

Chapter 2

Complement Regulators and Inhibitors in Health and Disease: A Structural Perspective

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Abstract The complement system is an important effector within the innate immune system as a defence against pathogens and maintaining homeostasis. Detection of pathogen- and damage-associated molecular patterns triggers the proteolytic cascade in complement. In healthy self-tissues effector proteins are tightly controlled by proteins acting as regulators of complement activation, and absence or malfunction of these regulators contribute to pathogenesis in a number of disease conditions in humans. Complement is highly relevant to nanomedicine due its role in adverse reactions on polymers and nanoparticle drug carriers, but also since complement hyperactivation contributes to pathogenesis in many disease conditions that are frequently addressed within nanomedicine. We review here the regulatory mechanisms that modulate complement activation and some of the most prominent cases linking complement dysregulation/deficiencies to pathogenesis as well as the strategies that have been considered for the development of therapeutic complement inhibitors and modulators to alleviate complement-mediated detrimental effects. In addition, this chapter summarizes the wealth of strategies adopted by pathogens to evade complement, such as inhibition of the proteolytic cascade, degradation of complement effector molecules and interference with transmembrane signaling by effectors, and highlights how structural and functional insight into their mode of function now provides leads for the development of novel complement therapeutics.

Keywords Innate immunity • Complement • Structural biology • Therapeutics

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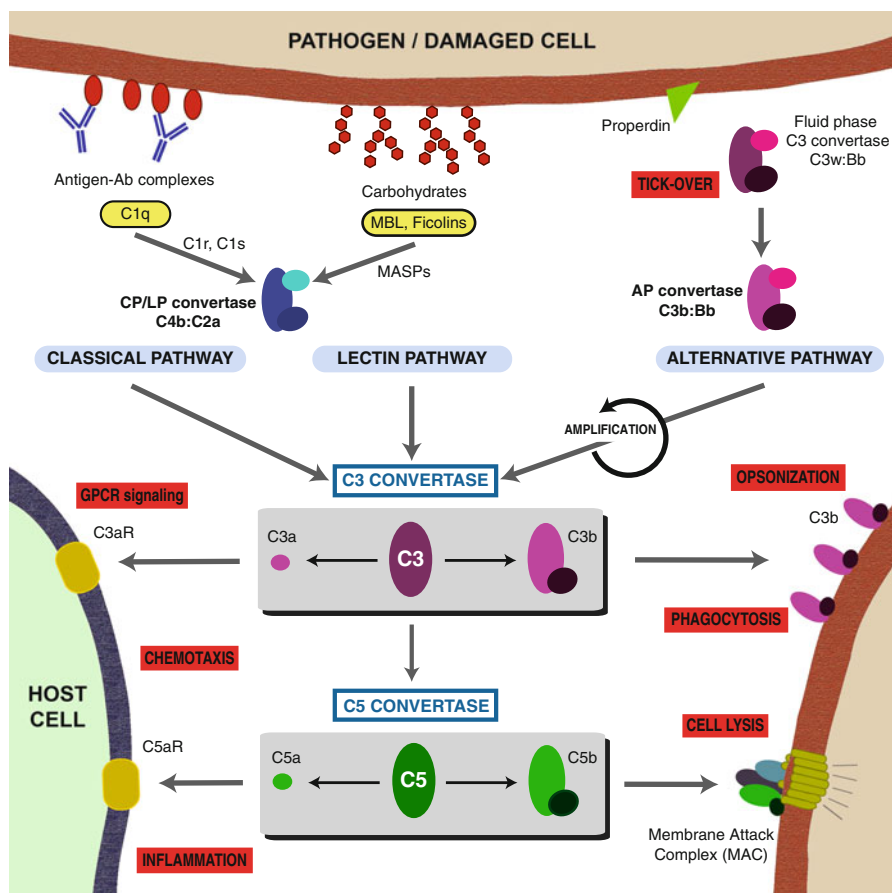


Fig. 2.1 General overview of the complement cascade emphasizing the initiation of the proteolytic cascade upon pattern recognition and the effector molecules acting on both host cells and pathogen/damaged cells

2.1 Introduction to Complement and Associated Diseases

2.1.1 Overview of the Complement Cascade

Complement is one of the major effectors of the innate immune system and is in the first line of defence against invading pathogens. Complement not only protects against infectious organisms, but also disposes of immune complexes, products of inflammatory injury and bridges the innate and the adaptive immunity [1–3]. Complement is a complex network of more than 50 circulating and membrane-bound proteins that can be activated through three different pathways: the classical (CP), lectin (LP) and alternative (AP) pathways (Fig. 2.1). The classical pathway is

initiated upon recognition by the C1 complex of antibody (IgG or IgM):antigen complexes or pentraxins (CRP, PTX3 and SAP) binding directly to activators [4, 5]. The C1 complex consists of the pattern-recognition molecule C1q and the associated serine proteases C1r and C1s that are activated upon pattern recognition [6]. C1s then initiates the cascade by proteolytic cleavage of C4 into C4a and C4b. Through an internal reactive thioester C4b is covalently linked to the activator [7], and C2 subsequently joins to form the proconvertase C4bC2, which is cleaved, also by C1s, resulting in the appearance of the CP C3 convertase C4bC2a [8]. The lectin pathway is similar to the CP but differs in the molecular patterns activating it. The recognition is achieved by mannan-binding lectin (MBL), ficolins, and collectin-11 binding to glycan moieties of a variety of glycoproteins and glycolipids specific to pathogens (bacteria, viruses and fungi) and damaged self [9, 10]. The pattern recognition molecules are associated to serine proteases MASP-1 and MASP-2, and recent work has established that MASP-1 may autoactivate and then cleaves MASP-2 resulting in an activated MASP-2 which can cleave C4. Further processing of the proconvertase may then be carried out by either MASP-1 or MASP-2 [11, 12].

C3 convertases are proteolytic complexes able to cleave the central complement component C3 into C3a and C3b [13]. The anaphylatoxin C3a recruits immune cells to the site of infection and initiates an acute inflammatory response [14] whereas C3b is the major opsonin of the complement system. Like C4b, it covalently attaches to the activator through its thioester [15] thereby “tagging” foreign and altered-self objects leading to opsonization. This also leads to initiation of the alternative pathway as activator-bound C3b recruits and binds factor B which is then cleaved by factor D, yielding the AP C3 convertase C3bBb [16]. The C3 convertase in turn generates more C3b from C3 and in this way creates a powerful amplification loop that accounts for 80–90 % of the outcome in the terminal pathway (see below) when complement is activated through the CP [17] or the LP [18]. However, AP activation may also occur spontaneously upon hydrolysis (tick-over) of the thioester bond within C3 generating a water-reacted molecule, C3(H₂O) capable of forming the fluid-phase C3 convertase [19], which can then be stabilized on microbial surfaces and apoptotic/necrotic host cells by properdin [20].

The C3 convertases may recruit a second molecule of C3b yielding the AP C5 convertase C3bBbC3b or the CP C5 convertase C4bC2aC3b [21]. These cleave C5 into C5a and C5b thereby initiating the terminal pathway (TP) [22]. The C5a anaphylatoxin signals through the G-protein coupled receptor, C5aR, and initiates inflammation (see below). C5b is devoid of the thioester and does not attach to activating surfaces but instead binds to C6, C7, C8 and multiple copies of C9, forming the (C5bC6C7C8)C9_n complex known as the membrane attack complex (MAC) [23]. Active MAC is able to insert into membranes and form pores resulting in cell lysis. Although MAC is potentially a powerful weapon against invading pathogens, deficiencies in the TP proteins primarily leads to *meningococci* infections [24].

