year later the first successful gene therapy in 3 children with severe combined immunodeficiency (SCID-X1, also: common γ-chain deficiency) was reported by Cavazzana-Calvo and Fischer at the Necker Hospital for Sick Children in Paris [240]. Since this initial report, however, several other patients were treated with the very same gene

therapy [241] and in total 4 out of 20 developed a leukaemia-like disease due to insertion of the used retrovirus into the promoter of a proto-oncogene [237, 242–244]; one patient even died following an unsuccessful allogeneic bone marrow transplantation. These results again dampened the initial enthusiasm for gene therapy.

Since these trials, much progress in vector technology has been achieved [245, 246]. Early recombinant viral vectors were inefficient, failed to persist in host cells and transgene expression was typically short-lived. The understanding of the molecular and cellular mechanisms has resulted in the development of highly sophisticated gene transfer tools with improved safety and therapeutic efficacy. This resulted in a series of Phase I/II trials in the past few years with excellent clinical results and no side effects reported so far [247].

Initially, gene therapy focused on orphan disease with monogenetic defects that require a straightforward but comparably easy approach, e.g. severe combined immunodeficiency (SCID) [247]. Early successes allowed the field to expand to acquired diseases [248] such as cancer [249, 250], cardiovascular diseases [251], neurodegenerative disorders [252] and infectious diseases [253].

Notable successes have been achieved in the treatment of hemophilia B [254] and lipoprotein lipase deficiency [255]. In a clinical trial, the group led by Nathwani could show not only long-term therapeutic factor IX expression but also prove safety of the therapy (AAV8 vector) [256, 257]. The treatment of patients with Leber's congenital amaurosis (due to a mutation in RPE56) also showed safety and efficacy of the treatment (AAV2 vector) [258–260].

Not a single gene therapy product has been approved by the Food and Drug Administration (FDA) so far, despite numerous clinical trials ([261], stand June 2015). In Europe, the first gene therapy, the AAV-based therapy Glybera was approved by the European Medicines Agency in 2012. Glybera is a treatment for a rare monogenetic disease, lipoprotein lipase deficiency (LPLD), and consists of the missing gene packaged into a recombinant AAV1 vector that is injected intramuscularly [262]. Glybera received marketing approval in the EU, albeit under "exceptional circumstances", i.e. only patients with detectable levels of LPL

protein [263]. [1] A complete list of ongoing and completed gene therapy trials can be found on the "Gene Therapy Net" website and links therein [264].

4.1.1.2 Vectors used in gene therapy

There are two ways to deliver genetic material into cells: viral and non-viral vectors. Viruses have evolved for thousands of years to specialise their molecular mechanism for optimal use of the cells' translational machineries. Non-viral vectors include all other vectors that do not use viral elements to enter a cell but use other methods (described in Sec. 4.1.1.2).

Viral vectors The viral genome is comprised of genes and *cis*-acting regulatory sequences which are spatially separated in most viruses. This arrangement is used to design viral vectors: the viral genes which are responsible for replication or caspid/envelope proteins are exchanged with therapeutic transgenes that are then flanked on both ends by the regulatory *cis*-acting sequences [248]. The deleted genes work in *trans* and can be provided either by the packaging cell line that has the viral genes incorporated into the genome or by heterologous plasmids that are co-transfected with the viral vector [265]. In this way, replication deficient virus particles harbouring the transgene can be produced that are able to transduce their target cell.

Viruses infect their natural host cell most efficiently. Pseudotyping involves exchanging the surface proteins that mediate cell entry with the ones from another virus in order to change the viral tropism. Examples for pseudotyping are the lentivirus pseudotyped with protein G of vesicular stomatitus virus (VSV G-pseudotyped lentivirus) that can transfect almost every cell and was first reported independently by three groups in 1996 [266–268]. In other cases, the tropism of a virus is limited to only the target cell which allows reduction of the vector dose administrated and prevents transgene expression outside the respective cell type, i.e expression only in the eye [269] or the liver [270–272]. Pseudotypes are also artificially generated i.e. libraries composed of DNA-shuffled AAV capsids with improved

[1] This is to ensure that no immune response against the transgene is elicited which could otherwise occur if a patient is completely deficient of the LPL protein.

tropism for human hepatocytes [273]. This way, it is hoped to overcome one of the main limitations of gene therapy in man (low vector uptake and transgene expression).

Vector types At present, there are 5 main classes of clinically applicable viral vector that are derived from γ-retroviruses, lentiviruses, herpes simplex-1 viruses (HSV-1), adenoviruses and adeno-associated viruses (AAVs) [248]. Viral vectors can be divided into integrating and non-integrating vectors. Integrating vectors such as retroviruses insert the transgene permanently into the host cellular chromosome. Non-integrating vectors mediate transgene expression from episomes. Compared to integrating vectors, which will be inherited to every daughter cell, non-integrating vectors will be quickly diluted out in rapidly dividing cells but also do not pose the risk of insertional mutagenesis. Therefore, retroviruses are usually used for transfection of e.g. haematopoietic stem cells which undergo rapid cell divisions and differentiation and non-integrating viruses are used for post-mitotic tissue such as the liver, muscle or eye. Choosing the vector involves careful and considerate balancing of risks and benefits.

Non-viral vectors Non-viral vectors do not have the disadvantages associated with viral vectors such as endogenous virus recombination, oncogenic effects and unexpected immune responses [274, 275] but are easy to use and to scale up. Unfortunately, they are not yet as efficient as viral vectors in transfecting cells and also, transgene expression is generally lower. DNA delivery can be achieved by physical and chemical methods. Physical methods include electroporation [276], ultrasound [277] and hydrodynamic injection. Chemical DNA delivery methods are mediated by lipid- [278], peptide- [279, 280] or polymer-gene carriers [281]. Recent advances in nanotechnology [282], material sciences [283] and nucleic acid chemistry [284] have yielded very promising new non-viral gene delivery systems [285]. Currently, 17.5% of all clinical trials use a naked plasmid DNA delivery approach while 5.2% use lipofection ([264], stand June 2015).

4.1.2 Expression of mouse complement Factor I in an adeno-associated virus expression system

Adeno-associated virus is a small, non-enveloped virus that consists of a linear single-stranded DNA genome with a packaging capacity of ≈ 4.7 kb [265, 286, 287]. [2] Its replicative cycle is dependent on co-infection of a helper virus that is able to complement missing genes for AAV replication [288], e.g adenovirus [3] [289], herpesvirus [290] or baculovirus [291]. The fact that AAV is a "naturally defective" virus adds a safety barrier to prevent inappropriate spread of viral vector in clinical applications [292]. Recombinant AAV vectors are generated by insertion of transgene between the two inverted terminal repeats (ITR) and co-transfection with plasmids encoding for all other essential genes. They can infect both dividing and quiescent cells and persist mainly in a episomal form as opposed to the wild-type AAV that preferably integrates at a specific site in human chromosome 19 [293]. If present in a dividing cell, the episomal AAV genome gets rapidly diluted out during cell division.

Although the *in vivo* integration rate is low, AAV vectors can integrate into the human genome but the primary source of rAAV-mediated gene expression is extrachromosomal, not integrated genomes [248]. In a model of murine hepatectomy, Nakai and collegues have shown that less than 10% of persistent vector genomes are integrated in the liver [294]. Even though the integration frequency is much lower than that of retroviruses, AAV also preferably integrates into active genes rather than non-coding regions [295] but integrated rAAV genomes are frequently associated with chromosomal rearrangements and deletions of large segments of chromosomal DNA [296]. Nevertheless, it should be pointed out that AAV, as opposed to retroviruses, is usually used to target non-dividing cells. Therefore, the likelihood of developing cancer is greatly reduced.

Up to date, over 100 different AAV serotypes have been identified [287, 297] but most vectors are based on AAV2 (i.e. transgene flanked by AAV2 ITR) [298]. Pseudotyping can be easily achieved by packaging the capsid sequence of another serotype into the helper plasmid. One of the major problems is that rAAV trials have revealed that there is little

[2] This small packaging capacity one of the major drawbacks of this vector

[3] believed to be the natural helper virus

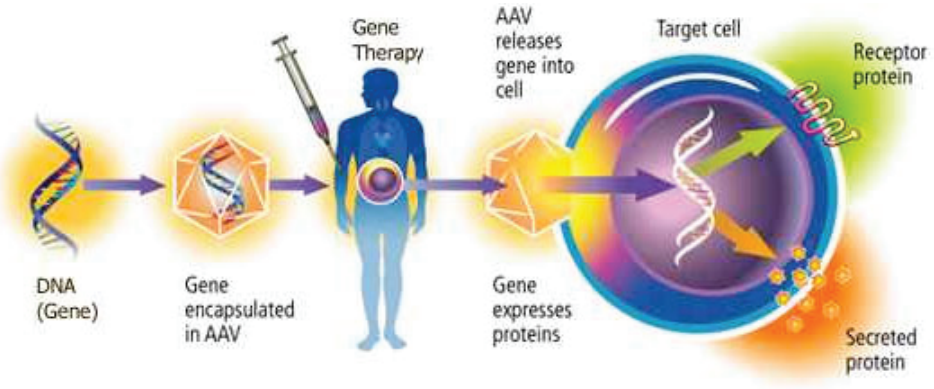

Fig. 4.1 AAV gene therapy

correlation between *in vitro* and *in vivo* transduction efficiency and in animal models versus human patients [273]. Therefore, several approaches to improve tropism and transgene expression have been taken: 1) construction of capsid libraries that consist of randomized capsid sequences [273], 2) insertion of amino acid sequences into the capsid of AAV2 (up to 30 kDa, possibility to insert a ScFv sequence to target AAV to a specific cell type) [299] and 3) rational design approach by combining knowledge of delivery mechanisms with AAV structural analyses [300].

AAV has an impressive safety record and has not been associated with any known human or animal diseases [265] although most humans ($> 70\%$) are seropositive for one or more serotypes [301, 302]. Apart from the generation of neutralizing antibodies that may affect re-administration, AAV vectors have not been associated with toxicity or an inflammatory response. By manipulating the capsid sequence, researchers aim to reduce these immune responses [303].

An increasing number of phase I-III clinical trials using AAV vectors reported positive results [300], promoting AAV to one of the most promising vectors for gene therapy .

4.1.3 The AAV-expression system used in this project

In this study, an AAV construct was used that was kindly provided by Professor Ian Alexander. It consists of an AAV2 viral backbone that was pseudotyped with the AAV8 capsid protein in order to confer liver tropism. Therefore, the virus mainly infects the liver and FI transgene is

over-expressed at its natural site. To further suppress extra-hepatic expression of FI transgene, an α-1-anti-trypsin promoter with two additional ApoE hepatic control regions was used (see Figure 4.2). All required genes are split up between three plasmids that are co-transfected into a cell line that provides the remaining missing genes for AAV packaging and will be further described below.

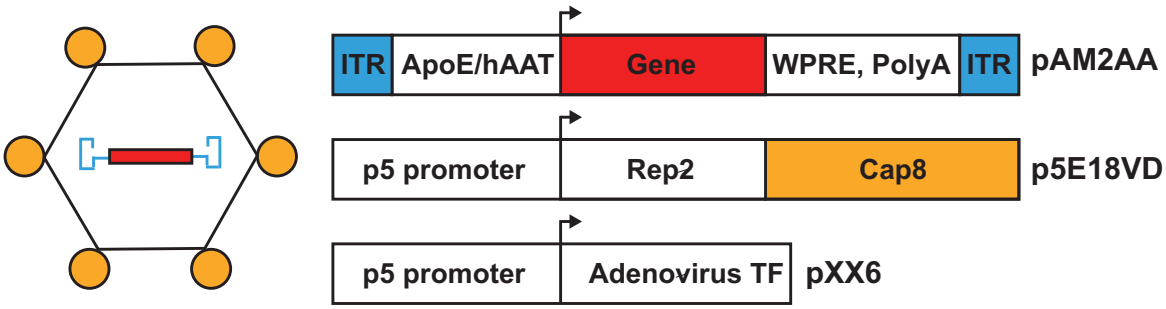

Fig. 4.2 AAV2/8 construct used for over expression of serum levels of FI. The transgene, here FI, is inserted between a promoter and a polyadenylation recognition site. Flanking on both sides are two inverted terminal repeats (ITR). The capsid sequence and further adenoviral genes are packed into other plasmids.

AAV (wild type): The 4.7 kb genome of wildtype AAV is characterized by two inverted terminal repeats (ITRs) and two sets of open reading frames (ORFs), which encode the replication (Rep) and capsid (Cap) proteins. The Rep ORFs encode four proteins (78, 68, 52, and 40 kDa) which function mainly in regulating AAV replication and integration. The Cap ORFs encode three structural proteins (85 kDa (VP1), 72 kDa (VP2) and 61 kDa (VP3)). VP1:VP2:VP3 ratios are approximately 1:1:8 or 1:1:10 in the capsid.

Recombinant AAV (rAAV): rAAV used in this project is AAV2 pseudotyped with AAV8 capsid (rAAV2/8) to confer liver tropism. The rAAV2/8 virions are packaged in HEK293 cells by triple transfection using the three plasmids described below:

1. pAM2AA (ITRs and transgene expression cassette): In this plasmid the 145 bp inverted terminal repeats (ITRs) from AAV2 flank the transgene cassette. The two ITRs are the only *cis* elements essential for all steps in the AAV life cycle. They function

as the origin of DNA replication, provide packaging and integration signals, and serve as regulatory elements for WT AAV gene expression. The pAM2AA cassette includes the human α-1-anti-trypsin promoter with two ApoE enhancers followed by the cDNA encoding the transgene. The 3'untranslated region (UTR) contains the woodchuck hepatitis post-transcriptional regulatory element (WPRE) and bovine growth hormone polyadenylation signal (BGH polyA). The packaging capacity of WT AAV is approximately 4.7 kb. In pAM2AA, the regulatory regions take up $\approx$ 2390 bp, leaving $\approx$ 2310 bp for insertion transgenes.

2. p5E18VD/8 (AAV helper sequences): The deleted viral coding sequences from AAV2, including Rep and Cap genes are present in this plasmid driven by the p5 AAV promoter. Here, the sequences encoding AAV2 capsid are replaced by sequences encoding AAV8 capsid. There is no homology between vector and helper sequences, reducing the possibility of generating wild type AAV recombinants.

3. pXX6 (adenoviral helper functions): The adenoviral genes essential for a productive AAV life cycle are the E1a, E1b, E2a, E4 and VA RNA genes. E1a serves as a transactivator which upregulates transcription of adenoviral genes as well as the AAV Rep and Cap genes. E1b interacts with E4 to facilitate transportation of viral mRNAs. E4 is also involved in facilitating AAV DNA replication. E2a and VA RNA act to enhance the viral mRNA stability and efficiency of translation especially for the Cap transcripts. pXX6 contains the essential adenoviral helper genes but lacks adenovirus structural and replication genes. Essential helper genes included in pXX6 are E2a, E4 and VA genes. Both E1a and E1b have been deleted, and the missing E1a and E1b genes are complemented by HEK293 cells.

4.2 Results and discussion

4.2.1 In vivo overexpression of complement Factor I by an adeno-associated virus delivery system

4.2.1.1 Preparation of construct and injection of mice

In order to enable cloning of FI into the pAM2AA expression vector, the restrictions sites had to be changed into compatible enzyme recognition sites (Primer sequences in A.1). The new sequences were attached to the mouse FI cDNA via PCR amplification. After purification of the amplified construct, it was ligated into pAM2AA. Virus preparation was kindly performed by Dr. Szun Tay in Professor Ian Alexander's lab at the Children's Medical Research Institute in Westmead (Australia). Once the vector had been prepared, an AAV_GFP vector was treated the same way alongside all subsequent virus packaging and purification steps. This ensures that no errors occurred since expression of hepatic GFP can easily be tracked visually under a fluorescence microscope.

12 mice were divided into 4 groups and were injected with different doses of either AAV_FI (low, medium and high dose were $5x10^9$, $5x10^{10}$ and $5x10^{11}$ virus particles, respectively) or AAV_GFP ($5x10^{11}$ virus particles). Four weeks after injection, mice were tail bled and after eight weeks, they were culled and serum was sent to the UK for analysis.

4.2.1.2 Quantification of elevated Factor I levels

In order to quantify FI levels, a polyclonal custom-made α-mFI antiserum was ordered. In the meantime, a western blot was performed. Here, α-FI (Santa Cruz) was used as detecting antibody to roughly quantify FI levels. Serum was diluted in sequential steps to reduce pipetting errors. The relative concentration of FI was determined as follows: AAV_GFP < AAV_FI low < AAV_FI medium < AAV_FI high (Fig. 4.3).

Once the antiserum arrived, a much more sensitive assay, an inhibition ELISA, was performed. Serum was pre-incubated with a known concentration of α-mFI-antiserum and then incubated on a microtiter plate coated with recombinant mFI. Preparation and binding

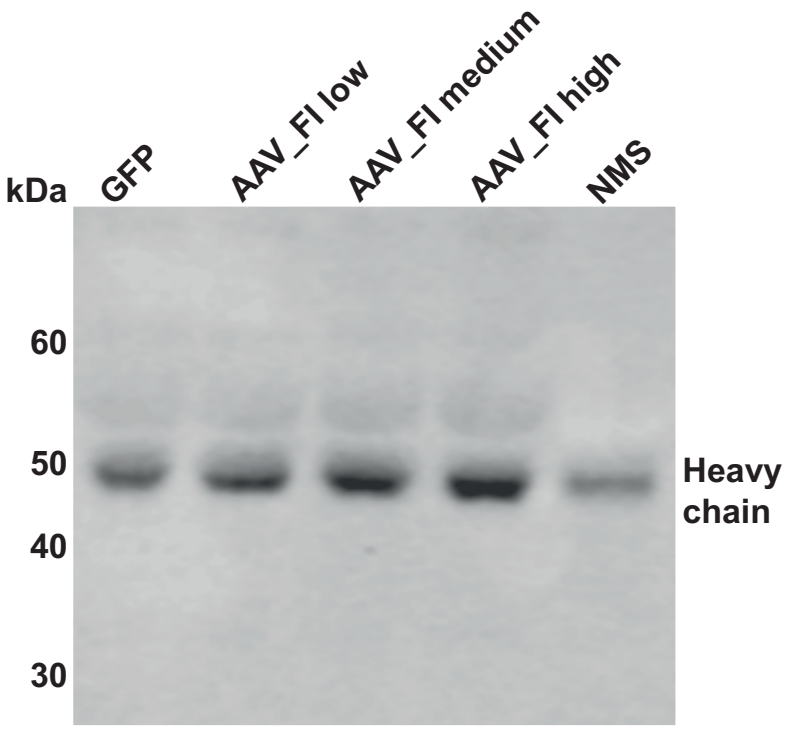

Fig. 4.3 Elevated FI levels measured by western blot. Mouse serum was diluted to 5% and 10 μl were separated by SDS PAGE. FI was detected with an α-FI antibody (1:500) that reacts with the heavy chain of mFI.The experiment was performed twice.

specificity of the α-mFI-antiserum are described in Sec. 2.1.1. Only unbound antibodies are able to react with the immobilised antigene on the plate. Therefore, the assay only gives a positive result if the amount of α-mFI-antibodies in the antiserum exceeds the mFI present in serum. The assay is calibrated with known FI concentrations.

It was found that antiserum concentrations above 75 μg/ml give a strong positive signal when the microtiter plate is coated with 1 μg mFI per well (not shown). As seen from Fig. 4.4a, the assay gives a positive signal at concentrations lower than 0.25 μg/ml. Using this calibration, the concentration of FI in a serum sample can be determined. First, a normal sample of wild-type mouse was tested and concentrations lower 0.625% gave a positive signal. Knowing that the threshold of a positive signal in this assay is 0.25 μg/ml mFI, the concentration of mFI in 100% serum can be calculated, i.e. 40 μg/ml. The same calculation was done for each of the mouse sera injected with the AAV-constructs and the concentration range per group was determined (see Tab. 4.1). Starting from 20-40μg/ml FI in the GFP

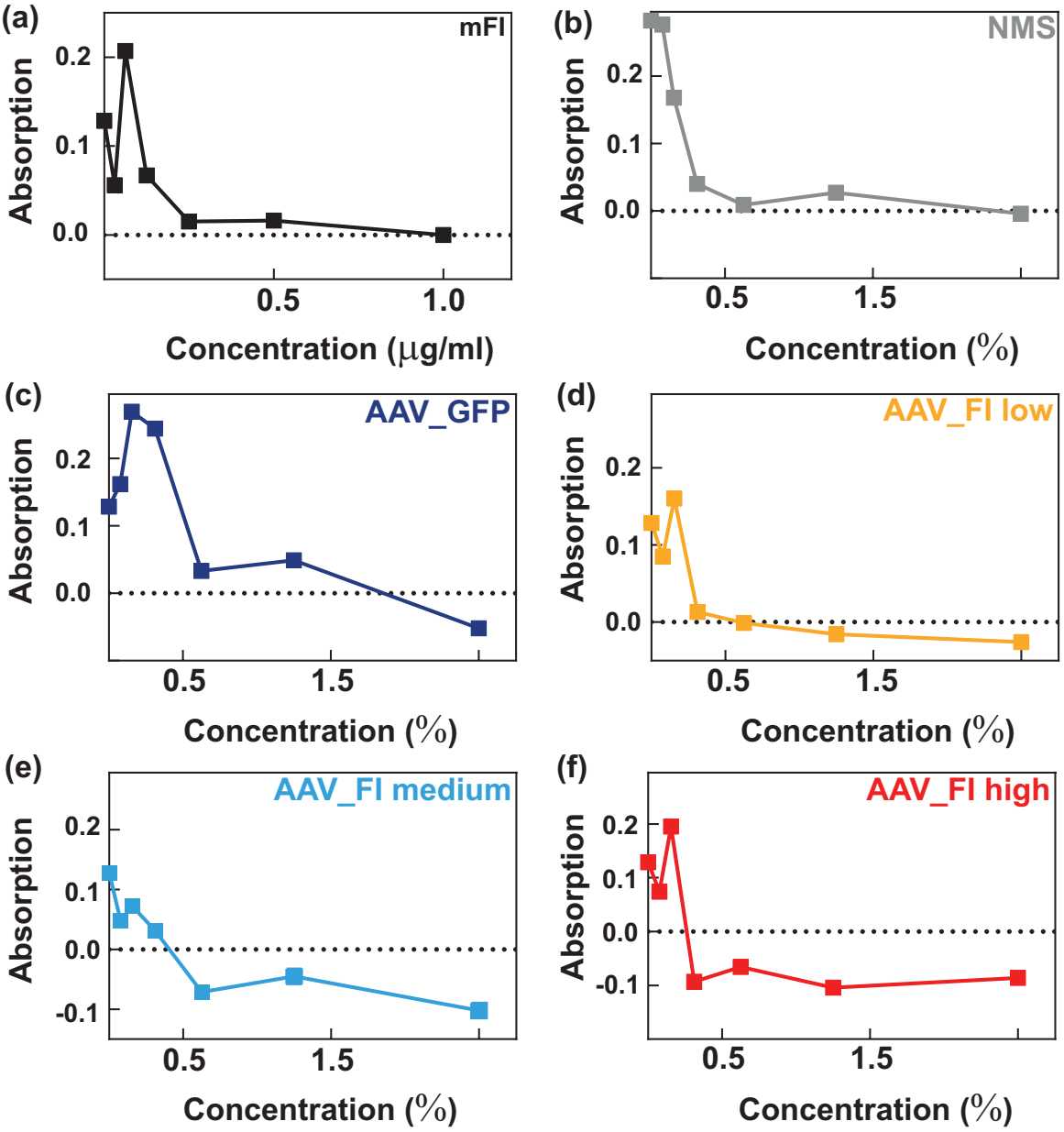

Fig. 4.4 Representative results from an inhibition ELISA. (a) The assay is calibrated with known concentrations of purified recombinant mFI. Concentrations lower than 0.25 μg/ml give a positive signal. (b) Normal mouse serum (NMS) is positive at concentrations lower than 0.625%. (c-d) Inhibition ELISA using sera from mice injected with AAV-constructs. Only one serum sample per group is shown here. Experiment was performed twice in triplicates.

control group, the concentration is 40-80μg/ml in the low-, 80μg/ml in the medium- and 80-160μg/ml FI in the high FI dose. Thus, the serum concentration can be raised up to 4x of normal levels by an AAV gene therapy.

Group	**FI μg/mL**	**FI increase**
AAV_GFP	20-40	1x
AAV_FI low	40-80	1-2x
AAV_FI medium	80	2x
AAV_FI high	80-160	2-4x

Table 4.1 Quantification of Factor I after over-expression by an adeno-associated virus expression system

4.2.1.3 Functional analysis of serum with elevated Factor I levels

The serum of the transgenic mice was analysed in a similar manner as the recombinant FI. Since the over-expressed protein will, theoretically, be processed and secreted as endogenous FI, the sera of the mice could be directly compared with each other. After FI increase has been confirmed by immunoblotting and by an inhibition ELISA, the functional activity of the over-expressed enzyme had to be confirmed. Therefore, the sera were analysed for FI functional activity by an *in vitro* C3b cleavage assay, a hemolysis assay and an iC3b deposition assay.

C3b and iC3b cleavage was measured in an *in vitro* assay as done before. Human C3 was digested to C3b with trypsin and incubated with hFH and serum from transgenic mice. After 30 minutes, 1 μg human C3 was loaded onto a SDS gel and blotted with clone 9. C3b is first cleaved into iC3b in a quick reaction (the antibody reacts with the C3α'1 chain of iC3b) and then further into C3dg in a much slower reaction. This second reaction can be speed up by increased FI concentrations. It is shown that human C3b incubated with human FH and serum from the different mouse groups was more efficiently cleaved to C3dg within the incubation period in the AAV_FI medium and AAV_FI high group (Fig. 4.5, lane 10-15). Mice that have been injected only with a control plasmid show no almost C3dg band within the incubation period.

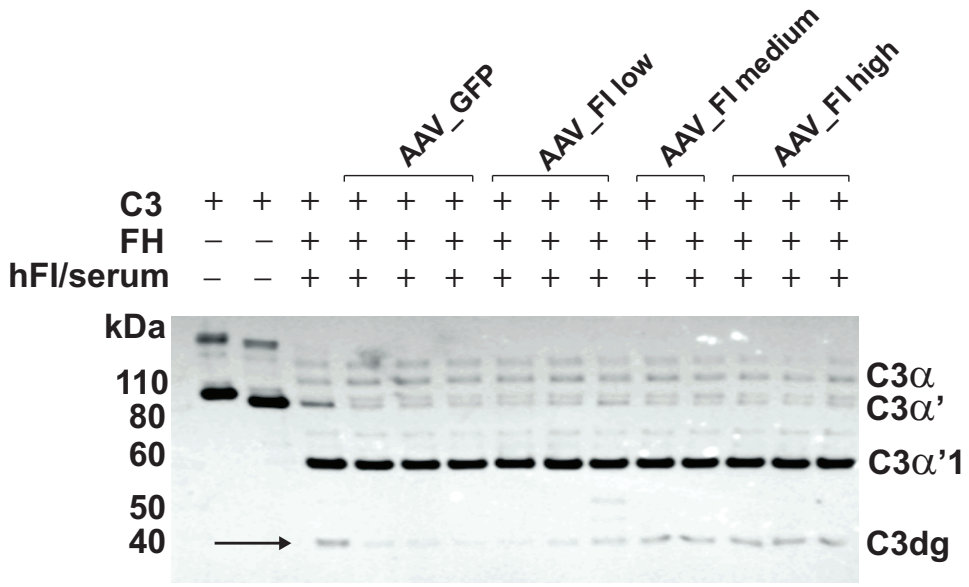

Fig. 4.5 C3b and iC3b *in vitro* cleavage assay to measure functional activity of over-expressed FI. Substrate. i.e. C3α'1 chain is generated quickly and the rate of its breakdown is compared, i.e. generation of C3dg. C3 cleavage was detected with α-hC3dg (1 μg/ml. Experiment was performed twice.

Next, a hemolytic assay was performed and the degree of lysis of rabbit erythrocytes was measured. Unfortunately, this assay turned out to be difficult to interpret when the standard protocol was used. This is because whole serum with serial dilutions was used but all sera differed in their initial colour, some of them quite severely. It was therefore not possible to analyse and compare the degree of lysis in the samples by simply measuring the colour of the supernatant alone. Therefore, the RBCs were spinned down and all liquid was removed from the wells. Next, the cells were lysed with H_2O, centrifuged again and the colour of the lysate was determined with a spectrometer.

This means, the more lysis had occurred, the less RBCs were still left intact when H_2O was added and therefore gave a lower reading. Conversely, if FI concentrations are elevated and protect RBCs from lysis, a greater number of intact RBCs is expected at the end of the incubation period and the signal after final lysis with H_2O will be higher. The final lysis step was only done after $\approx$ 18 hours incubation, after which there also will be some natural lysis of the RBCs. Unfortunately, no more serum samples were left to repeat the assay, so results of the hemolytic assay have to be analysed with care and were only added for completeness.

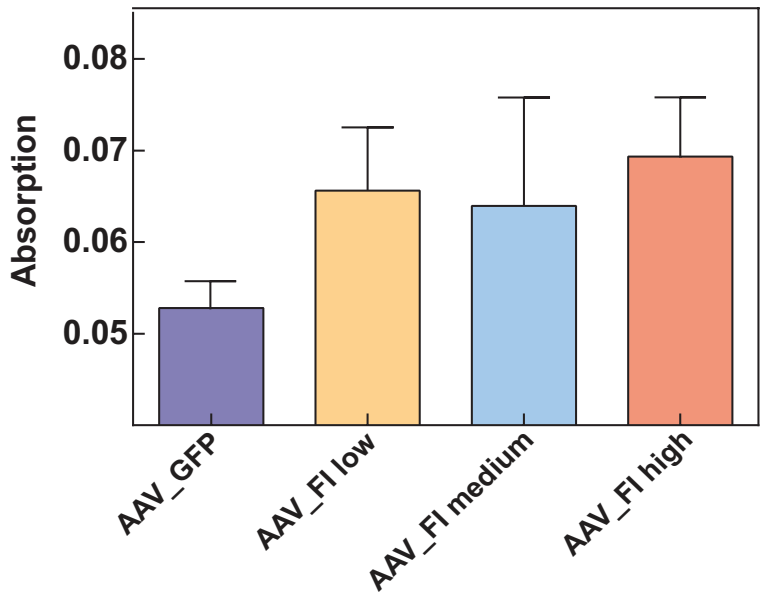

Fig. 4.6 Hemolytic assay to to measure functional activity of over-expressed Factor I. Rabbit RBCs were incubated for 3 hours at 37°C with serum from transgenic mice (diluted in alternative pathway complement fixation buffer). After the incubation period, RBCs were spun down and lysed with H_2O after removal of supernatant. Absorption at 490 nm was measured to determine the degree of lysis. Experiment was performed only once because not enough serum was left for repetition.

As expected, serum of AAV_GFP gave the lowest reading, while AAV_FI high gave the highest (Fig. 4.6). Sera of the low and medium dose of AAV-FI were intermediate. It should also be noted that lysis of the GFP control sera did not lyse as well as one would expect. It was thought that high level hepatic expression of a foreign protein, i.e. GFP, could influence expression of complement proteins required for lysis such as C6. In order to test whether this is indeed the reason for the lower hemolytic activity of the AAV_GFP sera, a C6 hemolytic assay was developed. Nevertheless, this assay showed fluctuation in C6 levels within the groups but no significant differences in the four groups were observed (Tab. 4.7). Therefore the reason for the controversies must lie somewhere else. Possible explanations will be discussed in the next section.

After showing that the sera differed in their degree of cleavage of purified C3b (and in lysis of rabbit erythrocytes whose results are slightly controversial), the next step was to show that over-expressed FI was also functionally active in whole serum. For this, a

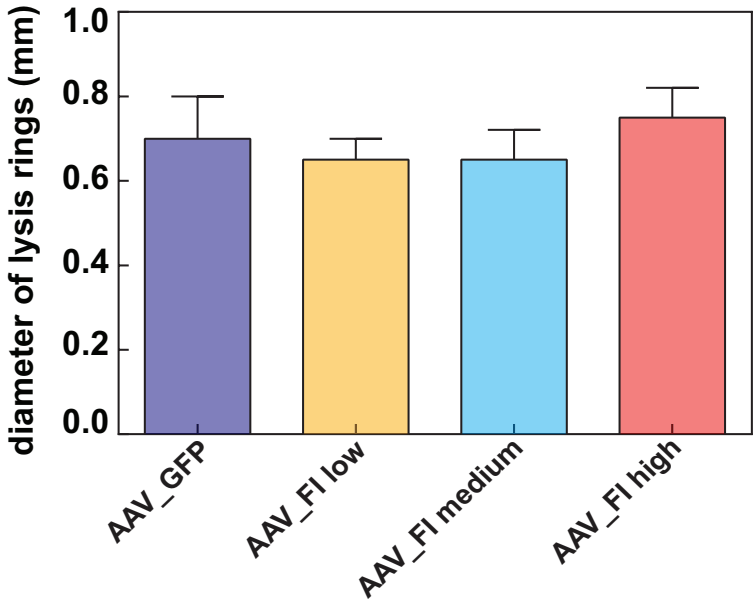

Fig. 4.7 Solid phase hemolytic agarose assay to measure C6 levels. An agarose plate containing C6 deficient rabbit serum and EA43 cells treated with antrypol is prepared and 10 μl mouse serum samples are added. Once C6 is restored by addition of test sera, hemolysis can be completed. Plates were incubated overnight at 4°C for diffusion of serum and then incubated for several hours at 37°C until hemolytic rings appeared. Ring diameter was measured. The experiment was performed twice in duplicates.

microtiter plate was coated with LPS and C3b/iC3b deposition was measured after incubation with transgenic mouse serum at 37°C. As with the recombinant protein, mice injected with AAV_mFI showed less C3b/iC3b deposition on LPS since their positive feedback loop of the alternative pathway was interrupted by FI (Fig. 4.8). C3b/iC3b deposition was reduced by 11% in the AAV_low FI, by 38% in the AAV_medium FI and by 50% in the AAV% high FI group.