2.1.2 Complement Regulation

Obviously, uncontrolled C3b deposition causes inflammation and cytolysis. For this reason complement has to be tightly regulated on healthy tissues. Host cells express cell-surface and soluble regulators (Fig. 2.2). Low concentration/absence or mutations in these proteins are at the heart of pathogenesis when complement is involved. The serpin C1 esterase inhibitor (C1-INH) blocks the C1 complex and also the LP proteases MASP-1 and MASP-2 [25], but it is not specific to complement as it also targets plasmin, thrombin, factor Xa and kallikrein. In the AP, most regulators function at the level of the C3 convertases by stimulating their dissociation (decay acceleration activity) or by promoting the proteolytic degradation of C3b into iC3b by the serine protease factor I [26] (co-factor activity). iC3b cannot associate with factor B and is thus irreversibly unable to form the C3 convertase. iC3b may be further degraded into C3dg and finally C3d by factor I and plasmin [27].

Factor H (fH) is the major AP regulator and exhibits both decay and co-factor activity for both C3bBb and C3(H₂O)Bb C3 convertases [28]. A pathogen cell opsonized with C3b but not capable of stabilizing fH binding will bind fH weakly preventing inactivation of C3b, whereas a non-activating host cell presenting the appropriate glycosaminoglycans will associate efficiently with fH yielding protection through C3b degradation [29]. fH consists of 20 complement control protein (CCP) domains (Fig. 2.2a). X-ray crystallography and NMR have deciphered the three-dimensional structures of all fH CCP domains but CCPs 9, 14 and 17, while full length fH has been studied by solution scattering [30–34]. Most of the structure-function research has focused on CCPs 1–4 and 19–20 binding C3b, and CCPs 6–8 and 19–20 associating with self-surfaces [34–36].

There are six other proteins related to fH that bind to C3b or C3d, the fH-like protein 1 (a splice variant of fH) and five fH-related proteins (CFHR1–5). All these are also composed entirely of CCP domains with different degrees of sequence identity with fH (Fig. 2.2a). CFHR1 can associate into a homodimer as well as into a heterodimer with CFHR2 or CFHR5 [37]. CFHR1 inhibits the C5 convertase as well as MAC formation [38] by binding to C3b through its N-terminal homodimer-forming moiety, but CFHR1 lacks both co-factor and decay activity [39]. CFHR2 inhibits the formation of the C3 convertase [40] but does not compete with fH for the binding to C3b [40]. CFHR3 competes with fH for the binding to C3b but its role in complement control is not settled [41]. The activity of CFHR4 is also unclear, but it binds C3b and has cofactor activity. CHFR5 possesses both cofactor and decay activities and is also recruited to damaged self-surfaces [42].

C4b-binding protein (C4BP) is a large plasma-circulating glycoprotein. The major form of C4BP consists of seven identical α chains and one β chain, both consisting of CCP domains [43]. The chains are connected by disulfide bridges and the structure is spider-like with seven elongated subunits attached to a relatively small central body [44]. Each α chain contains a binding site for C4b. Once bound to C4BP, C4b serves as a substrate for factor I. C4BP can also act as cofactor in factor I-mediated proteolysis of C3b, although fH and CR1 (see below) are more efficient

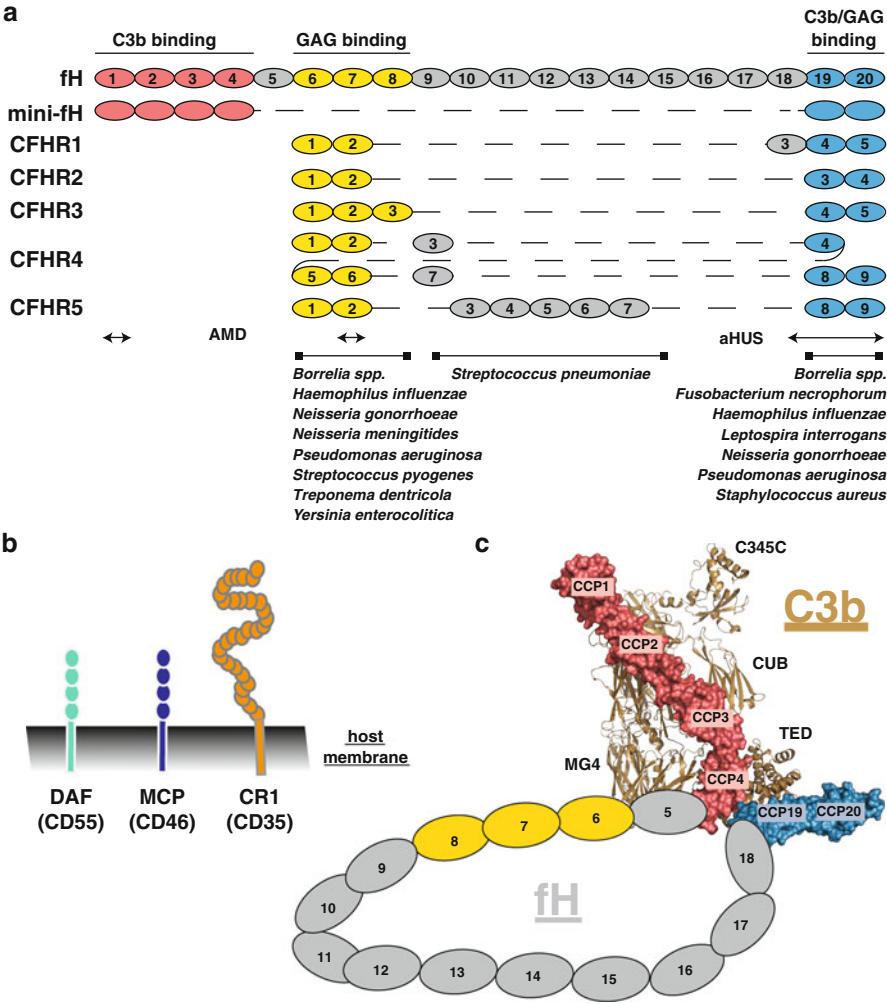


Fig. 2.2 Complement regulators built on complement control protein (CCP) modules. **(a)** Domain organization of human factor H. CCP domains involved in C3b binding are colored *red*, glycosaminoglycan (GAG) binding moieties *yellow*, CCPs binding C3d/GAG *blue*. Therapeutic molecule mini-fH is presented below. Factor H CCPs are conserved in CFHRs and are presented directly below. Regions mutated in AMD and aHUS are delimited with *arrows*. Bacterial species evading complement by binding fH are listed below with the respective fH binding regions. **(b)** Domain organization of the most prominent membrane-bound host regulators. **(c)** Structural model of fH binding to C3b [34, 36] (Color online)

cofactors for C3b. Humans have three CCP-based membrane-bound complement regulators as well (Fig. 2.2b). The first is CD46, also known as the membrane cofactor protein (MCP). It is a cofactor for factor I-mediated cleavage of C3b and C4b into iC3b and iC4b. CD46 is ubiquitously expressed on all nucleated cells, thus only

erythrocytes lack CD46 [45]. The extracellular part of CD46 contains four CCP domains that harbor the C3b and C4b binding sites. The second membrane-bound complement regulator is CD55 or decay accelerating factor (DAF). This is a 70 kDa GPI-anchored glycoprotein expressed on a variety of cells and tissues [46]. Membrane-bound DAF exerts its complement-inhibitory properties by disrupting both AP and CP C3 and C5 convertases, and this function resides within its four CCP domains [47].

Another important complement regulator is complement receptor 1 (CR1). It is a type 1 transmembrane protein composed of numerous CCP domains (44 for the longest allelic variant). CR1 is expressed on almost all peripheral blood cells except platelets, NK- and T-cells [48, 49]. Apart from peripheral blood cells, CR1 is found in some tissues. It plays an important role in the germinal centers of the lymph nodes where it is found on follicular dendritic cells capturing complement-opsonized antigens that serve to stimulate B-cells [50, 51]. CR1 can bind to both C3b and C4b with high affinity and to iC3b and C3d(g) with somewhat lower affinity [52]. Both CP and AP C3 and C5 convertases are inhibited by CR1 via its decay-accelerating activity. It also serves as cofactor for factor I-mediated degradation of C3b and C4b.

Three proteins not based on CCP domains function as MAC assembly inhibitors. Vitronectin (S-protein) binds to C5b-7 through the C5b-7 membrane-binding site and the resulting SC5b-7 complex associates with C8 and three molecules of C9 to form the 1 MDa soluble SC5b-9 complex [53, 54]. Another regulator is clusterin (also called SP-40,40 or apolipoprotein J) which impedes C5b-7 membrane association and the addition of C9 to C5b-8 and C5b-9 [55]. Whereas vitronectin and clusterin are soluble proteins, the third inhibitor of MAC assembly, CD59, is a 20 kDa, GPI-anchored and heavily glycosylated protein widely expressed on almost all tissues and circulating cells [56].

2.1.3 Complement-related Diseases

Uncontrolled and excessive complement activation leads to tissue damage and pathogenesis. The molecular details of how complement proteins contribute to a wide variety of disease conditions and how control of complement may be re-established has recently been extensively reviewed [57–59]. Here we will present some prominent conditions in which animal models, studies of individuals with mutations in complement proteins, and clinical usage of a C5 antibody suggest that therapeutic control of complement is clinically relevant.

The most frequent cause of blindness (50 %) affecting elderly persons is age-related macular degeneration (AMD) with more than 30 million people affected worldwide. AMD causes significant changes in the retinal anatomy and deposition of drusen. This can develop into either neovascular/wet AMD with invasion of blood vessels into the retina or dry AMD with constriction of blood vessels, photoreceptor degeneration, and geographic atrophy [60]. The dry form is currently untreatable whereas wet AMD is treated with intravitreal injections of VEGF antibody.

AMD is strongly correlated with insufficient control of the AP caused by mutations in fH, C3, and fI but also in other complement proteins [61–64]. The best characterized mutant, fH Tyr402His, accounts for 50 % of the heritability of AMD, and causes the altered fH to bind more weakly to host cell glycans as the tyrosine is located in the ligand binding site of fH [32]. Since fH has AP convertase decay and co-factor activity for fI in C3b degradation, host cells are less protected than in the presence of normal fH. The same effect is caused by two C3 mutations for which fH co-factor activity is reduced [65], and fI variants which are less expressed and secreted [66]. Further supporting the link between AMD and high convertase activity, mutations in fB causing weaker association with C3b provide protection against AMD [67]. Despite these connections between AMD and complement, clinical trials with complement inhibitors targeting either the AP (C3 and factor D) or the TP (C5) have so far been disappointing, and so far no complement based therapeutic for AMD has reached phase III clinical trials [60].

Sepsis is a systemic inflammatory condition established by infectious agents, which leads to an excessive immune response resulting in host damage [68]. A ‘cytokine storm’ occurs and together with intravascular coagulation triggers multi-organ failure. The two C5a receptors C5aR and C5L2 have been implicated in the pathogenesis of sepsis by contributing to the cytokine storm and suppression of the oxidative burst in neutrophils, thereby, hampering the elimination of the infectious agent [69]. In a rat cecal ligation and puncture (CLP) sepsis model, C5a antibodies effectively decreased bacteremia, increased survival, restored H_2O_2 release by blood neutrophils and reduced coagulation [70, 71]. Neutrophil function was also restored in a mouse CLP model upon treatment with a cyclic peptide C5aR antagonist [72]. Furthermore, in a CLP model of sepsis simultaneous blockade of both C5aR and C5L2 with antibodies or a C5aR/C5L2 antagonist (C5a-based antagonist (see below)) increased survival compared to inhibition of the receptors one at a time [73].

Ischemia–reperfusion (I/R) injuries are caused by a reduction of blood flow to tissues and organs followed by re-establishment of the blood flow during which there is an accumulation of leukocytes in the vascular epithelium, upregulation of vascular pro-inflammatory molecules and reactive oxygen species [74]. Complement activation has been observed in many different organs undergoing I/R, including the gastrointestinal system, brain, lung, and kidneys [75]. I/R damage occurs during kidney transplantation, and locally-produced complement proteins are the important mediators of damage in this case [76, 77]. The molecular mechanism of complement-mediated damage during I/R appears to be intricate and tissue-dependent. In renal I/R, the TP plays a central role since both inhibition of C5b-9 assembly in C6 deficient mice and inhibition of C5aR with a small molecule antagonist reduce I/R damage [78, 79]. Whereas MBL-deficient mice are protected [80], absence of C4 does not provide protection [78], suggesting that C3 cleavage bypasses the C4b-based CP C3 convertase. In mouse models of myocardial and gastro-intestinal I/R injury both knockout of MASP-2 and MASP-2 inhibition with antibodies conferred protection and again C3 deposition was found to be independent of C4 [81]. Once C3b has been generated, further amplification takes place through the AP explaining the benefits in a mouse ischemic stroke model of fB absence,

but with no protection afforded by C6 knockout [82]. In a mouse model of myocardial I/R injury absence of MBL and MASP-2 [81, 83] attenuates injury, and interestingly, when given at pharmacological doses, the naturally occurring MAP-1 (Map44) protein competing with MASP-1/2 for binding to MBL and ficolins also inhibits I/R injury in addition to inhibiting thrombosis [84].

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory condition conferring synovial joint damage [85] to which complement TP has been acknowledged as an important contributor [86]. Aggregation of self-antigen-antibody complexes leads to the formation of complement-activating immune complexes in the synovial tissue [87]. Several complement proteins are involved in RA pathogenesis, but C5 is likely to play a pivotal role in complement-mediated tissue damage within RA [86]. Studies on C5-deficient murine models with type II collagen-induced arthritis (CIA), suggest a role of C5 in the pathogenesis [88], and administration of anti-C5 antibodies in C5-sufficient murine models prevents the onset of CIA and significantly reduces the severity of the disease during active CIA [89]. Signaling of C5a through C5aR and C5L2 is believed to play a significant role in the pathogenesis of RA [90–92] and vaccination with a C5a-fusion protein reduced arthritis severity and incidence in a mouse model [93]. However, somewhat disappointing considering these results from animal models, administration of the C5aR antagonist PMX53 failed to reduce synovial inflammation in RA patients [94].