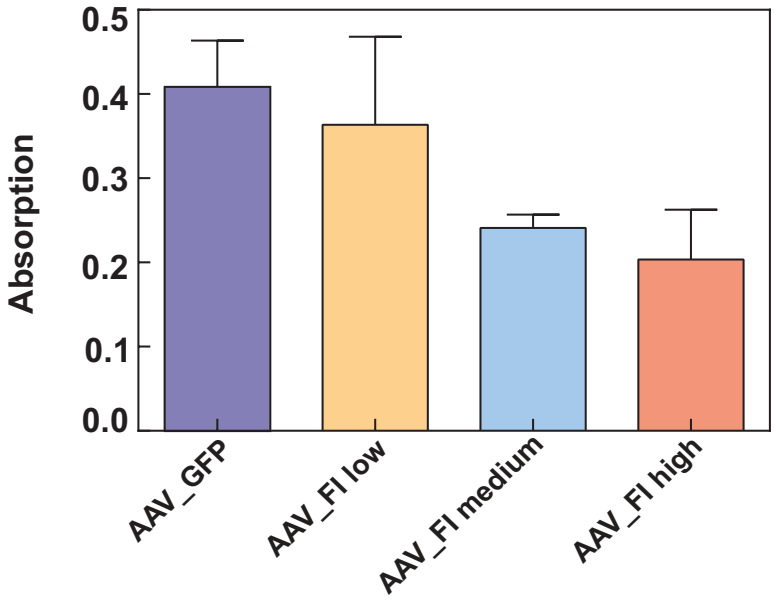

Fig. 4.8 iC3b deposition assay to test functional activity of over-expressed Factor I. Serum of injected mice was diluted to 25% in alternative pathway complement fixation buffer and loaded onto an LPS coated plate. After incubation for 1 hour at 37°C, bound iC3b was detected with an α-human C3c antibody. Absorption was measured at 415 nm. Experiment was performed twice times in duplicates

4.2.2 Discussion

Gene therapy is a new and exciting therapeutic option for the treatment of various diseases. It enables *in vivo* expression of proteins after transfection of the respective cell with a construct harbouring the DNA sequence of the protein of interest. This can be achieved by both viral and non-viral vectors. Theoretically, gene therapy can be utilised for expression of every protein, although practically, there are usually limitations such as e.g. length of the sequence. Missing proteins can be replaced, they can be expressed at different sites or their concentration can simply be elevated. Replacing absent proteins nevertheless always carries risk of immunogenicity but this risk is abolished if concentrations of normal proteins are simply raised (albeit probably only to a certain extent). In this study we wanted to elucidate whether complement FI can be over-expressed by gene therapy to levels which cause significant down-regulation of the alternative pathway.

In order to accomplish our aims, we started a collaboration with a leading group in gene therapy (Professor Ian Alexander, Children's Medical Research Institute in Westmead

[Sydney, Australia]). This turned out to be a very fruitful collaboration and we quickly succeeded in generating a vector for transgenic expression of mouse complement FI.

The viral expression system used is based on AAV2 and its capsid is derived from AAV8. Together this AAV2/8 construct mainly results in liver transduction in mice, although virus particles can be also found in extra-hepatic tissue. To increase liver specific expression, the transgene is under the control of the α-1-anti-trypsin promoter with two ApoE hepatic control regions for high-level and specific expression in hepatocytes. Therefore, transgene expression should only occur in hepatocyes. Sometimes although, it can happen that there is very little transgene expression in pancreatic tissues which is a very closely related tissue (personal communication from Prof. Ian Alexander).

Using this generated vector, I could demonstrate that over-expression of FI by the AAV expression system used clearly has an effect on complement down-regulation and secondly that this effect is titrate-able and becomes more profound as the vector dose is increased. In order to measure the total FI increase, a polyclonal antibody against mouse FI was raised in a rabbit (kindly performed by Dr. Wei Zhang, Absea Biotechnology Ltd., China) since commercially available α-FI antibodies were found unsuitable for measuring mouse FI concentrations in an ELISA (they do not react with native FI but only with the denatured protein in a western blot). Using this polyclonal custom-made antibody it was possible to show that serum concentrations were increased up to 4x of normal levels, i.e. from 20-40μg/ml in control mice to 80-160μg/ml FI in transgenic mice.

In the iC3b deposition assay, it was noted that the difference between the medium and high AAV_FI dose is not as much as one would expect given the fact that the latter has an injection dose that is ten fold higher. Since the inhibition ELISA showed that there is an increase in the AAV_high-FI compared to the AAV_medium-FI, results from the iC3b deposition assay could be explained by an exhaustion or saturation of the cellular protein manufacture machinery, so that the over-expressed protein cannot be processed properly. This phenomenon (incorrect folding/processing) has also been observed with recombinant FI expressed in cell culture. The inhibition ELISA does not distinguish between native and

misfolded protein as long as the antibody still recognizes its epitope. Also, the effect on the synthesis cannot be expected necessarily to be entirely linear with the amount of virus given.

Alternatively, the results obtained could also be due to the assay and its kinetics. Lectin pathway recognition molecules that function in the absence of Ca^{2+} can cause background C3b deposition. This initial C3b deposition is done by e.g. Ficolins using the lectin pathway but FI inhibits the subsequent feedback amplification. More mice have to be injected to determine a range of FI increase for each dose.

The validity of the use of GFP as a control should also be discussed in more depth. Even though GFP is an established control in many fields of biology, it could be that putting GFP into the liver may stop or at least inhibit it from producing other proteins at a normal rate. This concern is derived from the hemolytic assay data where serum of the GFP-control mice does not behave as well as expected. It was suspected that some of the proteins required for the terminal pathway, such as C6, C8 or C9 which are all made in the liver, may be down-regulated by over-expression of an intracellular, non-endogenous protein such as GFP. The complement system is a highly sensitive network of proteins and its effector function can easily be shifted by minor changes. Thus, it was hypothesized that this phenomenon is highly likely not to be due to any effect of FI but rather to the expression system/GFP control which could theoretically cause reduction in C6 expression. Even though these transgenic mice injected with AAV2/8_GFP have been examined for liver impairment by standard tests (measurement of ALT and AST levels), these tests did not indicate any liver damage but there still could be changes in gene expression. Nevertheless, when C6 levels were compared between the groups, no significant differences could be detected. Another reason could be caused by improper handling when serum was taken or during shipment from Australia. Mouse complement is extremely sensitive and easily becomes hemolytically inactive.

Another interesting question is whether transgene expression is further increased in an acute phase reaction. Since in the construct used, FI is under the control of the α-1-anti-trypsin promoter (α-1-anti-trypsin and FI are both positive acute phase reactants), it can be expected that transgene expression will also be increased in an acute phase reaction. This could be tested in a straightforward animal experiment that could be easily performed by

injection of LPS or IL6 into wildtype and transgenic mice and then compare the rate of FI increase. Assuming both, endogenous and transgenic FI expression would increase, then this could be a solution to reduce the initial virus load during AAV administration but with the option to increase expression is required.

Gene therapy has been quite successful in animal studies but one of the remaining problems in human AAV gene therapy is that results gained from animal models do not reflect what would happen in man. In particular, transfection efficiency is much lower in human [273] which requires development of new and better vectors. Since there are no cell lines that stably express all required helper functions for AAV packaging, a triple transfection is the current standard protocol. This is not only a tedious, time consuming process but can also only be done on a small scale due to otherwise exploding costs. Another problem is that other factors such as host immunity prevent the widespread use of AAV in man. Once exposed to AAV, people develop neutralizing antibodies that block gene delivery. By engineering of the capsid proteins, new variations can be generated that have higher transduction efficiencies and are not recognised by neutralising antibodies. New approaches to limit these immune responses are therefore undertaken [304]. Recent clinical studies show success of a single infusion of an AAV vector leading to two years of therapeutic levels of FIX in men with severe hemophilia B [305], although it should be pointed out that an increase of only 1% of normal FIX levels, substantially ameliorates the severe bleeding phenotype in hemolphilia B patients [256]. Before, AAV transgene expression of FIX in skeletal muscle was shown to persist for 10 years, although in this case, circulating FIX levels remained subtherapeutic (< 1%) [306].

In conclusion, the amplitude of the gained effect forms a sufficient basis for moving on to studies in which the effect of FI increase in disease can be studied. Expressed in numbers, an increase of FI by 50% maximal is what is aimed for therapy in human studies. This is by far exceeded using the AAV_FI-construct and the iC3b deposition assay shows an a reduction of up to 50% less C3b deposition in the high AAV_FI dose. Our preclinical data show that 50% more FI converts the activity of an high-risk complotype to the activity of a low-risk complotype [112]. Since both of these complotypes are extremely rare and the majority of

people will be within both extremes, an effect for risk reduction is only required within the range of 50% FI increase.

Gene therapy is a rapidly growing field that is now finally about to overcome initial failings and hurdles and although it still has some limitations, it also has many advantages over traditional recombinant protein production.

Chapter 5

Generation of a biomarker for the detection of bound iC3b/C3dg in vivo for diagnosis and follow up of early dry age-related macular degeneration

5.1 Introduction

The next chapter will describe the development of a biomarker for detection of early age-related macular degeneration. First, the disease pathology will be explained before the role of the complement system in disease progression will be discussed and then, the single-chain Fv biomarker construct which has been constructed will be introduced.

5.1.1 Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in elderly people in western countries [307]. The prevalence of AMD increases steadily with age: 2% of the population is affected at age 40, 10-18% between 65-70 years, while it is 25-30% by age 80 [308, 309]. It is a chronic, progressive disease which manifests itself particularly in the

macula and can have a significant debilitating effect on daily life of affected individuals. It causes no pain and diagnosis is based on visual dysfunction and characteristic macular findings [310].

5.1.1.1 The structure of the retina

A schematic diagram of a human retina is shown in Fig. 5.1. The retina is the innermost layer of the eye and is considered part of the central nervous system. In vertebrate embryonic development, the retina and the optic nerve originate as outgrowths of the developing brain [311]. The retina consists of several layers of neurons (photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells) from which the outermost layer (furthest away from the center of the eye) is made from photoreceptor cells. There are two types of photoreceptor cells: rods (black-white vision, motion) and cones (colour vision, acuity and resolution). Below the photoreceptor cells, there is a monolayer of retinal pigment epithelium (RPE) that associates with the outer segment of the photoreceptor cells. Thereby, the photoreceptors are supplied with nutrients and oxygen and at the same time, waste products can be removed. RPE are polarized hexagonal cells that are densely packed with pigment granules (melanosomes) [310]. The RPE lies on an extracellular matrix, called Bruch's membrane. Immediately below Bruch's membrane is a network of fine capillaries, known as the choriocapillaris, which supplies nutrients and oxygen to the RPE. [1] The choricapillaris is the outer layer of the choroid and consists of highly fenestrated capillaries that enable leakage of blood-borne factors and nutrients. Thus, the RPE layer is a blood-retinal-barrier that prevents non-specific diffusion and transport of material from the choroid. The Bruch's membrane and the fenestration of the choriocapillaris present further barriers to the movement of large macromolecules between the choroidal circulation and the RPE [312]. It is now known that the molecular, structural and functional properties of Bruch's membrane are dependent on age, genetics, environmental factors, location of the drusen and disease state [310]. It is interesting to note that the glomerular basement membrane in the kidney and the Bruch's membrane in the eye are the only two extracellular basement membranes of

[1] The inner retinal cells are supplied by a separate vasculature.

the human body that are in direct contact with the circulation. This may already partially explain why these two structures are extremely susceptible to complement deregulation.

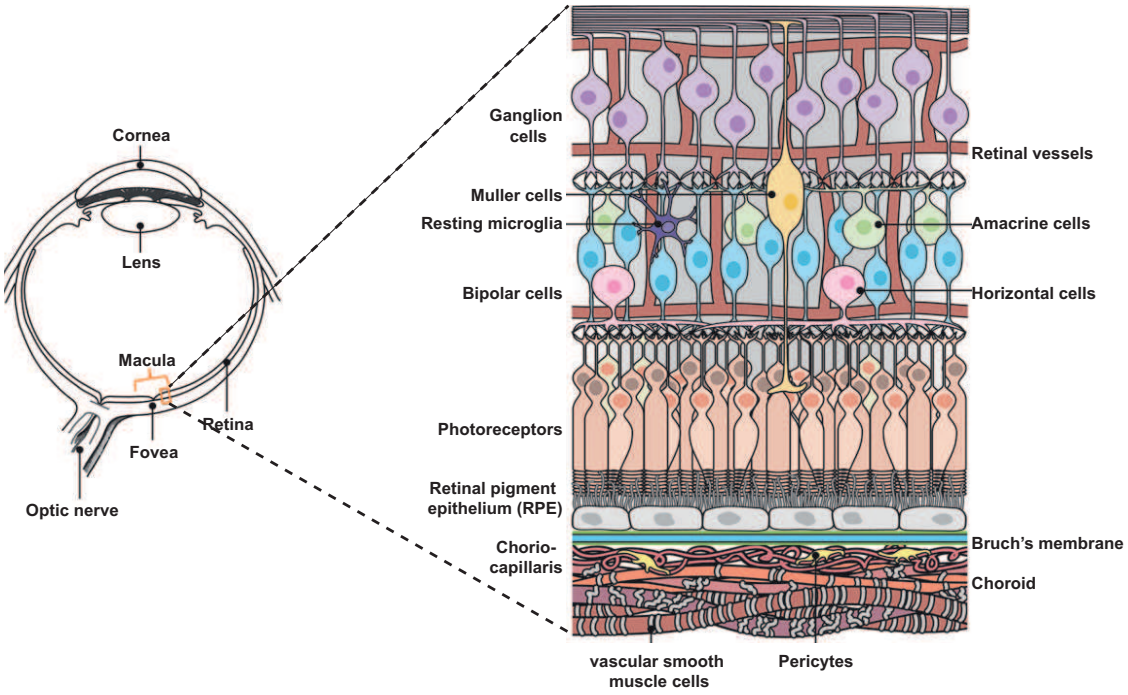

Fig. 5.1 Schematic diagram of the human eye and macula. The macula is a region of the retina close to the optic nerve and it contains primarily cone photoreceptors. It consists of several layers and celltypes which are shown in the diagram. Figure adapted from [313]

The extensive vasculature of the retina is required because the retina is one of the most-oxygen consuming tissues of the human body [314]. [2] Most of the oxygen is consumed by the photoreceptors and it is the RPE cells that remove accumulating oxidative and photo-oxidative waste products. Moreover, the RPE phagocytoses $\approx$ 10% of the outer segments of photoreceptors that are shed every day [311] [3] One RPE cell is responsible for 20-30 photoreceptor outer segments [310] and with little or no turnover of RPE cells, there is a progressive build up of indigestible residues within these cells [312]. One of these waste products is lipofuscin which could be one of the factors predisposing to AMD [312].

[2] In fact, oxygen consumption of the retina is $\approx$ 50% higher than that of the brain or kidneys when adjusted for its weight [314].

[3] Omega-3-fatty acids are one of the essential nutrients that are required for building photoreceptor outer segment membranes [315].

The centre of the retina is the macula which is about 6 mm in diameter. Its central region (fovea) contains almost exclusively cones and is responsible for highly detailed central vision. Thus, degradation of photoreceptor cells within the macula has a bigger impact on our vision as opposed to their loss in other, peripheral parts of the retina. Another reason for the macula being the location of greatest injury in AMD is that light is focused in the fovea and cones in this region face the most photo-oxidative associated products and stress.

5.1.1.2 Disease pathology

In a normal healthy eye, there is a symbiotic relationship between the complex of photoreceptors, RPE, Bruch's membrane and choriocapillaris [310]. Disturbances of this balance can cause pathological changes such as macular degeneration and it is known that the type of AMD depends on which component in the complex is affected first, although exact pathogenesis is still unclear. Age-related macular degeneration can be separated in early and late stages of disease which will now be described and are depicted in Fig.5.2.

Drusen and basal deposits, the hallmark of early AMD, are partially composed of indigestible waste products (lipids and proteins) from the overlying outer segment of photoreceptors and RPE [317]. Proteomic analyses of drusen showed that they are composed of multiple proteins and lipids, including proteins derived from the choroidal circulation (e.g. complement and coagulation proteins, amyloid-β,...) but also proteins/lipids secreted from RPE (e.g. lipofuscin, oxidated proteins and lipids,...) [318–321]. The accumulation of hydrophobic material in and around Bruch's membrane is considered to inhibit normal transport to and from the choriocapillaris [316] and therefore predisposes to development of later AMD stages. Other features of early AMD are pigment changes in the RPE, thinning and atrophy of the choriocapillaris [310, 322].

Early AMD (Fig. 5.2a) is associated with formation of large, soft (weakly defined) drusen between the RPE and Bruch's membrane and basal deposits within Bruch's membrane [312]. Appearance of small, hard (clearly defined) drusen, however, are common in the elderly population and do not confer AMD risk [316, 323].

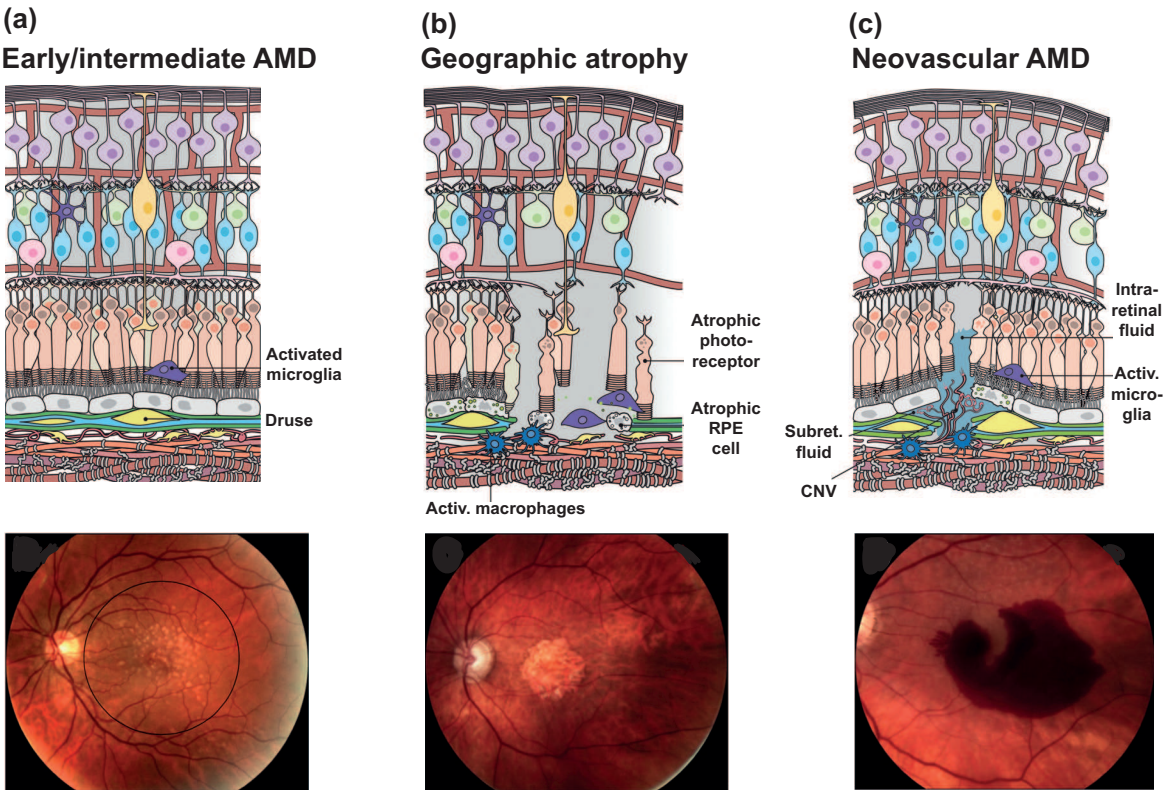

Fig. 5.2 Different stages of age-related macular degeneration. The upper figures show a schematic diagram of disease pathology, while the lower figures are clinical photographs of the posterior retina. (a) Early and intermediate AMD is characterized by the presence of drusen that form between the RPE and Bruch's membrane. In a photograph, they appear as yellow-whitish deposits in the macula. (b) In geographic atrophy, there is disruption of Bruch's membrane and loss of photoreceptor cells which is visible as white structure and loss of pigmentation. (c) In wet or neovascular AMD, new, leaky blood vessels grow from the choriocapillaris into the subretinal space which can be seen as haemorrhage in a photograph. Figure adapted from [316] and [313].