In humans the terminal pathway and MAC formation is an important contributor to pathophysiology in paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria [95], and atypical hemolytic urelytic syndrome (aHUS) [96]. PNH is caused by a *PIGA* gene mutation in haemopoietic stem cells resulting in deficiency of all GPI-anchored proteins on progeny cells [97–99] including CD59 and DAF. In PNH patients up to 90 % of erythrocytes are lysed by MAC assembly [100] resulting in anemia, intravascular hemolysis, and thrombosis. PNH is routinely treated by administration of a humanized monoclonal antibody (Eculizumab), preventing C5 cleavage and, thus, the assembly of MAC and the formation of C5a [101]. Eculizumab-treated patients are much more susceptible to neisserial infections and need to be immunized prior to treatment with the C5 antibody.

Asthma is a chronic airway disease characterized by inflammation of the upper respiratory tract, reversible airway obstruction, mucus cell hyperplasia and airway hyperresponsiveness (AHR) [102]. These outcomes are mediated by a T helper type 2 (Th2) polarized immune response, and the anaphylatoxins C3a and C5a have been implicated in both the sensitizing and the effector phases of the disease by regulating the adaptive immune response to allergens [103]. Through studies targeting C5aR by monoclonal antibodies or a C5a-based antagonist, it was suggested that C5a signaling mediates tolerance to aeroallergens by altering the ratio of immunogenic myeloid dendritic cells (mDCs) to tolerogenic plasmacytoid dendritic cells (pDCs), suppressing the Th2 immune response [104]. In contrast to C5a, C3aR knock-out mice develop less pronounced AHR when treated with ovalbumin (OVA) [105], and blocking of complement activation with the recombinant soluble form of the rodent-specific complement regulator Crry decreases airway inflammation in already sensitized mice, by a decrease in both pulmonary eosinophils and immunogenic

Th2 cytokine levels in bronchoalveolar lavage fluid (BALF) [106]. Increased levels of both C3a and C5a were found in BALF from asthmatic patients when compared to normal individuals [107], suggesting that both anaphylatoxins function during the effector phase [106, 108].

2.2 Inhibitors Targeting Complement

2.2.1 Inhibitors Targeting the Convertases

Convertases are obvious targets for complement inhibition, although therapeutics targeting these will in most complement-related disease conditions have to be given systemically, thus, requiring high doses. Convertases targeting has been exploited by pathogens to evade complement and in research and drug development for controlling complement activation at either C3 or C5 cleavage stage [109]. To understand in detail the mechanism of known convertase inhibitors and how to develop new ones, it is helpful to investigate the structure of convertases, their substrates and the complexes these molecules form with their inhibitors (Fig. 2.3). Considerable structural information has been generated concerning the AP C3 convertase. Structures of the proconvertase C3bB by EM and crystallography revealed how fB, in a MIDAS-Mg²⁺ dependent manner, associates with the C-terminal C345c domain of C3b, that is flexibly attached to the remaining relatively rigid part of C3b [110, 111]. The proconvertase can exist in two states, open and closed, differing by a rotation of the fB SP domain, and only in the open conformation is the scissile bond region exposed and accessible to fD, which binds primarily through a fB exosite located 25 Å from the scissile bond [110]. The relevance of this exosite is evidenced by the ability of the anti-factor D mAb, developed for localized complement inhibition in the eye by Genentech, to prevent fD binding to this exosite by steric hindrance [112].

Once the activation of the convertase has taken place it dissociates in minutes, but the *S. aureus* protein SCIN (see below) binds tightly to the AP C3 convertase and prevents it from binding C3. A dimeric form of the C3bBb-SCIN complex in which two SCIN molecules bridge two C3bBb complexes were crystallized and revealed the basic architecture of C3bBb. The only contact here is between the C3b C345c domain and the Bb von Willebrand factor type A (vWA) domain, whereas, the SP domain is extending away from C3b [113]. Deeper insight into substrate recognition by the convertases was obtained with the structure of C5 in complex with the C3b homolog cobra venom factor (CVF). C5 and CVF interact in a head-to-head manner with the long axis of the two molecules aligned and with two separated points of contact. The largest of these is formed between the MG4 and MG5 domains from both proteins. The remaining intermolecular contacts are formed between the C5 MG7 domain and the CVF MG6 and MG7 domains [114]. Compared to the structure of both unbound C5 [115] and human C3 [116], CVF-bound C5 undergoes a significant conformational change that is necessary to establish the two-points interaction, and this conformation has also been captured by crystal

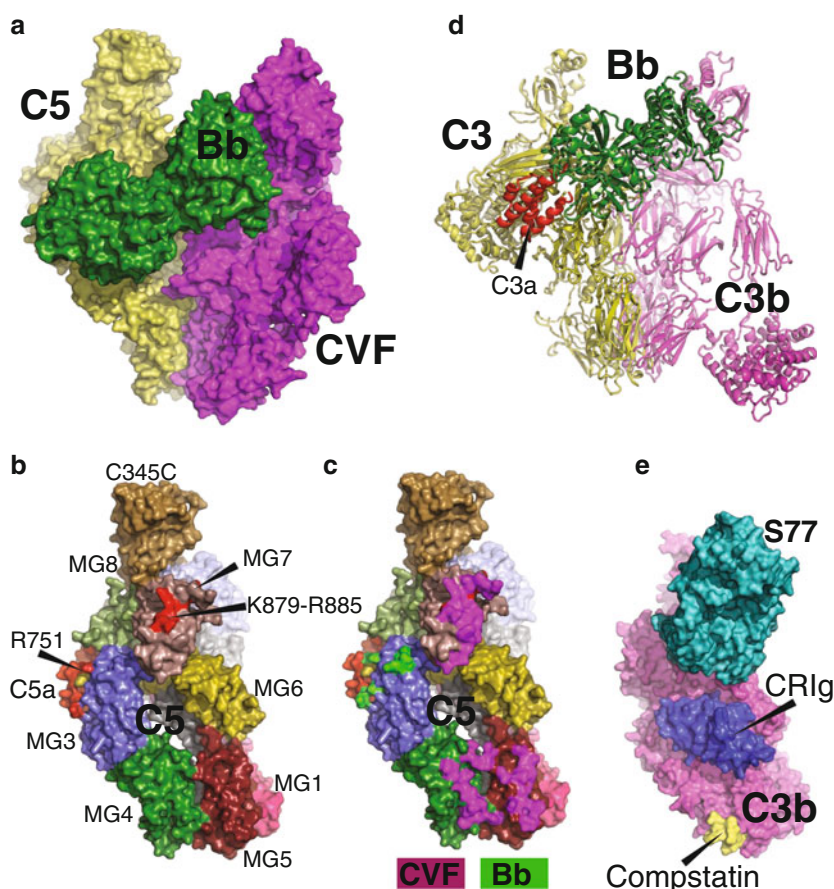


Fig. 2.3 A general model for convertase-substrate complexes explains the inhibition mechanism for convertase inhibitors. **(a)** The model of the CVFBb convertase bound to C5 [114]. **(b)** Complement C5 colored and labelled according to its domains as seen from CVF, the Eculizumab epitope K879-R885 is indicated. **(c)** As in *panel b*, but surface areas in contact with CVF and Bb in *panel a* are colored *magenta* and *green*, respectively. Notice the overlap between the Eculizumab epitope and the CVF-interacting area of the C5 MG7 domain explaining how the antibody prevents C5 cleavage. **(d)** A model of the C3 substrate bound to the AP convertase C3bBb. The C3a domain released is marked in *red*. **(e)** C3b within the AP C3 convertase as seen from the C3 substrate. The three convertase inhibitors S77, CRIg and Compstatin effectively prevent substrate recognition by steric hindrance (Color online)