The late forms of AMD include "dry" geographic atrophy (GA)[4] and "wet" choroidal neovascularisation (CNV). It is yet not clear why some individuals develop GA while others CNV but in both conditions, drusen are the leading trigger [324].

In GA (Fig. 5.2b), there is loss of RPE cells which occurs in the area of confluent drusen and it has been observed that drusen regress once atrophy has developed [325]. As opposed to CNV, disease progression and loss of vision occurs gradually. It has been reported that in GA, dysfunction of RPE and Bruch's membrane happens first and causes

[4] In fact, both, early/intermediate and geographic atrophy are referred to as dry AMD.

secondary dysfunction/death of photoreceptors and choriocapillaris [310]. Wherever these photoreceptors are lost, they cause a black spot to appear in the vision. It is thought that several early changes in the outer retina occur prior to RPE loss. These are thickening of Bruch's membrane, deposition of waste products, lipofuscin accumulation in the RPE, RPE hyperpigmentation and drusen formation beneath the RPE monolayer [313]. Up to now, there is no treatment for dry AMD, although there are promising clinical trials.

In wet AMD (Fig.5.2c), there is growth of new vessels from the choroid which break through Bruch's membrane into the intraretinal space. These delicate vessels have a tendency to break and cause fluid accumulation between the RPE and Bruch's membrane. Bleeding of these new vessels can cause a sudden and rapid loss of vision and can result in scar formation. It is assumed that the initial event is loss of choriocapillaris caused by vessel stenosis which results from age and diet [322, 326, 327]. The result is a reduction in blood supply and hypoxic RPEs secrete pro-angiogenic substances such as vascular endothelial growth factor (VEGF) which subsequently causes choroidal neovascularisation. In fact, loss of chariocapillaris with an intact RPE layer has been observed [327]. Reduction in choriocapillaris could further stimulate drusen formation due to limitations in the waste removal system [310]. In contrast to dry AMD, the wet form can be treated with VEGF inhibitors or antibodies and anti-VEGF therapies have shown considerable success in preventing further loss of vision in wet AMD [328].

"Dry" and "wet" AMD, although having similar symptoms in their early stages, probably have different underlying disease mechanism. It therefore remains to be clarified, whether early dry AMD should be seen as the first step in disease, with GA being the default pathway and wet AMD only occurring at a low percentage of affected patients, or whether there are differences in early stages of both diseases that we are not yet aware of.

5.1.1.3 Risk factors for age-related macular degeneration

AMD is a multi-factorial disease but it is now clear that the risk of becoming affected is associated with environmental, clinical and genetic factors [316], see Fig. 5.3a and b. These risk factors are followed by smoking (the highest, modifiable risk factor), age, a family history of

AMD and previous cataract surgery [329]. Other risk factors include high fat diet, high body mass index, history of cardiovascular disease and hypertension [316, 330]. The rate of AMD prevalence has been found to vary widely between different ethnic groups and individuals with a Caucasoid background have a much higher AMD risk (Fig. 5.3b) [331]. Since 2005, it is known that there also is an association to the complement system, e.g. a polymorphism of *FH* (Y402H) was found that increases the risk by several fold [113–116]. This SNP lies in CCP7 of *FH*, a region that is responsible for FH binding to glycosaminoglycans (GAGs), i.e. heparan and dermatan sulphate [332] (see also Fig. 1.5. Interestingly, it was also shown that there is a $\approx$ 60% reduction in heparan sulphates in old vs. young [333] which would explain why there is a sharp increase in AMD prevalence in individuals over 80 years (see Fig 5.3a). Around 30% of white Europeans are heterozygous for this FH risk variant [324]. Since 2005, many other AMD risk-associated polymorphism and mutations have been found. Affected genes are: *HTRA/ARMS2* locus, *CFH*, *CFHR*, *CFB*, *C2*, *C3*, *CFI* of the complement system, genes of the lipid metabolism, genes associated with apoptosis and the extracellular matrix [313]. Rare mutations in the *CFI* gene were found that cause a decrease of its plasma concentration and affected individuals have a higher risk of advance AMD [119, 334].

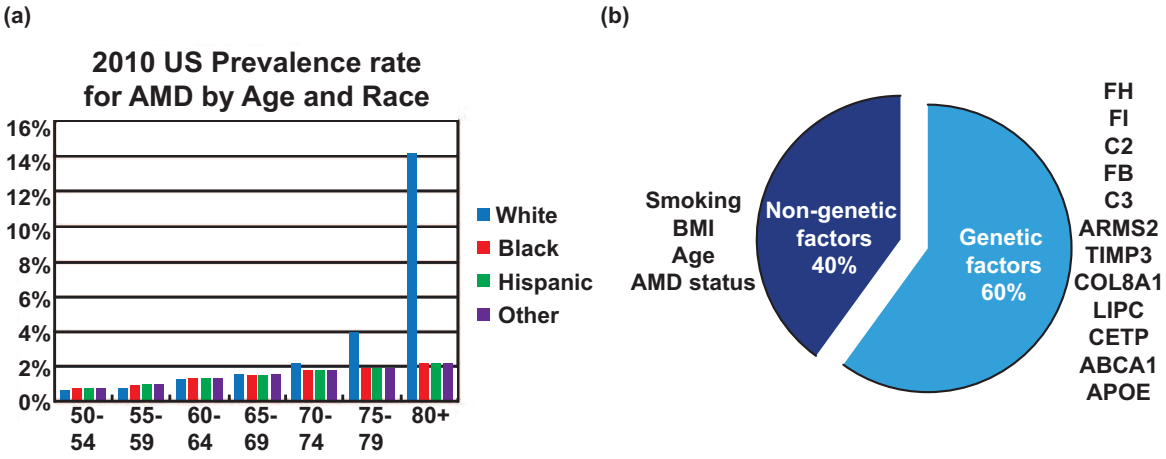

Fig. 5.3 Prevalence of age-related macular degeneration and its risk factors. (a) US prevalence rate for AMD by age and race. Individuals with a white background have a 2x higher risk to develop AMD by age 75-79 but this risk is 7x higher by the age of 80 or higher. (b) Risk factors for AMD are separated into genetic and non-genetic causes. Figure adapted from the National Eye Institute [335].

Further, it is long known that there is a link between AMD and atherosclerosis [336], in that both diseases are linked to age, systemic complement deregulation, endothelial dysfunction, oxidative stress as underlying dominator and formation of drusen with amyloid-β being a major component [337]. In total, genetic factors are responsible for 60% of AMD cases while environmental factors account for the remaining 40% (Fig. 5.3b).

5.1.1.4 Complement deregulation in age-related macular degeneration

Even though there is a clear association of the complement system to AMD, the exact pathological mechanisms are still unclear. Drusen, the hallmark of early dry AMD contain complement proteins and regulators [21]. Risk-associated polymorphism were primarily associated with the alternative pathway of the complement system [21] and are introduced in Sec. 1.4.1.

Studies showed that there is deposition of terminal complement components, i.e. MAC, primarily in the choriocapillaris but also in Bruch's membrane of affected individuals [338, 339]. MAC deposition also occurs in unaffected individuals but increases steadily with age and was already detected in donor eyes as young as five years [338, 339]. Thus, endothelial cells of the choriocapillaris are exposed to MAC for decades before onset of early AMD. In contrast to RPE cells that can express membrane-bound complement regulators such as CD46, endothelial cells from the choriocapillaris rely mainly on recruitment of FH from the circulation. The Y402H SNP demonstrates this importance of FH binding to GAGs in the eye (i.e. to heparan sulphates) for tissue protection and homeostasis [332] because patients homozygous for 402H (susceptible) have more MAC deposition [322, 340]. Further, a deletion in *CFHR1/CFHR3* was associated with a reduced risk for AMD was reported [117] which can be explained by the fact that FH-related proteins have a role in complement regulation as they compete with FH for binding to C3b [136, 341]. Reduced expression of CD46 has been described on RPE cells in eyes with advancing geographic atrophy which further suggests that with age, the retina become more suceptible to complement attacks [342].

Retinal metabolic demands require an oxygen-rich environment and also produce a substantial amount of photo-oxidative waste products that combined with age-related impairment of autophagy, accumulation of pro-oxidative lipofuscin and oxidative damage to RPE, result in a local inflammatory milieu [319, 21, 343, 344]. In other words, MAC deposition is presumably required for opsonisation and proper removal of this debris but with increasing age and loss of RPE cells due to normal ageing, the beneficial role is overwhelmed and the pro-inflammatory effects become dominating [322]. The Alzheimer's disease protein amyloid-β was found to be deposited in drusen as well [320] and it is known that amyloid-β inhibits FI function [345]. Many studies so far have demonstrated that RPE cells are able to synthesize complement proteins and together with proteins sequestered from the choroidal circulation, they promote further inflammation [21].

It is currently unknown what exactly activates complement in AMD in the first place but small changes (congenital or acquired with age) in systemic complement regulation, especially in the alternative pathway, can tip the delicate balance towards self-damage. Repeated episodes of complement activation by gram-negative bacteria or other stimuli may cause gradual damage over the years. Thus, the exact complotype most probably has a great impact on an individual's risk to develop AMD within lifetime. A therapeutic approach using complement inhibitors will very likely delay onset of AMD symptoms. One such study (MAHALO study, performed by Genentech/Roche) has already started a phase III clinical study using an inhibitory antibody to Factor D (Lampalizumab). In the previous phase II study, a 20% reduction in lesion growth in patients with GA compared to shams was detected in an 18-month study [346]. In this study it was found that patients with a biomarker in *CFI* progress much faster in GA development than patients without the biomarker but also responded much better to treatment with Lampalizumab (44% reduction in lesion growth compared to sham patients with the FI biomarker).

Theoretically everyone would develop AMD eventually but depending on the exact genotype and environmental circumstances, disease onset either begins in the last decades of life or the individual dies because of other reasons before AMD symptoms can manifest (personal communication with Paul Bishop and Simon Clark, University of Manchester). If

retinal, RPE or immune system physiology can be modified by a treatment that slows the rate of disease progression by as little as 10–20%, and thereby delays the onset of vision loss by 5–10 years, the impact on human health will be substantial [312]. With regards to amyloid-β, a study has already shown that increasing FI concentrations could restore C3b cleavage in the presence of amyloid-β [345].

5.1.2 A potential biomarker for complement activation in early age-related macular degeneration

It is clear that AMD is a multi-factorial disease and that it will be difficult to control all parameters. Nevertheless, there is a clear association to complement involvement, especially in early disease, and its deposition in drusen. In another project of our lab I collaborated, the aim is to prevent or slow down development of early AMD by inhibiting excessive complement activation [112]. As discussed in great detail in the introduction to this thesis, the strategy is to promote the breakdown cycle of the alternative pathway by increasing the concentration of FI which shows highly promising results in *in vitro* experiments and hopefully results in clinical trials in the near future.

A problem of AMD clinical trials is that slow disease progress results in long trials and heterogenity of progression requires large number of patients [347]. This does not only take much valuable time to evaluate therapeutic efficiency but also has a high cost. Currently, the FDA approved guideline for a clinical trial in dry AMD evaluation is that the growth rate of atrophic lesions must be reduced (Peter Adamson, personal communication). We propose a new approach of evaluation of therapy in AMD which is based on the fact that complement is activated very early but throughout disease progress and therefore represents a potential disease biomarker.

There is already an antibody which was generated in the lab of Peter Lachmann that exclusively reacts with an epitope in C3g that is accessible only in iC3b and C3dg [141]. This antibody is called clone 9 and was already used in previous chapters. As stated in Sec. 2.1.1, clone 9 reacts exclusively with iC3b and C3dg in experiments using native

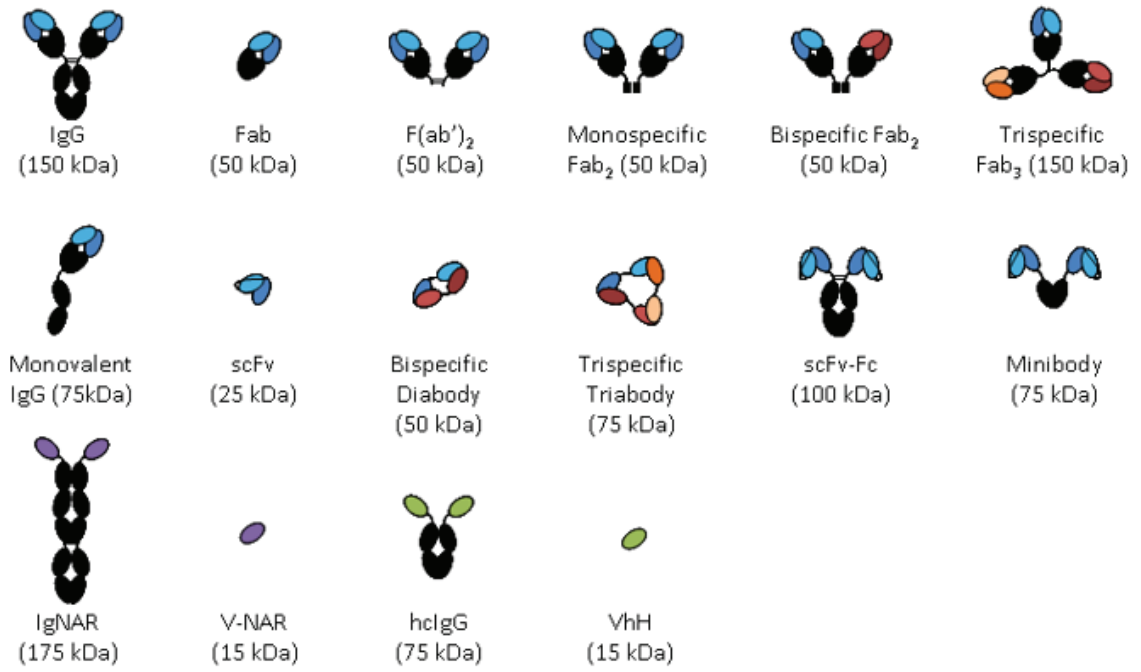

Fig. 5.4 Schematic representation of different antibody formats showing the classical IgG molecule, camelid heavy chain IgG (hcIgG) and shark IgNAR alongside antibody fragments generated from these. Figure taken from [348].

protein. It therefore does not bind to abundantly available C3 in plasma and not to the short-lived C3b. For any therapeutic, it is essential to reach the site of action and the biomarker therefore has to be able to cross the blood-retina barrier. Across Bruch's membrane, there is significant transport of individual molecular species, including dissolved gases, nutrients, cytokines, waste products but also serum proteins driven by passive diffusion [349]. However, this transport rate declines linearly with age for all molecules measured and in particular proteins larger than 100 kDa have significantly decreased flux through Bruch's membrane of older individuals [350]. Another important factor for a retinal biomarker is retention and the bigger a molecule, the longer it is retained in the body before being excreted by the kidneys. An IgG antibody of 150 kDa will first of all have reduced access to the retina in older individuals but would also have a longer half life. However, prolonged retention of an antibody, particularly due to the Fc-tail at a location with an already established inflammatory milieu could further exacerbate disease. With a size of $\approx$ 30 kDa, a ScFv is small enough to enter the choriocapillaries and traverse Bruch's membrane but will also be quickly be

removed from circulation by the kidneys. ScFvs are small fragments of antibodies that only contain the Fv region of the antibody that harbours the three complementary determining regions (CDRs). Usually, the Fv region of heavy and light chain of an antibody molecule are fused by a linker and the resulting ScFv retains specificity of its mother antibody while being only a fraction of its size. By tagging clone 9-ScFvs with a flourescent molecule, a one-step immuno-detection is enabled. Therefore, ScFv formats, including monomeric, dimeric ScFvs or ScFv-hinge-C_H3 constructs, exhibit the best tissue penetration to retention ratio and thus are the ideal candidates for a biomarker.

In this chapter, the development of such a clone 9-ScFv is described and its clinical operational-ability will be discussed.

5.2 Results and discussion

5.2.1 Production of a single-chain variable fragment (ScFv) antibody

A schematic diagram of the produced ScFv is shown is Fig. 5.5a and b. The single chain Fv of clone 9 consists of its heavy and light chain linked by a linker peptide of 15 amino acids [$(G_4S)_3$]. The construct was expressed in e.coli and a periplasmic signal sequence was added N-terminally. The oxidising environment of the periplasm allows formation of disulfide bonds which does not occur in the reducing environment of the cytoplasm. The periplasm of e.coli further contains two foldases that catalyse the formation of S-S bonds. A C-terminal cysteine was added downstream of the light chain so that two ScFvs can be dimerised to a diabody which has increased avidity. Dibromobinane (DBB) is a fluorescent thiol-specific labelling reagent that can dimerise two molecules by binding to the SH-groups of cysteines. DBB only fluoresces when bound on both sides, a property that can be exploited to separate ScFv diabodies by FACS. At later stages in this project, a construct with the cysteine mutated to a serine was generated and therefore, the designation ScFv-S will be used for the construct which cannot be dimerised. A histidin tag was added to enable easy purification of the recombinant ScFv by affinity chromatography on a nickel column.

5.2.2 Preparation of a ScFv construct of an α-hC3g antibody (= clone 9)

Clone 9 hybridomas were generously provided by Professor Christina Rada and the late Professor Michael Neuberger (Cambridge, LMB Unit). The cells were grown up from frozen stocks, secreted antibody in the supernatant was isotyped and revealed the presence of an IgG1 heavy chain and various light chain isotypes (κ and λ), all of which could be the relevant variable region. Therefore, primers were designed that would amplify all these isotypes.

After several trials using different sets of primers, the variable regions could be amplified. The primers for this first PCR were designed to bind in the framework 1 (FR1) and 4 (FR4) region or beginning of the constant region (primer sequences in Tab. A.2). Thus, the PCR products might be longer than actually needed. Next, the PCR products were subcloned

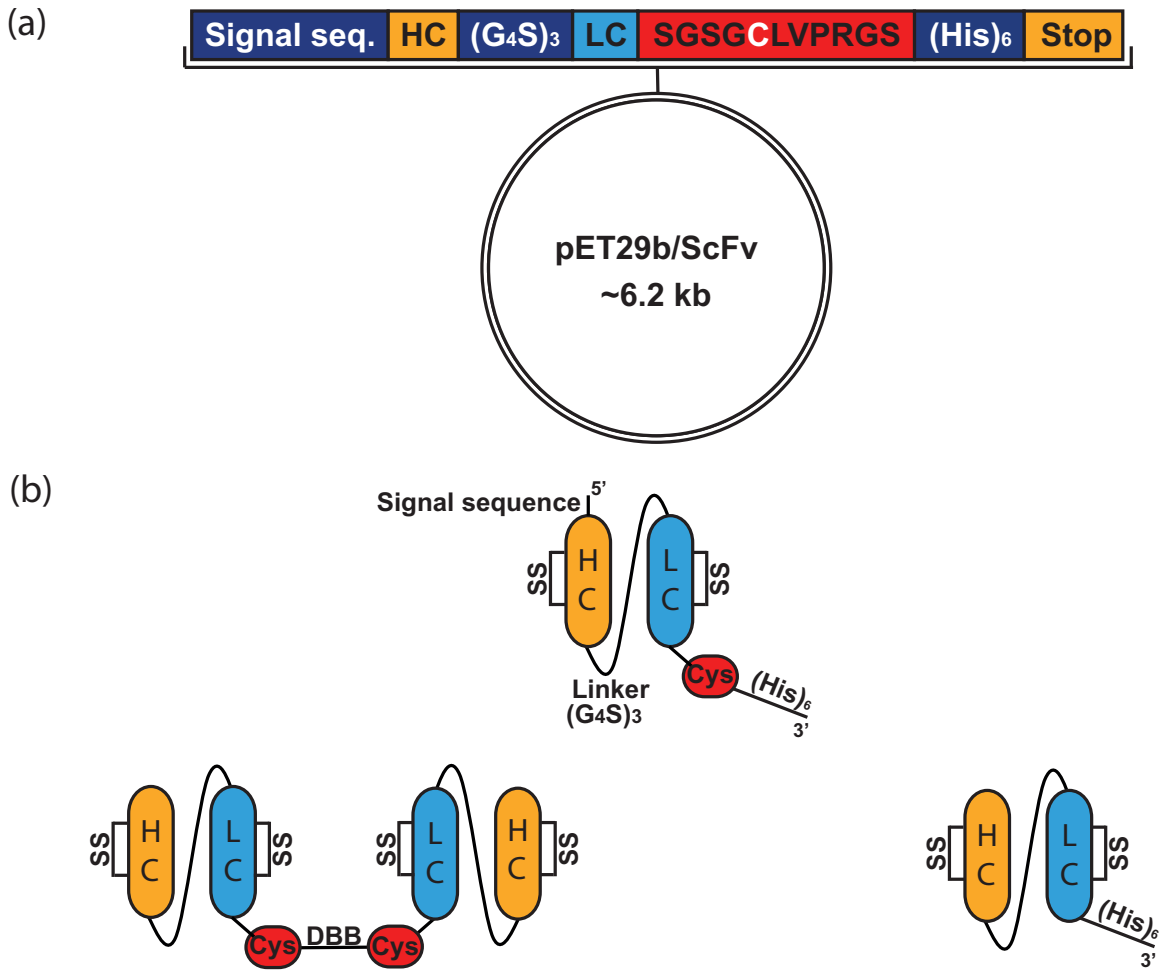

Fig. 5.5 Schematic depiction of ScFv-clone 9 construct. (a) ScFv consists of signal sequence, heavy chain (HC), linker peptide ($(G_4S)_3$), light chain (LC), short linker (GSGS), cysteine (C, white) for dimerisation, thrombin removal sequence (LVPRGS), histidin tag (His_6) and stop codon (Stop). (b) Arrangement of domains in folded ScFv. Upper: monomeric Scv, lower left: dimerised ScFv without signal sequence, lower right: monomeric ScFv without signal sequence.

into the cloning vector pJET1.2 blunt. The primers for the second PCR were designed to amplify only the variable regions (primer sequences in Tab. A.3). While only one heavy chain was amplified, three different light chains were isolated which belonged to the λ-1 and -2 isotypes (Fi. 5.6a, lane 1-3). In order to determine the right light chain, the parent antibody was sent for N-terminal sequencing to identify the relevant variable regions but also the translational start point. It was confirmed that λ-2 isotype was the relevant light chain and that the translated sequences started with EVQLVESG (heavy chain) and YELIQPPSA (light

chain). Therefore, λ-1 isotype could be ruled out but the other two candidates differed only in one base which was either deleted or inserted and therefore led to a frame shift mutation. Considering these two possibilities, both variants were amplified and fused with the heavy chain. They were designated as λ-chain 1 (LC-1) and 2 (LC-2), yielding ScFv-1 and -2 after fusion with the heavy chain (Fig. 5.6, lane 4 and 5). Later, ScFv-1 was ruled out because it contained 3 cysteines and would not lead to a balanced formation of disulfide bridges in the native, refolded protein. Heavy and light chain were fused in an overlap PCR separated by a linker peptide consisting of glycines and serines to improve flexibility and solubility (primer sequences in Tab. A.4).

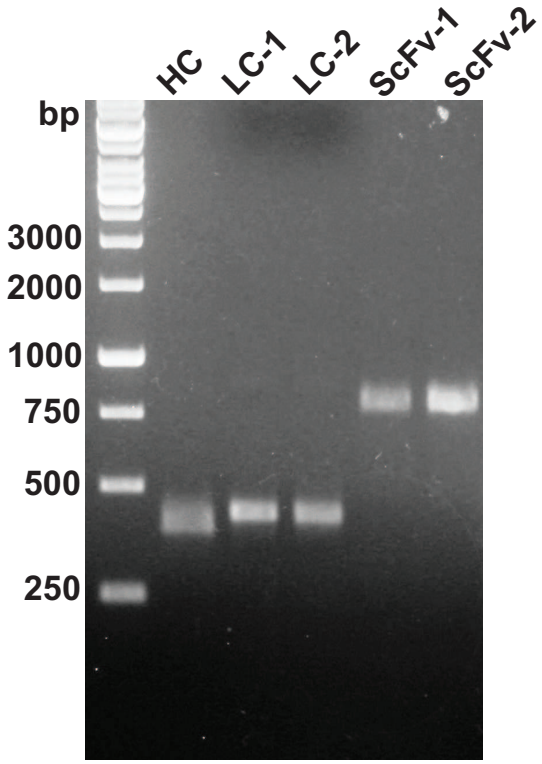

Fig. 5.6 Agarose gel after PCR purification of variable regions of clone 9 antibody. The variable regions of heavy and light chain (both around 400bp) were amplified and fused together to yield a ScFv (800bp). One heavy chain and two light chains were isolated.

First ligation and transformation efforts into OneShot competent cells were not successful and this was probably caused by the NdeI restriction site which contains the start codon but which also has been described of having poor ligation efficiency [351]. After prolonged

incubation and increased concentrations of insert, the construct could be ligated with its expression vector, pRSET-A.

5.2.2.1 Expression of ScFv antibody fragments in a bacterial expression system

First expressions in BL21(DE3) cells failed to induce any recombinant protein expression at all tried IPTG concentrations. Therefore, the construct was cloned into another expression plasmid, pET29b which has a lac operon to repress expression of both the inserted gene and the T7 RNA polymerase in the absence of an inducer, i.e. IPTG. Expression prior to induction was further repressed by using the BL21(DE3)LysS strain. LysS is a selective inhibitor of the T7 RNA polymerase and therefore reduces background expression of genes that are under control of a T7 promoter. Rosetta(DE3)LysS cells which carry an additional plasmid coding for rare codons in e.coli, were also tested for ScFv expression. Since they gave the same yield but were growing much slower, BL21(DE3)LysS cells were used for all further experiments.

Next, ScFv expression was confirmed in a time course experiment in a SDS PAGE (Fig. 5.7) and in a western blot (Fig. 5.8). In brief, gene expression was induced with 1 mM IPTG, samples were taken at several time points and normalized according to their OD600 before loading. It could be confirmed that the ScFv is expressed and that its expression increases with time, i.e. it is not degraded but accumulated inside the bacteria.

5.2.2.2 Purification of ScFvs

Once expression of ScFv was confirmed, the recombinant protein had to be purified. First, the exact location of the recombinant protein had to be found so that purification methods could be adjusted. A cellular fractionation revealed that ScFvs were not, as planned, in the periplasm but rather residing as insoluble inclusion bodies in the cytoplasm. Even though, a periplasmic signal sequence, PelB, was attached to the N-terminus, the recombinant protein could not be directed there. Various ways to slow down protein expression were examined so that saturation of the bottleneck (= translocation through the periplasmic membrane by the Sec translocon) would be avoided: the temperature was decreased to 24°C, different

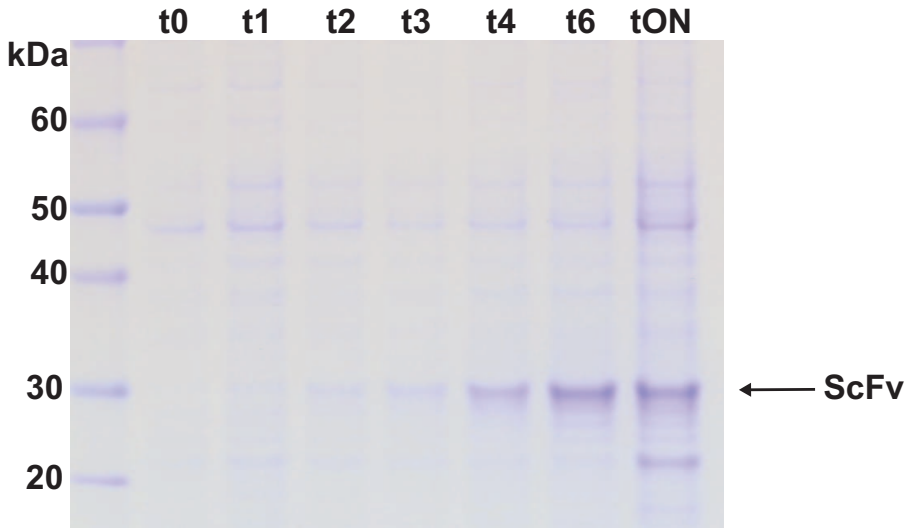

Fig. 5.7 SDS PAGE analysis of ScFv expression (reduced) after induction with IPTG. The ScFv has a molecular weight of $\approx$ 30 kDa.

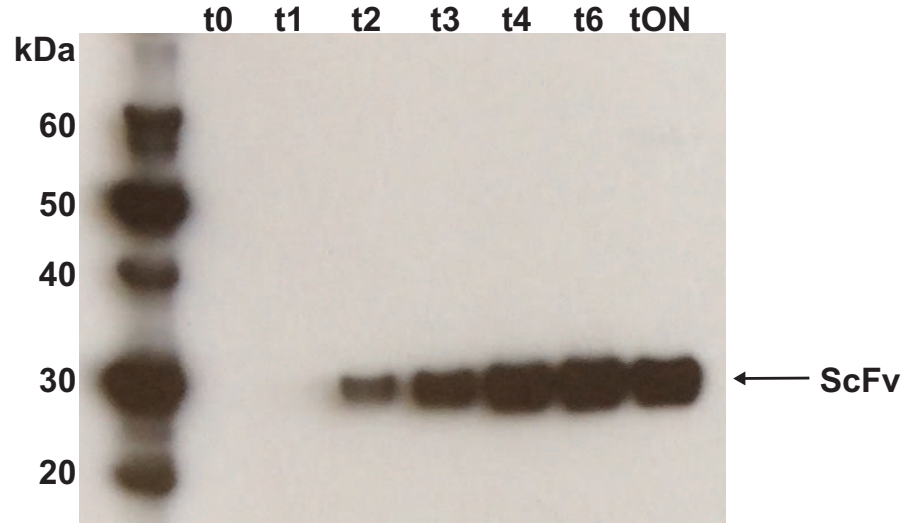

Fig. 5.8 Western blot analysis of ScFv expression. ScFv was separated by SDS PAGE and detected with an α-His antibody (1:4000).

concentrations of IPTG were used, glucose was added to repress the lac promoter before IPTG induction or sucrose to increase the osmotic pressure which should promote protein solubility because of accumulation of osmoprotectants in the cell. No periplasmic expression could be achieved, though. Thus, the signal sequence of the recombinant protein was changed into the one of the periplasmic proteins DsbA and OmpA. Using a different set of forward primers, constructs with the new signal sequences were generated (Fig. 5.9, primer sequences in tab. A.5). Additionally, a construct without a signal sequence was generated as well. Unfortunately, this construct could not be expressed in sufficient amounts but was rapidly degraded once expressed. Expression could be confirmed by western blotting but the protein was not present in sufficient amounts for purification and could not be seen as distinct band on SDS PAGE.

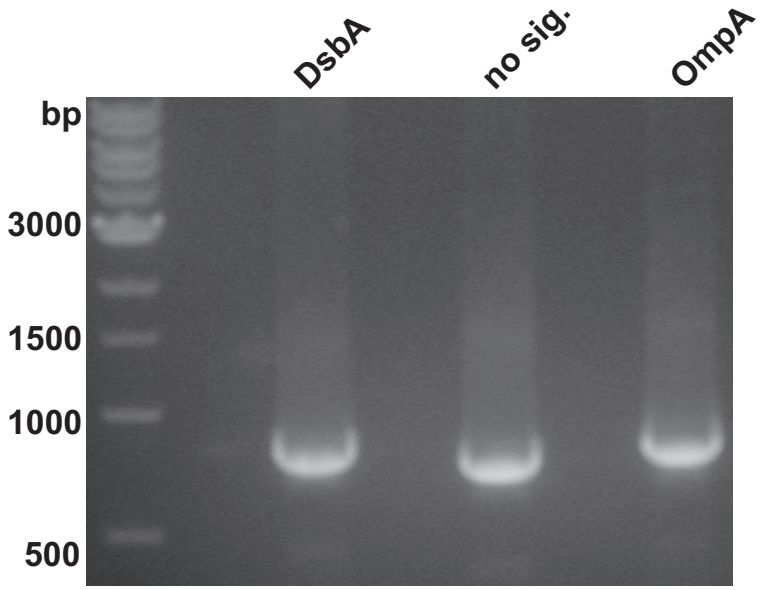

Fig. 5.9 Change of signal sequence of ScFv constructs. Primers encoding for the signal sequence of the periplasmic proteins DsbA and OmpA were designed and fused with the ScFv sequence (Lane 1 and 3). A construct without a signal sequence was prepared as well (Lane 2).

In a new experiment, cellular compartments of both constructs, DsbA and OmpA signal sequences, were fractionated. ScFv could then be detected in the periplasm and cytoplasm although the majority of recombinant protein was still found in inclusion bodies (Fig. 5.10).

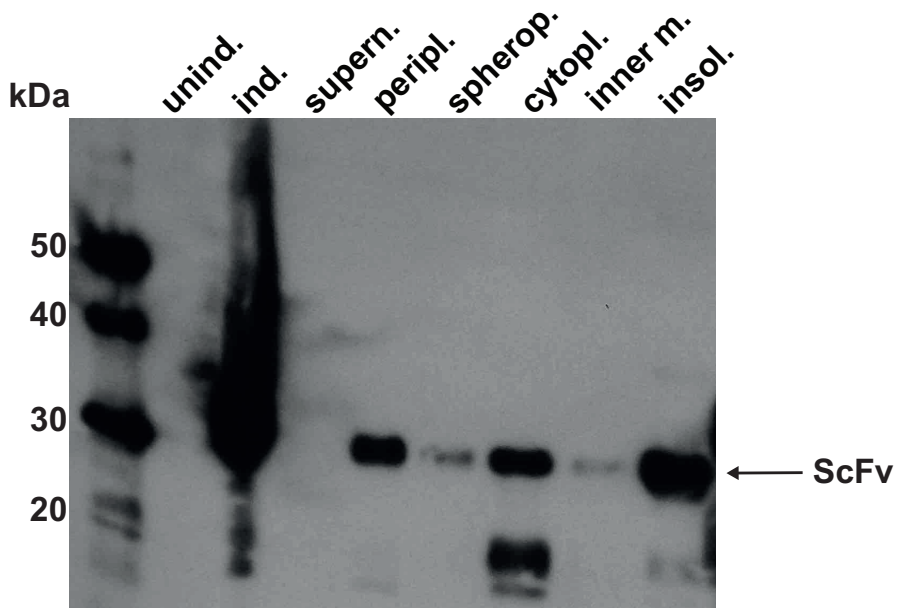

Fig. 5.10 Cellular fractionation to locate ScFv. Western blot of cellular fractionation of ScFv-DsbA. Lane 1-3 are uninduced and induced bacteria and supernatant. Bacteria were then fractionated into periplasmic-, spheroblast-, cytoplasmic-fraction, inner membrane and insoluble proteins (= inclusion bodies). ScFv was detected with α-His, 1:3000.