packing for bovine C3 [117]. The combination of the SCIN-stabilized C3bBb structure and the C5-CVF structure led to the formulation of a general model for convertase-substrate interactions (Fig. 2.3). Since the catalytic subunit C2a/Bb is common for the CP/AP C3 and C5 convertases, it was suggested that the orientation of the substrates C3 and C5 with respect to the catalytic subunit is similar in the two types of convertases [114], although covalent or non-covalent association of either C3

convertase with C3b switches the specificity from C3 to C5 [118–120]. The additional C3b molecule lowers the K_m value for the C5 substrate by a factor of 100–1000 [121, 122], but whether this is through a direct interaction with C5 or induced conformational change in the C3 convertase remains open. The validity of this convertase-substrate model is emphasized by its ability to rationalize how some of the best characterized man-made and microbial complement inhibitors exert their effect. Prominent examples are the antibodies S77 and Eculizumab, the CRIg ectodomain, and the cyclic compstatin peptide, as described in the following.

The S77 mAb interferes with both AP convertases [123], which can be explained by its binding to C3b MG6 and MG7 domains (Fig. 2.3e), that are both predicted to recognize the substrate C3/C5 MG7 domain [114]. A similar inhibitor is the ectodomain of the CRIg complement receptor. This receptor is found on tissue resident macrophages and plays an important function in clearance of pathogens from circulation through interaction with C3b and iC3b on complement-opsonized activators [124]. The binding site on C3b has been mapped to the MG3, MG4, MG5, MG6 domains and the LNK region (Fig. 2.3e), and the ectodomain inhibits AP C3 and C5 convertase activity [125]. The Compstatin peptide is frequently used as a general complement inhibitor and through its binding to C3 it blocks both the CP and AP C3 convertases by interfering with binding of C3 to the convertases. Compstatin also binds to C3b, iC3b and C3c [126]. One disadvantage of compstatin is the high C3 plasma concentration (7 μ M) and the rapid clearance of peptides, but new compstatin analogues with sub-nanomolar K_d have substantially increased half-lives [127]. The peptide binds in a groove between the MG4 and MG5 domains of C3 or its fragments [128] (Fig. 2.3e). As these domains in the substrate C3 are predicted to be recognized by the convertase, this explains the ability of compstatins to suppress C3 cleavage by either AP or CP convertases. Like compstatin, the C5 antibody Eculizumab hinders the binding of the substrate C5 to the convertase and, thereby, prevents its cleavage. However, Eculizumab binds to an epitope far from the convertase cleavage site in the MG7 domain centered on residues 879–885 [129, 130], which overlaps substantially with the area of C5 in contact with CVF in their complex [114] (Fig. 2.3b, c).

2.2.2 Regulator-derived C3 and C5 Inhibitors

Over the past years, the development of complement-targeted therapeutics has been inspired by natural complement regulators. The idea behind the rational design of such therapeutics is the use of naturally-occurring host proteins. Strategies involving complement targeting with systemically-administered inhibitors of such type have, however, a few drawbacks. Firstly, inhibiting complement systemically could have detrimental consequences in regard to the host immune defence. Secondly, due to the relatively low affinity of these molecules (μ M range) the administered dose would need to be very high. Thus, a more focused strategy is needed. Indeed, direct targeting of the surfaces where complement activation occurs is a more intelligent

and less costly approach. fH would be very attractive in therapeutics as it is a complement modulator that is a self-molecule, acts in the fluid-phase as well as on surfaces and does not interfere with complement defence against pathogens. Purified fH was shown to be able to control the C3 convertase in a mouse model of fH deficiency [131]. In vitro assays in the presence of aHUS-associated anti-fH autoantibodies showed that fH protected self-cells from complement. However, due to its size (155 kDa), presence of glycans and 40 disulfide bridges, recombinant production of full-length fH is challenging for routine administration to patients.

By the use of protein engineering it has been possible to develop mini-fH, a compact version of fH harbouring fH CCP 1–4 linked to CCP 19–20 (Fig. 2.2a). This molecule contains fH binding sites to C3b/iC3b/C3d, retains its decay acceleration and cofactor activity and, very importantly, has the ability of fH to recognize host surface-specific glycans. Interestingly, this molecule surpasses fH in both affinity towards C3 activation products and the ability to control AP activation [132, 133]. Mini-fH has potential as a therapeutic molecule in complement-mediated diseases such as aHUS, PNH or C3 glomerulopathies although its clinical properties remain to be investigated. Another inhibitor based on the fH ability to bind C3b is TT30 [134]. It is a chimeric protein developed by Alexion containing fH regulatory domains CCP 1–5 and CR2 CCP 1–4 responsible for binding to the C3b thioester domain. TT30 was extensively tested in the case of complement-mediated hemolysis and found to inhibit MAC formation resulting from AP but not CP or LP activation.

Part of the recent research in complement therapeutics has been focused on preventing complement activation on blood-exposed materials, such as implants. A great deal of research has been evolving around heparin as it has been expected to recruit fH and inhibit complement activation on the surface of biomaterials. As expected, coating the biomaterials with high densities of heparin resulted in inhibition of complement activation [135–137] but it was later found that this inhibition was fH-independent [138]. An alternative approach, employed recently, consists of coating the bioimplant surfaces with fH binding peptides but not interfering with its activity as complement regulator [139] but this approach remains to be tested in a clinical setting.

Since CR1 possesses cofactor activity for factor I-mediated proteolysis of C3b and C4b, its potential in therapeutics has been extensively studied. Soluble recombinant CR1 (sCR1) was studied in animal models of autoimmune and inflammatory disorders such as glomerulonephritis [140], myocardial infarction [141] and autoimmune thyroiditis [142]. In mice, administration of sCR1 resulted in resolution of inflammation and these encouraging results drove Avant Immunotherapeutics to develop sCR1 (TP10) for managing complement activation following coronary artery bypass graft surgery. TP10 was shown to be safe and well-tolerated in patients. However, its efficacy was far greater in male patients, and this formulation has been withdrawn from the market but its potential as a therapeutic agent has not been disputed. In fact, it has been successfully used in I/R injury when administered intravenously [143]. Nevertheless, since it is produced in Chinese hamster ovary cells as a 240 kDa glycoprotein [141, 144] and has to be injected systemically, it is

an expensive therapeutic agent. An alternative approach has been used for the development of Microcept [145], a molecule readily produced in *E. coli* containing only the first 3 CCP domains of CR1, sufficient for inhibition of C3 and C5 activation by the AP and CP. Microcept contains a synthetic thiol-reactive myristoylated basic peptide tail attached through a C-terminal cysteine residue allowing its insertion into biological membranes and, thus, targeting and protecting the cells directly.