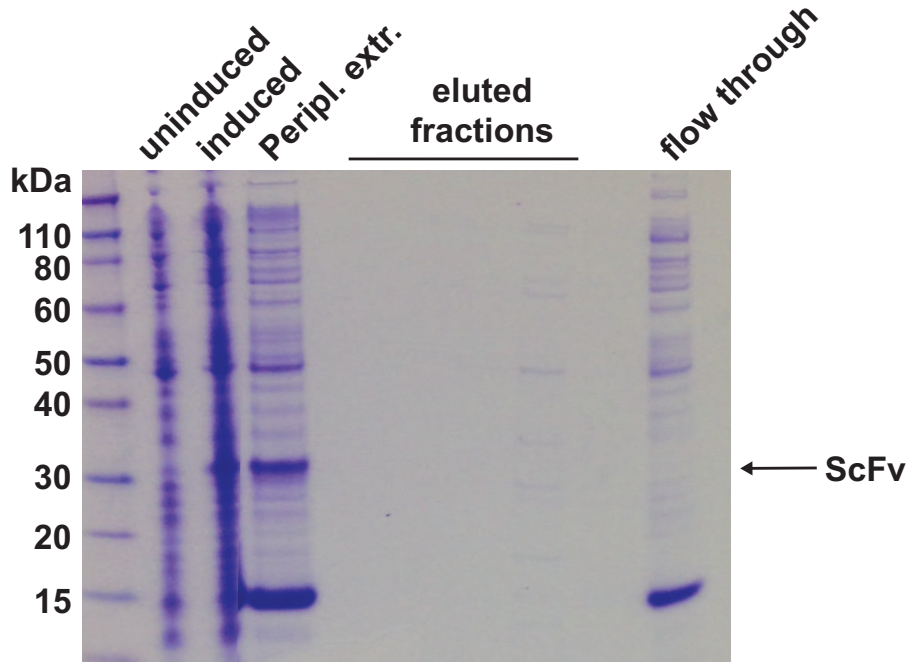

Fig. 5.11 Periplasmic purification of ScFv. Periplasmic fraction was purified and dialysed overnight (lane 3). All samples were loaded under reducing conditions. No ScFv could be eluted from a nickel column.

An SDS PAGE from a periplasmic fractionation shows that the ScFv can be extracted (Fig. 5.11 lane 3) but once the solution was cleared by centrifugation and microfiltration, almost all ScFv has disappeared i.e. was insoluble and aggregated. No ScFv could be eluted from a nickel column and the same results happened when the cytoplasmic fraction was purified. It was then tried to increase the amount of soluble recombinant protein production by lowering of the temperature, and variation of the IPTG concentration, addition of glucose or sucrose as described before. Nevertheless, all these modifications did not change location or solubility of the ScFv significantly. Also, the construct was transformed into a SHuffle expression strain which are engineered to promote disulfide bond formation in the cytoplasm but no significant differences in protein solubility could be seen. After many unsuccessful trials of ScFv purification and modifications to get soluble protein expression, the protein was purified from the inclusion bodies which have the advantage, first of all, that they can be isolated to high purity before purification and second, that they are protected from protease degradation. The main disadvantage, though, is that they require denaturation and subsequent refolding. A protocol was established that involved complete denaturation of inclusion bodies and a subsequent DEAE sepharose purification step. At pH 8, clone-9-ScFv is almost neutrally loaded and does not bind to the column while contaminants do. Thus, the ScFvs could be purified to almost 100% purity (Fig. 5.12).

5.2.2.3 Refolding and final purification of ScFvs

Once purified by DEAE sepharose chromatography, the ScFvs had to be refolded sinto their native structure in order to be functionally active. Refolding is an empirical process and can be achieved by several methods: by dialysis, dilution or when the target protein is bound to a column. All three methods were tried and will be shortly summarised below.

First, it was tried to refold by dialysis. Both, one- (8→ 0 M urea) and sequential-step (8→4→2→1→0.5→ 0 M urea) dialysis were performed but only using the latter, ScFvs could be, at least, partially refolded (Fig. 5.13). In the non reducing lanes, several bands are seen which correspond to monomeric but also dimeric and higher oligomeric, aggregated ScFv. To test whether the ScFv was refolded into a correct native state, it was tested for C3

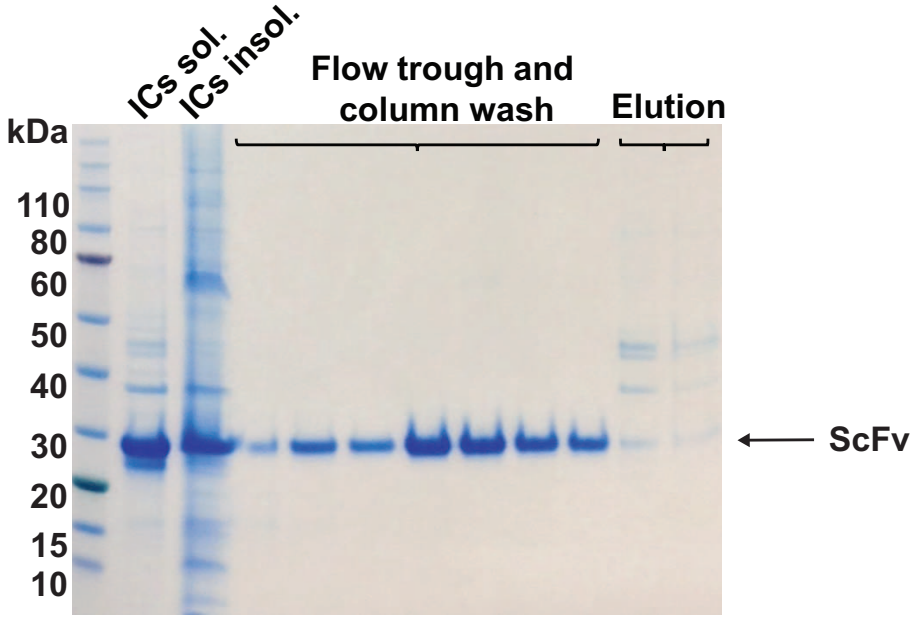

Fig. 5.12 SDS PAGE of ScFv after DEAE purification. Solubilised ICs were loaded onto a DEAE sepharose column. ScFv is almost neutrally loaded at pH 8 and is found in the flow trough and first washes. Elution with 1M NaCl removes bound contaminants from the column. Samples were loaded under reducing conditions.

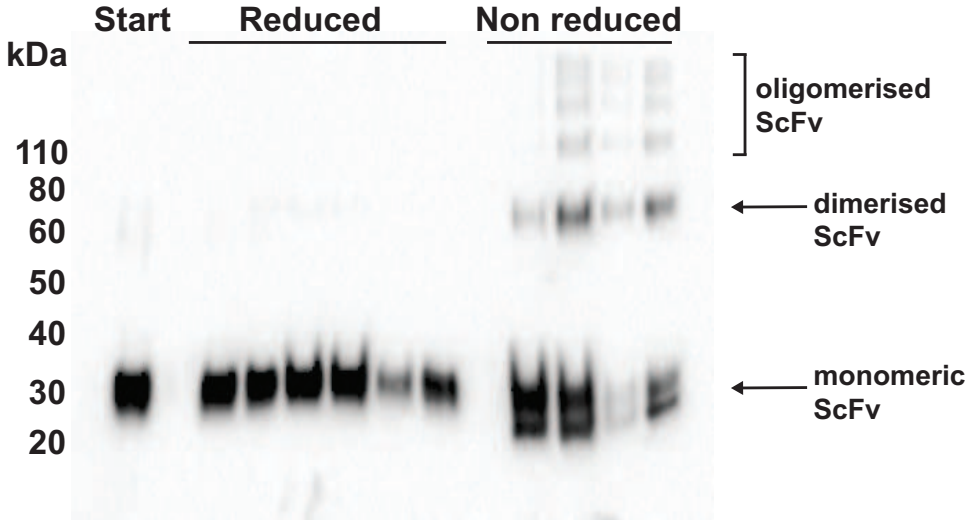

Fig. 5.13 Refolding by sequential step dialysis. Western blot after refolding and concentration. ScFv loaded reduced and non-reduced and detected with α-His 1:2000. Experiment was repeated twice.

binding. Using the sequential refolded batch in a western blot, C3 binding occurred but only very weakly. Therefore, the refolding protocol was further improved.

Since the ScFv could not be refolded to a higher yield, I thought that the C-terminal cysteine (for dimerisation of two ScFv) might cause havoc during refolding. There are two cysteines in both, the heavy and the light chain which have to form a disulfide bond in the native antibody protein and also the ScFv. The C-terminal cysteines on the other side which will be used for dimerisation of two ScFv with DBB, should stay unbound during the whole expression and purification process. In order to facilitate refolding, a new construct was made that has the C-terminal cysteine exchanged to a serine (S). The new construct is designated as ScFv-S. The amino acid change was achieved by a PCR reaction and a new reverse primer that would change the nucleotides of the cysteine to a serine (TGC→ACG, primers in Tab. A.5). It was also tested if this new construct would be directed to the periplasm but as before, the recombinant protein was mainly accumulated as insoluble inclusion body in the cytoplasm.

After the C→S mutation, the ScFvs can no longer be dimerised and therefore avidity will be reduced by half. To test whether the binding charaacteristics of the parental antibody are sufficient to ensure strong antigen recognition with only one Fv region, a Fab of clone 9 was prepared with a papain digest and biotinylated. Nevertheless, it was shown to behave exactly like the bivalent whole antibody molecule (Fig. 5.16b). A monomeric ScFv on clone 9 should therefore still bind strongly enough that it can be used as diagnosing antibody reagent. ScFv-S was used from now on because, theoretically, refolding should be achieved easier since no interfering cysteines that must remain unbound during refolding, are present. The ScFv was prepared and purified using a DEAE column as described before. All three refolding methods were tested again and it was found that refolding by dilution works best for refolding of the ScFv-S. Using the buffer recipes from the commercially available Athena QuickFold Protein Refolding kit, buffer 14 was able to provide the best refolding environment from all buffers not containing DTT. This buffer was modified further to optimize refolding. First, triton was exchanged to n-octyl-β- L-glucopyranoside and later to CHAPS, because these two detergents have a higher critical micelle concentration and can therefore be dialysed

out even at higher concentrations. Second, the redox-couple GSH:GSSH was found to be optimal at a ratio 1:5 instead 1:10. Third, the pH was adjusted to pH 8 at the temperature during the refolding process because S-S bonds form better at this pH. Fourth, an increase of the Tris buffer concentration from 50 to 100 mM was found to further improve refolding. Using this modified buffer composition, it was possible to refold the ScFv-S.

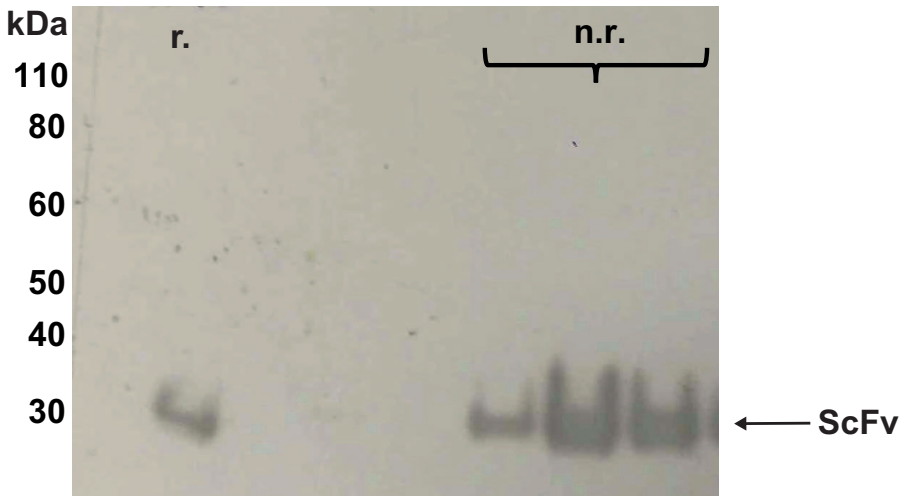

Fig. 5.14 Western blot of ScFv refolded by dilution. Protein was loaded reduced and non-reduced and detected with α-His 1:3000. Refolded protein can be detected by a single band in the non-reducing lanes. Experiment was performed three times.

The next step was to concentrate the refolded ScFv-S again (refolding was performed at a concentration of 10 μg/ml). The diluted protein could not be concentrated by binding to a nickel column because arginine interferes with binding to nickel. Both, an ammonium sulphate cut and amicon concentration was done but only the latter did not precipitate the ScFv-S. After concentration and dialysis, the protein was purified with a nickel column which has the advantage that it only binds if the His tag is freely available, i.e. only not aggregated protein. A western blot and silver staining were performed to confirm that ScFv-S was folded correctly, without forming aggregates (Fig. 5.14 and 5.15).

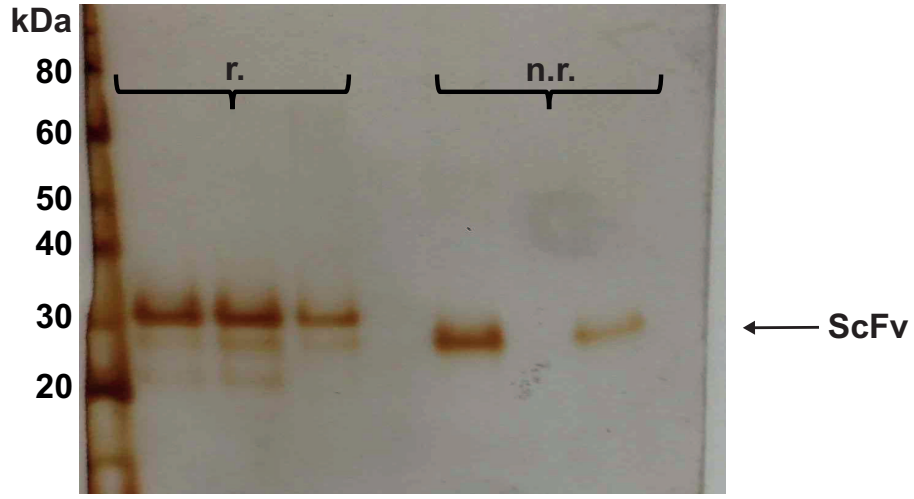

Fig. 5.15 Silver staining of refolded ScFv. Protein was loaded reduced and non-reduced and stained with silver nitrate. Refolded protein can be detected by a single band in the non-reducing lanes. Experiment was performed twice.

5.2.3 Functional testing of the ScFv-S antibody

The refolded ScFv-S was tested for C3 binding in a western blot. In order to demonstrate correct binding of the ScFv-S, the "mother" antibody clone 9 and clone 9-Fabs were used alongside the ScFv-S.

10 μl of 1% normal human serum were separated reduced and non-reduced by SDS PAGE and blotted. Next purified and refolded ScFv-S (5 μg/ml), clone 9-Fabs (2 μg/ml) or clone 9 (1 μg/ml) were added. Extravidin or α-His antibodies were used as secondary antibodies for detection.

The western blot showed that, as described in previous chapters, clone 9 reacts with the α-chain of C3 in reduced and non-reduced form and additionally with a band at $\approx$ 47 kDa (Fig. 5.16a). There is also a diffuse band around 60 kDa which is highly likely to be unspecific binding of antibody to albumin, the most abundant protein in serum. Likewise, the Fab also binds to the same C3 bands (Fig. 5.16b). The ScFv-S is shown to react with C3 as well (Fig. 5.16c). It strongly binds to the denatured α-chain of C3 and also reacts with the "47 kDa" fragment. Non-specific binding of antibody to BSA at $\approx$ 60 kDa is seen in both, clone 9 and the ScFv-S but also slightly with Fabs.