CD59 is an important complement regulator as evidenced by PNH patients, and the protein has been explored as a therapeutic agent since it only blocks MAC formation. Although CD59 has little effect as MAC inhibitor when administered systemically in a soluble form, if targeted to the sites of MAC formation, recombinant CD59 could be an effective complement modulator. Several strategies have explored generating CD59 chimeric proteins. One of them consisted of fusing CD59 to DAF and a GPI anchor, thus allowing its insertion into the membranes and its localization where C3 and C5 convertases are present [146]. Another strategy involved fusing CD59 to CR2 and DAF to CR2 [147]. These CR2 fusion proteins were efficient complement modulators in mouse models of lupus nephritis. In conclusion, fusing CD59 to a cell-surface targeting moiety may be an efficient therapeutic strategy in disease conditions where only MAC formation needs to be inhibited.

2.2.3 *Inhibitors Targeting the Anaphylatoxin/Receptor Axis*

The anaphylatoxins C3a and C5a produced by the complement proteolytic cascade and their associated receptors are important targets for the therapeutical treatment of inflammatory disorders. C3a and C5a mediate their pro-inflammatory effects by signaling through the G-coupled protein receptors C3aR and C5aR, respectively. This leads to chemotaxis, oxidative burst, production of pro-inflammatory molecules and activation of the adaptive immune system [148]. C3a is generally considered as a weaker pro-inflammatory inducer than C5a. To control the signaling exerted by both anaphylatoxins, carboxypeptidases cleave their C-terminal arginine resulting in C5a-desArg and C3a-desArg. C5a-desArg partially maintains C5aR binding and signaling activity whereas C3a-desArg is devoid of any signaling through C3aR [149, 150]. The 7TM receptor C5L2 binding C5a, C5a-desArg, C3a, and C3a-desArg, has long been considered as a decoy receptor, but there are now reports suggesting that it actively participates in orchestrating pro-inflammatory events [151]. Plasma levels of anaphylatoxins are elevated in various disease settings, and they can be used as biomarkers in a number of inflammatory disorders [148].

Targeting of the anaphylatoxin-receptor axis provides a selective way of down-regulating complement-mediated inflammation and is, therefore, an attractive therapeutic strategy since opsonization and MAC formation are preserved. Focus has so far been given to the C5a-C5aR axis, although C3aR antagonists are also in development as extensively reviewed recently [152]. In humans, C5a is a glycosylated protein that adopts either a compact four-helix bundle core with a flexible C-terminal extension [153] or a three-helix bundle for C5a-desArg [154]. The proposed binding

interface for C5aR on C5a is hidden within the C5 molecule [115]. Thus targeting molecules can mostly be directed either towards the exposed C5a surface present on C5 or towards the C5aR binding surface only available on the released anaphylatoxin. The first strategy presents more risks since it can generate global C5 inhibitors, if large inhibitory molecules are employed impairing C5 cleavage and, thereby, the entire TP. This effect is in some clinical contexts undesirable, e.g. in the treatment of sepsis. Inhibitors directly interfering with C5a-C5aR interaction while preserving other C5 functions are, therefore, more appealing.

Several groups and companies have developed potent C5a monoclonal antibodies [155]. The use of C5a antibodies for preventing multiple organ failure and improving survival rate in sepsis has been documented since the 1980s [156]. Of interest among these, the anti-C5a mAb 137-26 directly binds to the C5a moiety on C5 without inhibiting C5 cleavage and subsequent MAC formation [157]. It is commercialized as TNX558 by Tanox/Genentech and has been in preclinical development for inflammatory diseases [158]. Another humanized C5a monoclonal antibody, CaCP29 (IFX-1), developed by InflaRx GmbH, has passed Phase I clinical trials in Germany for human sepsis [159]. Aptamer approaches have also been considered for the blockade of C5a function. NOXXON Pharma has developed a class of aptamers called Spiegelmers® [160] built on nucleotides containing L-ribose making them the mirror images of D-ribose containing RNA. As therapeutics, Spiegelmers are much more resistant to nuclease degradation compared to conventional aptamers giving high stability in the blood. C5a has been efficiently targeted by Spiegelmers® NOX-D19 and NOX-D20, which have shown promising results in reducing vascular injuries after transplantation and in attenuating organ damage during sepsis [151, 161]. NOX-D20 binds to both human and murine C5a with picomolar affinities but also human C5 with similar affinity although NOX-D20 does not prevent C5 cleavage. These mirror-image L-RNA aptamers, therefore, appear to be promising anti-C5a therapeutics. An alternative strategy involving immunization with a recombinant MBP-C5a fusion protein resulted in the production of neutralizing C5a antibodies [93]. A new study instead used a recombinant C5a molecule modified with unnatural amino acids. A single replacement was sufficient to induce the production of anti-C5a antibodies capable of blocking the C5a-C5aR interaction, leading to significant relief of the clinical symptoms in a mouse model of rheumatoid arthritis [162].

In relation to C5a-targeting molecules the anaphylatoxin receptors have also been a focus for inhibitor development. A large number of C5aR inhibitors have been developed. A well described cyclic peptide antagonist known as PMX53 or 3D53 targets C5aR and competes with C5a binding to human polymorphonuclear neutrophils (PMNs) with an IC_{50} value of 300 nM, but does not bind human C5L2 [148, 163]. The antagonistic activity of PMX53 was measured, showing an inhibition of myeloperoxidase release from PMNs with an IC_{50} of 20 nM [163]. NMR studies of PMX53 suggests a β -turn motif in the molecule [164]. Mitsubishi Pharmaceuticals Company developed the orally active small molecule C5aR antagonist W54011 with a K_i of 2.2 nM. It inhibits calcium mobilization in human neutrophils and C5a-induced neutropenia in gerbils, and shows no inhibition of

C5a-binding to C5L2 [165, 166]. Another small molecule, NDT9513727, specifically targeting C5aR, reduces the constitutive GTP γ [³⁵S] binding of human C5aR-coupled G-proteins, and, therefore, functions as an inverse agonist [167].

Proteins have also been selected to target C5aR. Developing human C5aR knock-in mice responding to endogenously produced C5a facilitated the selection of anti-C5aR antibodies showing promising results in preventing and reversing serum-induced inflammation in the knock-in mice. The most potent anti-C5aR antibodies bind to the second extracellular loop of the receptor [90], which was previously identified as important for balancing the activity of C5aR, since mutations in this region resulted in constitutively active receptors [168]. The C5a molecule has also been exploited as a scaffold to generate potent competitors of C5a receptors, and from a phage library an antagonist called Δ pIII-A8 was selected which reduced intestinal injury and lung vascular permeability and increased survival of (I/R) injured mice [169]. A shortened version (A8 ^{Δ 71-73}) targeting both C5aR and C5L2 was developed later and, interestingly, it was found that one particular residue determines agonism versus antagonism of A8 related proteins. In C5a, this amino acid is an aspartate and in A8 ^{Δ 71-73} it is mutated to an arginine [170].

For C3aR inhibitor developments have mainly focused on peptide analogs of the C-terminal region of C3a. Hexapeptides mimicking the C-terminus of C3a were synthesized resulting in both agonists and antagonists. The most potent agonists shared an N-terminal phenylalanine, a tryptophan or leucine at the second position and the highly conserved C-terminal sequence Leu-Ala-Arg. Substituting the fourth leucine to the bulky cyclohexylalanine resulted in antagonists [171]. By NMR, one of the most potent agonistic peptides was found to adopt a β -turn motif similar to C5aR ligands [172]. As an alternative to peptides, small-molecule compounds targeting C3aR have also been discovered, with an example being the functional antagonist SB290157 with an IC₅₀ of 200 nM [173]. Although this drug showed anti-inflammatory activity in a guinea pig model [174], a more recent paper found this molecule to have partial agonistic activity, a conflict that might be explained by differences in receptor density in the systems used [175]. Numerous other drugs including proteins, peptides and small molecules have been developed targeting the anaphylatoxin receptors and have been extensively reviewed elsewhere [148, 152, 176].

2.3 Bacterial Strategies for Immune Evasion: What Can Be Learnt from Them

Complement primary function resides in the host defence against pathogens, but many pathogens successfully evade complement [177]. One can learn from these evasion strategies on how to inhibit complement at various stages of the cascade and apply this knowledge to design inhibitors for therapeutic applications. Many pathogen inhibitors have also proven useful to gain structural insights into complement mechanistics by freezing complement proteins and their complexes in specific functional states with the ability of the *S. aureus* protein SCIN to stabilize the

otherwise rapidly dissociating C3 convertase as the best example. We will review here some of the best characterized pathogen inhibitors and try to comprehend how their interaction with complement may inspire attempts to make potent, selective inhibitors of complement.

2.3.1 Pathogen Inhibitors Targeting C3

Staphylococci are quite versatile organisms with respect to complement evasion. Targets of choice for staphylococcal proteins are the C3 and C5 convertases [178] as already described for SCIN above. Three other potent complement inhibitors from *Staphylococcus aureus* - Efb, the closely related Ehp, and Sbi - are binders of C3 and its cleavage products, C3b and iC3b, through preferential interaction with the C3 thioester domain, with the C3b degradation product C3d as the minimal binding partner. Crystal structures of the Ehc:C3d, Efb-C:C3d and Sbi:C3d complexes revealed that their primary binding site on C3d is partially inaccessible within the intact C3b molecule [179–181] (Fig. 2.4a). As a consequence of their binding to this hindered site, Efb and Ehp induce an altered overall conformation of C3 and C3b. Displacement of the C3d thioester domain is relayed to the rest of the molecule, therefore preventing its further participation in the downstream events of the complement cascade, including formation of the AP convertases and covalent deposition on the pathogen surface. In the case of Sbi, a secondary binding site on C3d allows the inhibitory protein to form a covalent adduct with activated C3 forms (C3b and/or C3(H₂O)), thereby directly interfering with AP activation [181] (Fig. 2.4a). In addition, all three inhibitors compete with complement receptor 2 for the binding of C3d and, therefore, impede the stimulation of B cells mediated by this receptor [182].

2.3.2 Pathogen Inhibitors Targeting C5

Several pathogen inhibitors block the C5 convertase by interacting with C5. OmCI, a small protein from the soft tick *Ornithodoros moubata*, binds directly to C5 and inhibits its cleavage into C5a and C5b [183]. Structural studies of the OmCI:C5 complex suggested that OmCI binding to C5 fixes the C5 C345c domain and inhibits convertase binding to C5 by impairing the conformational flexibility of this domain [115]. A recombinant version of OmCI, rev576, has shown promising results in models of auto-immune neuromuscular diseases and sepsis [184–186]. The SSL7 protein from *Staphylococcus aureus* likewise binds C5 and prevents its proteolytic processing by the C5 convertase, thereby impairing MAC-mediated bacteriolysis and C5a release. Its mechanism of action was elucidated through structural analysis of the C5:SSL7 complex [187]. SSL7 binds to a surface patch quite distant from the C5 cleavage site suggesting a more complex mechanism than

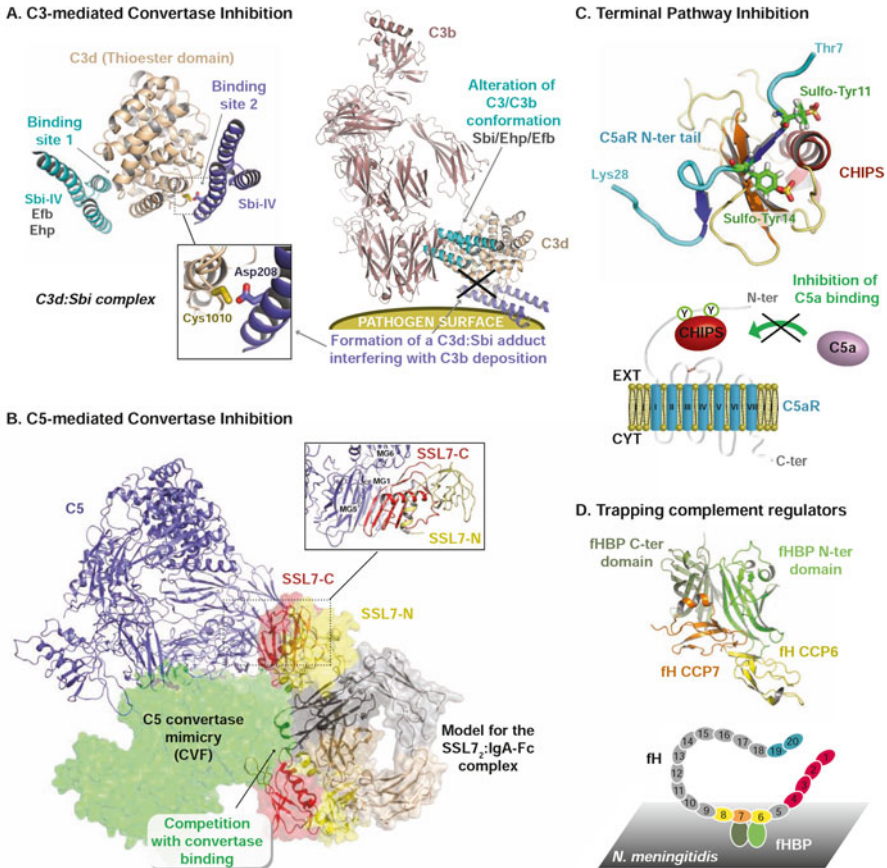


Fig. 2.4 Complement evasion by pathogens. (a) The two binding modes of staphylococcal protein Sbi onto C3d [181] and their docking onto C3b [210], revealing the structural model for their inhibition of the C3 convertase function. (b) Structural model for SSL7 inhibition of the C5 convertase by dual recruitment of C5 and IgA, based on the C5:SSL7 [187], C5:CVF [114] and SSL7:IgA-Fc [211] complex structures. (c) Structure of the inhibitor CHIPS in complex with a peptidic fragment of C5aR [193] and model for its inhibition of C5a binding by blocking the C5aR N-terminal docking site around the two sulfo-tyrosines. (d) Structure of the complex between fH CCPs 6–7 and fH-binding protein (fHBP) from *N. meningitidis* [199] and scheme for fH trapping on the pathogen surface

simple steric hindrance of the C5 cleavage site. This was later confirmed by the structure of the ternary complex between C5, SSL7 and CVF [114]. Together these studies explained how simultaneous recruitment of C5 by SSL7 C-terminal domain and IgA by SSL7 N-terminal domain prevented convertase recognition of C5 (Fig. 2.4b). Interestingly, the isolated C-terminal domain of SSL7 devoid of affinity for IgA, did not prevent C5 cleavage and permitted bacteriolysis while preserving a very low hemolytic activity on erythrocytes [187] suggesting that this domain may

indicate a direction for development of a therapeutic agent targeting complement-dependent hemolytic diseases.