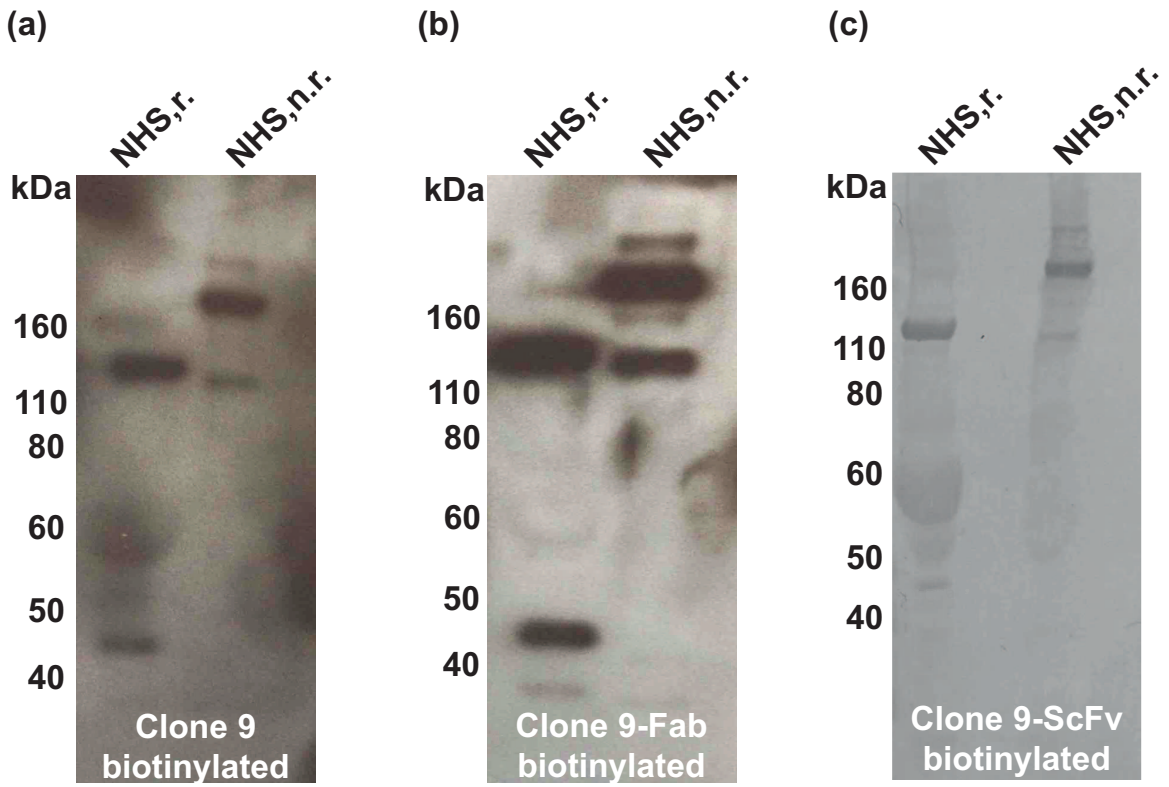

Fig. 5.16 Binding analysis of clone 9, Fab and ScFv-S. 1% NHS was loaded and detected with all three antibody formats that were bioyinylated (a, b and c).

5.2.4 Eukaryotic expression of ScFv-S

Because refolding of ScFv turned out to be slightly unpredictable, expression of ScFv in eukaryotic cells was also examined. By comparison of results from the amplification of the variable regions and N-terminal sequencing, it was possible to deduce the native signal peptide of clone 9. Primers were designed to add the appropriate restriction sites and the native signal sequence for cloning into pcDNA3.1 (primer sequences in Tab. A.5). The construct was cloned into pcDNA3.1/Hygro(+) and both HEK and CHO cells were transfected as described before. Unfortunately, ScFv expression could not be detected in any cell line which is probably caused by instability of the construct (further details in discussion).

5.2.5 Discussion

Age-related macular generation in a leading cause of blindness and also causes a great burden to our health system. It is important to prevent or at least slow down disease progress and a number of clinical trials could show positive results for either wet AMD (α-VEGF therapy) or geographic atrophy (α-FD antibodies). It is therefore necessary to quickly be able to monitor disease and/or therapy progress which justifies the development of a new diagnostic *in vivo* biomarker that could be applied intravenously and rapidly show inflammation in the retina. It would, although, not be limited to the retina but could also find application in diagnosis of complement-associated kidney diseases, provided the ScFv can be detected with e.g. an infra-red label. The intended application would nevertheless be detection of inflammation in various eye diseases. This biomarker is a ScFv antibody that binds to the complement activation products iC3b and C3dg. Clone 9 is an ideal candidate because it is highly specific and does not get consumed in the circulation by binding to C3. A ScFv of clone 9 is even better because the smaller a construct, the shorter the circulating half-life and the better the tissue penetration and also ScFvs do not have an Fc-tail that would cause further inflammatory reactions or effector functions.

5.2.5.1 Expression of a ScFv of clone 9

At the time clone 9 was generated in the early 1980ies, NSO cells which nowadays are commonly used for generation of hybridomas, were not yet available. The rat myeloma cell line Y3 Ag 1.2.3 was used as an immortal fusion partner for spleen cells of rats that have been immunized with purified human C3 [141].

Once the variable regions were amplified, several sequences were isolated, so the relevant chains had to be identified. Even though, hybridomas are considered to express “monoclonal” antibodies, they may encode more than one functional or even non-functional, rearranged abortive variable regions. They sometimes transcribe several variable sequences of which only a few are translated because of stop codons in the non-relevant chains [352]. Indeed, several sequences for the light chain could be identified which either belonged to the myeloma

fusion partner or the to B-cell which was fused. The right sequences and also the first amino acids of secreted clone 9 antibodies could finally be identified after N-terminal sequencing.

Bacteria were chosen as expression system because they have been described extensively before for ScFv production and since they do not process proteins post-translationally like eukaryotic cells, they fulfil all requirements for a diagnostic antibody fragment which should not have a long serum half-life due to increased retention because of glycosylation. Nevertheless, e.coli expression systems also have inherent drawbacks for the expression of eukaryotic proteins which be be discussed below.

One such problem is the lack of disulfide bond formation, although the periplasm of e.coli is known to provide an oxidative environment that enables formation of S-S bonds and therefore folding of proteins with cysteines. Recombinant proteins can be directed to the periplasm by addition of an N-terminal leader sequence which will be clipped off once the protein is translocated. In this project, periplasmic expression did not work as well as expected even though three different signal sequences were tested. Cellular fractionation showed that ScFv could be found in almost all fractions which could be either explained by limitations of the fractionation protocol or confirm that the ScFv is indeed found at several cellular locations. During peri- or spheroplasmic purification, osmotic pressure can disrupt the peptidoglycan layer which causes leakage of cytoplasmic proteins. More than 95% of expressed ScFvs were found as insoluble inclusion bodies in the cytoplasm. The presence of positively charged amino acids within 20-30 positions downstream of the signal peptide is thought to inhibit protein translocation to the periplasm [353] and both lysines and arginines are found in the clone 9-ScFv sequence. It is also reported that presence of a signal sequence on proteins is often toxic to a cell [354]. It is known that most eukaryotic proteins are insoluble in bacteria due to their inability to provide an oxidative milieu in the cytoplasm but some can be expressed as soluble proteins. Another factor is that eukaryotic proteins usually contain a high percentage of codons and amino acids that are less frequently or rarely used in e.coli [355, 356]. Codon optimization can have a great impact on solubility of ScFv [357] but in this project, although 10% were rare codons, the use of codon-optimized bacterial strains (i.e. Rosetta(DE3)LysS) did not improve expression of soluble ScFvs and mutagenesis to

exchange these rare codons was not performed. This is a further frequent limitation of ScFvs, in that on the one side, in e.coli they can be expressed cheaply and in large quantities, but on the other side, subsequent refolding into an active molecule can be time-consuming and is an important rate-limiting step. Especially for ScFvs, it is reported that they tend to expose hydrophobic patches on the surfaces of their *in vivo* folding intermediates which favours protein aggregation [358, 359]. It should not be generalized but often, ScFvs derived from hybridomas are more likely to be aggregated than those selected from phage displays which is most probably caused by prior enrichment and selection of soluble antibody fragments [357]. Thus, the majority of ScFvs expressed in bacteria have to be refolded *in vitro* after purification. A problem with generation of ScFvs is that every ScFv behaves differently because amino acid sequences can vary dramatically in the hyper-variable CDR regions. There is therefore no standard protocol for expression and refolding of ScFvs but it has to be optimized for every single ScFv. After testing various different protocols, refolding conditions could be established that promote, at least partially, formation of functionally active clone 9-ScFv-S antibody fragments.

In summary, a ScFv antibody fragment was generated that binds to complement iC3b and C3dg and can be used as an *in vivo* biomarker for detection of inflammation. In order to be a useful diagnostic tool, the ScFv has to be further optimised and expressed at a GMP-location. Improvement of the ScFv can be achieved by several ways such as optimization of the primary sequence, of refolding techniques or the expression system could be changed. Future work includes generation of a ScFv-Fc construct (or ScFv-hinge-C_H3 construct) which is known to enable stable expression of the construct in a eukaryotic expression.

Together with promising clinical trials in age-related macular degenerations, this diagnostic biomarker has the potential to greatly improve early disease diagnosis and therapy and thus improve quality of life of millions affected individuals.

Chapter 6

General discussion and conclusion

The complement system is an important defence system of our body and has various effector functions which are described in the introduction to this thesis (see Sec. 1.2). Complement activation at the right time and place is clearly of importance in host defence but also in normal physiological function. However, due to its enormous effector power, activation of complement in different situations can completely reverse its protective role, which can be seen in several immune, inflammatory, neurodegenerative, ischemic, and age-related diseases. The alternative pathway of the complement system consists of two competing cycles, the C3b amplification, a positive feedback loop to generate more C3b and the C3b breakdown cycle, an inhibiting mechanism that counterbalances the feedback loop by inactivation of generated C3b. As shown in Fig. 1.4, these two mechanism both have regulators that ensure proper balance and thereby homeostasis. Since the alternative pathway is continuously activated and maintained by the tickover, defective regulation at any step can have far-reaching consequences. The main aim of our overall project is down-regulation of the alternative pathway's feedback loop which has an important role in many pathogenic conditions, most importantly, in age-related macular degeneration. A steady increasing number of polymorphisms have been identified that confer increased risk to development of AMD (see Sec. 1.4.1). There are a few ways to manage uncontrolled alternative pathway activation which can be separated into either inhibition of C3b feedback amplification or promotion of the C3b breakdown cycle. The approach of this study is to increase the rate of

the C3b breakdown cycle into iC3b and C3dg. By increasing the plasma concentrations of the complement regulator Factor I, the feedback loop of the alternative pathway can be inhibited by cleavage and inactivation of C3b [112, 134, 135, 140]. Once cleaved, C3b can no longer take part in a C3 or C5 convertase and therefore in the feedback cycle or in terminal pathways of complement. Factor I also cleaves iC3b which is a main mediator of inflammation by its reaction with CR3 on (mainly) polymorphonuclear neutrophils. It should be stressed that under normal physiological circumstances, Factor I rapidly cleaves every C3b to iC3b that is not bound to a protected surface but the second cleavage to C3dg occurs only at a much slower speed, rendering iC3b the major opsonin of complement. Raising FI concentrations promotes fast iC3b cleavage in a dose-dependent manner [112].

6.1 General discussion

Age-related macular degeneration is the leading cause of blindness in the western world and affects $\approx$ 10% of 70 years old and its prevalence increases to almost 30% in 75-85 years old [308, 309]. The disease does not only affect the daily life of patients but also poses an enormous burden on the health system. Complement components were found in subretinal drusen, the hallmark of AMD [318, 21], and soon after that, many polymorphisms in Factor H predisposing to AMD were identified in genome-wide association studies (GWAS) [113–116]. MAC deposition is known to occur in the choriocapillaris but also in Bruch's membrane of affected individuals [338, 339]. Since this also occurs in unaffected individuals, presence of MAC demonstrate that there is constant complement activation and attack in the choriocapillaris. It is assumed that AMD is the result of long lasting low grade complement activation and inflammation. It is therefore possible that complement and/or drusen deposits are also found in other parts of the body but go unrecognised since they do not interfere with an important function such a central vision. Polymorphisms in genes that are involved in the feedback mechanism demonstrate that strict control of the alternative pathway is of utmost importance to keep homoeostasis and to prevent or slow down development of AMD.

Since, there is no perfect AMD mouse model, in this thesis, the effect of raising concentrations of complement Factor I was examined in another disease setting which depends on alternative pathway activation: a mouse model of renal ischemia reperfusion injury. Ischemia reperfusion is an inevitable and injurious event in clinical conditions such as infarction, sepsis and solid organ transplantation and occurs after insufficient local blood supply. It is believed that depletion of ATP, the cellular energy, is the initiating cause of cellular injury during ischemia. Once circulation is restored, oxygen and other substrates which are required for tissue regeneration and restoration of energy levels, are provided again. But reperfusion also exacerbates tissue injury and initiates a series of inflammatory events [187]. These include secretion of reactive oxygen species (ROS), pro-inflammatory cytokines and chemokines, recruitment of leukocytes and activation of the complement system. The exact activation mechanism of the complement system in renal ischemia reperfuion injury is still controversial but it is clear that during the reperfusion period, complement exacerbates local inflammation and tubular injury. Hence, its inhibition or dampening seems logical in reducing injury. Earlier studies of mouse renal ischemia models pointed towards a major role of the alternative pathway in complement-mediated damage, however recent studies highlight the role of the lectin pathway using CL-11 as recognition molecule and MASP-2 as associated protease that activates complement. In the study presented in this thesis, FI was shown to ameliorate renal damage and greatly reduce IRI-associated mortality (50% reduction in mortality). Nevertheless, it should be emphasised that this study was designed as a pilot study to explore a potential FI-based therapy and therefore only included a very small number of mice per group. For significant and publishable results, this promising pilot study has to be repeated. An explanation that Factor I treated mice do not show complete protection could be that recombinant Factor I only incompletely inhibits the alternative pathway due to reduced *in vivo* activity. Moreover, it also highlights the fact that the alternative pathway does not play the only role in renal IRI but that inhibition of the feedback cycle reduces at least some of the damage. It should be stressed that reduced activity or degradation of the injected recombinant protein are always possibilities which could limit its therapeutic effect. A combination therapy consisting of MASP-2 inhibition and administration of Factor I would

highly likely reduce complement-mediated damage to a minimum and should therefore be explored.

Elevated FI concentrations can be achieved using purified protein from plasma fractionation, recombinant techniques or achieved by *in vivo* expression. Since Factor I is a minor component of plasma, much mouse plasma would be needed for plasma fractionation so it was decided to prepare recombinant mFI. A gene therapy abrogates many of the problems associated with plasma purification or recombinant protein production. In order to find out how much FI increase can be achieved by *in vivo* over-expression in hepatocytes, an AAV based expression plasmid harbouring the mouse Factor I cDNA sequence was used. It was found that using this expression system, Factor I concentrations are titratable and can be increased up to 4x which is a lot more than is presumably needed in order to achieve a therapeutic effect (at least *in vitro* [140]).

Another issue that should be addressed is a discussion of potential disadvantages of a therapy that involves increase of Factor I plasma concentrations by injection or gene therapy. Inhibition of the complement system always carries the risk of increased infection especially *Neisseria*, which have to be killed by lysis. Regarding other pathogens, it is known that complement is essential for their control only in the first few years of life but later in life, antibodies have been developed that usually quickly opsonise common pathogens. Mice injected with AAV_mFI were not kept in special pathogen free environments and did not show any signs of infection, although the duration of the study was only 8 weeks. Also, mice transgenically expressing soluble Crry were not more prone to get infections and were protected from antibody-induced glomerular injury [360]. A gene therapy that increases FI levels is unlikely to have detrimental side effects because first of all, it only increases the concentration of a normal plasma protein and second, does not inhibit complement recognition and opsonisation of pathogens by the classical and lectin pathway. While conserving complement opsonisation, the inflammatory effects that cause damage in many chronic diseases are reduced.

The third part of this thesis describes the generation of a biomarker for detection of complement activation products in early dry AMD. The justification for this project is that

currently, there is no fast and easy way to assess therapy progress in an AMD clinical trial. The current FDA requirements are that the rate of areas of geographic atrophy must be reduced and vision must improve during a 18-month period which results in very long trials. A solution to this would be to measure inflammatory products directly as opposed to their effects, i.e. measure the level of C3 deposition in the retina vs. reduction of atrophic areas. The chosen target for this approach are the C3 cleavage products iC3b and C3dg which are known to be the longer-lasting opsonins, as opposed to C3b which is quickly cleaved by Factor I. An antibody to complement C3g which does not react with native C3 but selectively reacts with iC3b/C3dg and is fluorescently labelled would have all required properties for a biomarker. ScFv molecules are much smaller than IgGs, are able to quickly cross the blood-eye barrier and also have a much shorter half-life. These small mini-antibody constructs should be cleared at first pass and are therefore unlikely to be antigenic. However, it would be necessary to prepare these constructs to good manufacturing practices (GMP) which cannot be achieved using normal laboratory standards. The generation of such a biomarker has been achieved and *in vitro* has the same binding characteristics as its mother antibody, an α-human C3g antibody (= clone 9).

In order to succeed with a successful therapy and/or prevention of early dry AMD, further work is still required with respect to all three projects presented in this thesis. With regards to a Factor I therapy for prevention of AMD, a first clinical trial would most likely consist of a small group of patients which are given plasma purified Factor I, the easiest source available for a first small scale clinical trial. On the larger scale, a gene therapy would be much more economical and feasible because first of all, repeated injections and the risk of blood-borne infections would thereby be avoided and second, increased Factor I concentrations presumably not only ameliorate AMD pathogenesis but also other age-related conditions with a chronic inflammatory background and a gene therapy would ensure a continuous steady expression of Factor I. With the current speed of research progress in the gene therapy field, it is highly likely that in the near future, there are greatly improved vectors for expression of various proteins in human. The generated biomarker for iC3b deposition will also be of great use in the diagnosis of early AMD and its follow-up after

therapy. Nevertheless, until it is of clinical use, the protocol for the α-C3g-ScFv has to be simplified and optimized to increase the yield of correctly folded and active antibody. Since every ScFv consists mainly of the variable regions which by definition are different in every ScFv, there is no standard protocol for ScFv refolding and therefore it seems most logical to completely avoid *in vitro* refolding but use the inherent capacities of mammalian cells to refold the ScFvs. Thus, the expression system will be changed to a eukaryotic one which should theoretically overcome the problems of refolding the synthetic protein. Also, in the near future, the ischemia reperfusion injury mouse model will be repeated using the AAV_mFI expression system which first of all confirms previous results using recombinant Factor I but also further tests these transgenically engineered mice.

6.2 Conclusion

Since the beginning of the work for this thesis in January 2012 many new associations between uncontrolled alternative pathway activation and various diseases have been drawn. Most importantly, low levels of Factor I were shown to be a high risk factor for development of early advanced AMD [119] which not only isolates a group of potential patients for a Factor I therapy but also highlights the important role of FI's effector functions and iC3b in AMD. In the course of my PhD studies, I collaborated with *in vitro* work showing that relatively modest Factor I increase converts serum from at-risk genotypes to behave like serum from a protected genotype (results published in [112, 140]). The positive effects of increased Factor I concentrations are not limited to AMD but should presumably also improve overall outcome of other diseases or conditions, such as ischemia reperfusion injuries, strokes, C3 glomerolupathies, Alzheimer's disease, ...

A Factor I therapy has the potential to revolutionize therapies for conditions with underlying acute and chronic complement activation. In order to avoid repeated injections, continuous over-expression of Factor I by a gene therapy would be the way to go and also reduce the risk of infection or transmission of blood-borne viruses (in case of plasma purified Factor I). Nevertheless, a Factor I treatment does not treat the underlying cause of the disease

nor inhibit complement activation in the first place but ameliorates inflammatory effects by inhibiting complement amplification.

Similarly, the biomarker for iC3b deposition described in this dissertation would also change the current state of the art in AMD diagnosis and treatment. Together with better knowledge of at risk genotypes and the early detection of retinal changes, AMD treatment could start at a much earlier time point and aim to prevent disease progression to a stage that disables or at least prolongs outbreak of late-stage disease.

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Appendix A

Supplementary

A.1 Primer sequences

Primer name	Sequence
FI-F	5´- CAT CAT GGA TCC GCC ACC ATG AAG CTC GCT CAT CTC AGT - 3´
FI-R	5´- ATG ATG GCG GCC GCT CAA TGA TGA TGA TGA TGA TGG CCG GAG CCG GAG ACA TTG TGT TGA GAA ACA AG - 3´
FI-AAV-F	5´- TCT AGA GGA TCC GCC ACC ATG AAG CTC - 3´
FI-AAV-R	5´- AAG CTT GGC GGC CGC TCA GAC ATT GTG TTG AGA AAC AAG AGA CCT TC - 3´

Table A.1 Primers for expression of mouse complement Factor I

Primer name	Sequence
HC-F-1	5’ - ATG GAC ATC AGG CTC AGC TTG GCT TTC CTT GTC CTT CTC - 3’
HC-R-1	5’ - TCT TGT ACA GTA ATA AGT GGC CG - 3’
Lambda-F-1	5’ - ATG ACA TGG ACT CTA CTA TTC CTT - 3’
Lambda-1-R-1	5’ - GAA GAG CTG TCA ATG GAG C - 3’
Lambda-2-R-1	5’ - GTG TGG CTT TGT TTC CCT GG - 3’

Table A.2 Primers for ScFv PCR 1 - Amplification of immunoglobulin genes

Primer name	Sequence
HC-F-PelB	5’ - AAA CAT ATG AAA TAC CTG CTG CCG ACC GCT GCT GCT GGT CTG CTG CTC CTC GCT GCC CAG CCG GCG ATG GCC ATG GAC ATC AGG CTC AGC TT-3’
HC-R-2	5’ - CGC CGC TGC CGC CGC CGC CGC TGC CGC CGC CGC CTC TTG TAC AGT AAT AAG TGG CCG TGT - 3’
Lambda-F-2	5’ - GGC GGC GGC GGC AGC GGC GGC GGC GGC AGC TAT GAG CTG ATC CAA CCA CC - 3’
Lambda-2.1-R-2	5’ - AAG CTT CAG TGG TGG TGG TGG TGG TGG CTG CCC CTG GGC ACC AGG CAG CCG CTG CCG CTA GTG TGG GAG TGG ACT TGG - 3’
Lambda-2.2-R-2	5’ - AAG CTT CAG TGG TGG TGG TGG TGG TGG CTG CCC CTG GGC ACC AGG CAG CCG CTG CCG CTG AGT GTG GGA GTG GAC TTG GGC - 3’

Table A.3 Primers for ScFv PCR 2 - Amplification of variable regions

Primer name	Sequence
ScFv-F	5’ - AAA CAT ATG AAA TAC CTG CTG CCG ACC G - 3’
ScFv-R	5’ - AAA AAG CTT CAG TGG TGG TGG TGG TGG TG - 3’

Table A.4 Primers for ScFv PCR 3 - Overlap Extension

Primer name	Sequence
ScFv-DsbA-F	5’ - AAA CAT ATG AAA AAG ATT TGG CTG GCG CTG GCT GGT TTA GTT TTA GCG TTT AGC GCA TCG GCG GCG GAG GTG CAG CTG GTG GAG TCT - 3’
ScFv-OmpA-F	5’ - AAA CAT ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAG GTG CAG CTG GTG GAG TCT - 3’
ScFv-native Sign.-F	5’ - CAT GCT AGC ATG GAC ATC AGG CTC AGC TTG GCT TTC CTT GTC CTT CTC ATA AAA GGT GTC CAG TGT GAG GTG CAG CTG GTG GAG T - 3’

ScFv-C→S-R	5’ - TTT AAG CTT CAG TGG TGG TGG TGG TGG TGG CTG CCC CTG GGC ACC AGG CTG CCG CTG CC - 3’

Table A.5 Primers for ScFv 4 - Changing of signal sequences

A.2 Buffers

General buffers

Tris buffered saline (TBS)
50 mM Tris
150 mM NaCl
pH 8

Phosphate buffered saline (PBS)
PBS tablets (Gibco)

Molecular biology

Transformation buffer 1
100 mM RbCl
10 mM $CaCl_2$
50 mM $MnCl_2$
30 mM CH_3COOK
15% v/v glycerol

Transformation buffer 2
10 mM MOPS
10 mM RbCl
75 mM $CaCl_2$
15% v/v glycerol

SDS PAGE and Western Blot

SDS Running Buffer
500 mM MES Hydrate
500 mM Tris-Base
10 mM EDTA
35 mM SDS

Coomassie staining buffer
1.2 g Coomassie brilliant Blue
50% methanol
10% acetic acid glacial (v/v)
40% H_2O

Destaining buffer
45% methanol
10% acetic acid glacial (v/v)
45% H_2O

Transfer Buffer
25 mM Tris-Base
200 mM Glycine
20% MeOH

Blocking Buffer
5% Marvel (= skimmed milk powder) in PBS-
0,05% Tween (= PBS-T).

Wash Buffer:
1:10 dilution of blocking buffer in PBS-T

Final Wash Buffer
TBS-0.05% Tween (= TBS-T)
pH 8

ECL substrate
Stocks of 90 mM Coumaric acid and 250 mM Luminol, diluted in DMSO, store at -20°C. Mix solutions A and B only immediately prior to developing.

solution A
40 μl Luminol stock
18 μl Coumaric acid stock
200 μl 2 M Tris, pH 8.5
3.8 ml dH_2O

solution B
10 μl H_2O_2
200 μl 2 M Tris, pH 8.5
3.8 ml H_2O

Silver staining

Developer
3% sodium carbonate
600 μl formalin
200 μl H_2O

Farmer's reducer
0.8 g sodium thiosulphate USP
0.5 g potassium ferricyanide
50 ml H_2O

ELISA

Coating buffer
15 mM Na_2CO_3
35 mM $NaHCO_3$
pH 9.6

Wash buffer
10 mM Tris-HCl
140 mM NaCl
0.05% Tween-20
pH 7.4

Complement fixation buffer

Complement fixation diluent (CFD)
tablets (Oxoid), fill up with sterile, endotoxin-free water.

Veronal buffered saline (VBS)
145 mM NaCl
2.6 mM Sodium Barbitone
12.3 mM Barbital
pH 7.3

Alternative pathway complement fixation buffer
1x CFD or VBS
10 mM EGTA
2 mM $MgCl_2$
Fill up with sterile, endotoxin-free water.
Always prepare freshly.

Protein expression and purification buffer

Periplasmic extraction buffer
100 mM Tris
1 mM EDTA
20% sucrose
2 mM PMSF
pH 8

Spheroblast extraction buffer
100 mM Tris
5 mM $MgCl_2$
2 mM PMSF
pH 8

Bacterial resuspension buffer
100 mM Tris
2 mM PMSF
1 tablet protease inhibitor cocktail (Roche)
pH 8

IM solubilisation buffer
100 mM Tris
1% Triton X-100
10 mM $MgCl_2$
2 mM PMSF
pH 8

Pellet wash buffer
100 mM Tris
2 M Urea
1 mM EDTA
2%Triton X-100
pH 8

Inclusion body solubilisation buffer
50 mM Tris
8 M Urea
10 mM DTT
pH 8

Chromatography

IMAC wash buffer
20 mM Phosphate
500 mM NaCl
20 mM Imidazole
pH 8

IMAC wash buffer
20 mM Phosphate
500 mM NaCl
250 mM Imidazole
pH 8

Refolding Buffers

A.3 Refolding Buffers

Buffer	Ingredients
1	50 mM MES pH 6, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M guanidine-HCl, 0.5% Triton X-100, 1 mM DTT
2	50 mM MES pH 6, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
3	50 mM MES pH 6, 9.6 mM NaCl, 0.4 mM KCl,1 mM EDTA, 0.4 M sucrose, 0.75 M guanidine-HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM DTT
4	50 mM pH 6, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.5% Triton X-100, 1 mM GSH, 0.1 mM GSSH
5	50 mM MES pH 6, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.4 M sucrose, 0.75 M guanidine-HCl, 1 mM DTT

6	50 mM MES pH 6, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
7	50 mM MES pH 6, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M guanidine-HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT
8	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
9	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M guanidine-HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT
10	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M guanidine HCl, 1 mM GSH, 0.1 mM GSSH
11	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT
12	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
13	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M guanidine-HCl, 0.05% Triton X-100, 1 mM DTT
14	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M guanidine HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
15	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 1 mM DTT

Table A.6 Buffers - refolding by dilution

The rAAV delivery system

2x Hepes-buffered saline (2X HBS)
280 mM NaCl
50 mM HEPES
pH 7.14, autoclave.

Calcium Phosphate solution
0.15 M Na_2HPO_4
pH 7.1, autoclave

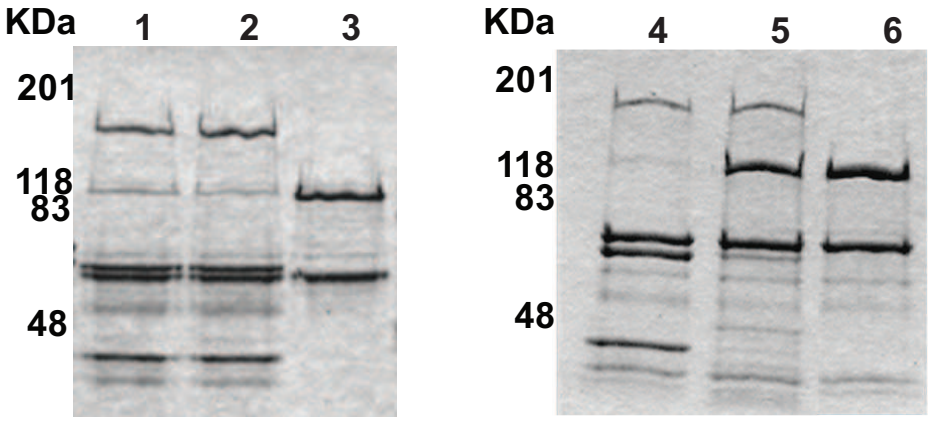
Saturated ammonium sulphate solution
530 g $(NH_4)_2SO_4$ in 1L water.
pH 7.0
Filter sterilise using a 0.45 μm cellulose filter (Nalgene).

Cesium chloride solution 1.37 g/ml in PBS
Dissolve 50 mg of CsCl in PBS (with Calcium and Magnesium; pH 7.5) and adjust to 100 ml.
Weigh 1 ml of the solution to check the density.
Filter sterilise through a 0.2 μm filter.

Cesium chloride solution 1.5 g/ml in PBS
Dissolve 33.75 g of CsCl in PBS (with Calcium and Magnesium) and adjust to 50 ml.
Weight 1 ml of the solution to check the density.
Filter sterilize through a 0.2 μm filter.

Dialysis Solution (TMN buffer) for *in vivo* work
10 mM Tris
100 mM NaCl
2 mM $MgCl_2$
pH 8.0
fill up with autoclaved water and filter through 0.2 μm filter.

A.4 Species specificity of Factor H and I in human and mouse serum



1. Mouse C3b + mouse FH + human FI
2. Mouse C3b + human FH + human FI
3. Mouse C3b
4. Human C3b + human FH + human FI
5. Human C3b + mouse FH + human FI
6. Human C3b

Fig. A.1 Species specificity of Factor H and I. C3b *in vitro* cleavage assay using purified components. Figure provided by Professor Peter Lachmann.

Appendix B

List of abbreviations

3MC	Malpuech, Michels and Mingarelli-Carnevale syndromes
aa	Amino acid
AAV	Adeno-associated virus
AD	Alzheimer's disease
aHUS	Atypical hemolytiv uremic syndrome
ALT	Alanine aminotransferase
AMD	Age-related Macular Degeneration
AP	Alkaline Phosphate or alternative pathway
Arg (or R)	Arginine
AS	Ammonium sulphate
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate
BBS	Barbital Buffer Saline
BGH Poly A	Bovine growth hormnone polyadenylation signal
bp	Base pair
BSA	Bovine Serum Albumin
C1-INH	C1-Inhibitor
C3aR	C3a Receptor
C4BP	C4-bBinding Protein
C5aR	C5a Receptor
Cap	Capsid
CCP	Complement Control Protein
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary
CL-11	Collectin-11

CP	Classical pathway
CPN	Carboxypeptidase N
CR	Complement Receptor
CRIg	Complement Receptor of the Immunoglobulin superfamily
CRP	C-reactive protein
Crry	Complement receptor 1–related protein y
CUB	Complement C1r/C1s, Uegf, Bone morphogenetic protein 1
CV	Column volume
DAF	Decay Accelerating Factor
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EA	Antibody sensitised erythrocytes
EA1423	EA opsonised with complement components C1, 4, 2 and 3
e.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol-bis-N,N,N,N-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FB	Factor B
Fc	Fragment crystallizable
FD	Factor D
FH	Factor H
FHL-1	Factor H-like Protein 1
FHR	Factor H-related protein
FI	Factor I
FI	Framework
GA	Geographic atrophy
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GMP	Good manufacturing practice
GPI	Glycosyl phosphatidylinositol
GWAS	Genome-wide association studies
h	Human
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High Fidelity
His	Histidine

HRP	Horse Radish Peroxidase
HSV	Herpes Simplex Virus
HUS	Hemolytic Uremic Syndrome
Ig	Immunoglobulin
IPTG	Isopropyl β- D-1-thiogalactopyranoside
LB	Luria-Bertani
LEA	Lectin pathway Effector Arm
LP	Lectin pathway
LTA	Lipoteichoic Acid
m	Mouse
mAB	Monoclonal antibody
MAC	Membrane Attack Complex
MAp	MBL-associated protein
MASP	MBL-Associated Serine Protease
MBL	Mannan Binding Lectin
MCP	Membrane Cofactor Protein
MPGN II	Membranoproliferative Glomerulonephritis Type II
NEF or C3-NEF	C3 nephritic factor
NHS	Normal Human Serum
OD	Optical Density
ORF	Open reading frame
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNH	Paroxysmal Nocturnal Haemoglobinuria
PTEC	Proximal epithelial tubular cell
R3	Guinea pig serum depleted of C3
RBC	Red blood cell
Rep	Replication
RPE	Retinal pigment epithelium
s.c.	Sub cutaneously
ScFv	Single-chain variable fragment
sCR1	Soluble Complement receptor 1
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Ser (or S)	Serine

SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SP	Serine protease
SPA	Surfactant protein A
SRCR	Scavenger Receptor Cysteine-Rich
TAE	Tris-base/Acetic acid/EDTA
TBS	Tris Buffered Saline
TBST	TBS Tween-20
Tfb	Transformation buffer
TNF-α	Tumour Necrosis Factor α
UTR	Untranslated region
WPRE	Woodchuck hepatitis post-transcriptional regulatory element
WT	Wild Type
Xgal	5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside

Table B.1 List of abbreviations

Appendix C

Already published papers that are in partial fulfilment of my PhD project

1. Further studies of the down-regulation by Factor I of the C3b feedback cycle using endotoxin as a soluble activator and red cells as a source of CR1 on sera of different complotype

   Lachmann, P J, Lay E., Seilly D., Buchberger A., Schwaeble W., Khadake J.

   Clinical and Experimental Immunology, 2015 CEI-2015-5577.R1

2. Complotype affects the extent of down-regulation by Factor I of the C3b feedback cycle in-vitro.

   Lay E., Nutland S., Smith J.E., Hiles I., Smith R.A., Seilly D.J., Buchberger A., Schwaeble W., Lachmann P.J.

   Clinical Experimental Immunology, 2014, doi:10.1111/cei.12437.

3. Low-dose recombinant properdin provides substantial protection against Streptococcus pneumoniae and Neisseria meningitidis infection

   Youssif Mohammed Ali, Azam Hayat, Bayad Mawlood Saeed, Kashif S. Haleem, Saleh Alshamrani, Hany I. Kenawy, Viviana P. Ferreira, Gurpanna Saggu, Anna Buchberger, Peter J. Lachmann, Robert B. Sim, Dimitrios Goundis, Peter W. Andrew, Nicholas J. Lynch, and Wilhelm J. Schwaeble

   PNAS 2014 111 (14) 5301-5306, doi:10.1073/pnas.1401011111

4. Therapeutic application of recombinant properdin confers a significant degree of protection during Neisseria meningitidis infection

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