Bacterial proteins that directly target MAC formation have also been reported. Streptococcal Inhibitor of Complement (SIC) and variants from other *Streptococcus* strains bind to the C5b67 complex hindering membrane insertion [188]. An outer membrane protein from *Escherichia coli* K12 strain, TraT, also impairs MAC formation by direct binding to C5b6 preventing C7 recruitment [189]. *Borrelia burgdorferi* produces a CD59-like protein that blocks C9 polymerization [190]. Pathogen proteases are also potent effectors in complement evasion and C5a peptidases are found in various organisms. In particular, group A *streptococci* produce a cell-envelope proteinase (ScpA or ScpB) cleaving off the seven last residues from the C5a C-terminus, thereby shifting C5a from agonistic to antagonistic activity [190]. Proteinases from *Porphyromonas gingivalis* are also capable of shedding off the N-terminal region of C5aR [191], thus inactivating the receptor. Direct inhibition of C5aR is also achieved by pathogenic virulence factors. A well-studied case is the secreted chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) binding C5aR with a K_d of 1.1 nM [192]. An NMR structure of CHIPS in complex with a peptide mimicking the N-terminus of C5aR containing *O*-sulfated tyrosines revealed how CHIPS competitively antagonizes the interaction of the core structure of C5a with the receptor [193] (Fig. 2.4c). CHIPS or derivatives of this was suggested to serve as an anti-inflammatory therapeutic agent but this was questioned due to the high immunogenicity of CHIPS [194]. However, mutated or shortened versions of CHIPS with lower immunogenicity maintain the C5aR antagonizing effect [195, 196].

2.3.3 Inhibition/Trapping of the Complement Regulators

Bacteria and viruses have also developed approaches to hijack complement components to their own advantage with complement regulators fH, fHL-1 and C4BP being the preferred victims. The plethora of bacterial fH binders includes complement regulator-acquiring surface proteins (CRASP) and outer surface protein E (OspE) from *Borrelia burgdorferi*, M proteins, Fba, Scl1.6 and Hic from *Streptococci*, and fH binding protein (fHBP) from *Neisseria meningitidis*. CRASP-1 binds to CCP5–7 of fH as a dimer and clamps the bound CCP domains, thereby enhancing scavenging of the complement regulator on the pathogen cell surface [197]. OspE and neisserial FHBP, on the other hand, use a protein mimicry of the host carbohydrates to sequester fH by targeting either CCP19–20 [198] or CCP6–7 [199] (Fig. 2.4d). In both cases, the binding epitopes on fH are similar to the ones proposed for glycosaminoglycans binding on endothelial cells. Finally, certain viruses also possess complement regulators, such as the vaccinia virus complement-control protein (VCP) and the smallpox inhibitor of complement enzyme (SPICE) which both show cofactor activity for degradation of C3b [200]. <http://www.nature.com/nrmicro/journal/v6/n2/full/nrmicro1824.html-B27>

2.4 Discussion and Concluding Remarks

Although the concept of complement as a cornerstone in defence against microbial invasion and in homeostasis still holds true, there is also clearly a dark side to this highly complex machinery. The capacity of complement to recognize danger-associated molecular patterns from both pathogens and host cells and its interconnectivity with other branches of the innate and adaptive immune system and even the coagulation system [2], adds on to difficulty of maintaining tight control of the system. Evidently, any breach in its regulation may disturb the fine balance between protection and damage. The list of diseases that implicate complement as one of the causative elements keeps growing and encompasses a broad panel of pathologies ranging from inflammatory diseases to neurodegenerative disorders and cancers [59, 201]. Our knowledge of the intricate mechanisms at work during complement activation and complement crosstalk with parallel defence systems has gained considerable depth over the last decade, not least due to the increasing amount of available structural and biochemical data allowing a comprehension of the complement cascade in atomic details.

The success of Eculizumab [101] has encouraged many new initiatives in the field. A constantly growing number of molecules arising from both academic and industrial research efforts are now under development, with already promising preliminary results in clinical studies for several of them, as reviewed here. Many classes of molecules have been considered for these drug candidates, including antibodies, aptamers, small chemical compounds and recombinant versions of naturally occurring proteic inhibitors, e.g. based on bacterial inhibitors or host regulators. The constantly improved understanding of the mode of action of complement regulators and receptors has led to the conception of new generations of multimodular inhibitors incorporating functionalities of one or several complement regulators for efficient convertase decay and/or opsonin degradation [57]. Although many regulatory concepts have already been exploited, new ideas are emerging thanks to the availability of both functional and structural data allowing comprehending the mechanistics of complement activation at the molecular level. As one example, recent structures of C4 and its complex with MASP-2 revealed an important exosite interaction of MASP-2 with the C4 C345c domain and it could be demonstrated that a recombinant version of this domain functions as a CP/LP pathway inhibitor [202].

Evidently, targeting of complement at specific stages of the cascade is highly desirable to allow selective containment of the dysregulated pathway while preserving protective functions of the overall immune system. While targeting of central complement components such as the C3 convertase will lead to complete shutdown of the system, more refined approaches can be directed towards the initiation or the terminal steps. Interfering with the initial steps of complement activation may, for example, offer a way to specifically target one of the activation pathways while retaining normal complement defence functions through the other

untargeted pathways—granted that the identified pathway is the major contributor to the pathological condition.

Targeting of the terminal steps of complement cascade has already been extensively exploited through the inhibition of C5a-mediated signaling [70, 152] and the blockade of C5 by Eculizumab [95, 101]. Nevertheless, improvement of the known strategies may still be needed as exemplified by Eculizumab. Although the antibody effectively prevents hemolytic activity by impairing MAC formation, it does not interfere with AP complement activation and subsequent opsonization of PNH cells, which thus are still preferentially marked for extravascular lysis [203]. Furthermore, the increased susceptibility to neisserial infections of the patients treated with Eculizumab still constitutes a disadvantage [204]. Another major concern for life-long Eculizumab treatment for PNH and for some aHUS patients is the annual cost of €460,000 for an adult [130]. Despite these drawbacks, no efficient substitute has been produced yet and clinical trials for the use of Eculizumab in acute inflammatory disorders, such as antibody-mediated transplant graft rejection, are currently conducted [205]. In such settings where C5aR-mediated signaling is seen as a major contributor to the underlying inflammation, a C5a or C5aR antagonist might simply be a more promising drug candidate [203].

An emerging idea is that the inhibitor design has to be rethought for each particular disease [57]. During acute inflammation (e.g. in sepsis), large amounts of complement effectors (C3b, C4b, C5b, anaphylatoxins) will be produced. Thus, efficient inhibitors should have fast, high affinity binding capacities towards their target and a slow dissociation rate, to allow rapid and complete blocking of the complement cascade. In chronic inflammation, on the other hand, a milder modulation of complement may be sufficient to re-establish a proper balance between protective function and injury—complete shutdown of the system being avoidable in that case. Another trend within complement inhibitors is the specific cell/tissue targeting of these [206]. Local delivery at the site of injury is highly desirable if one wants to preserve systemic complement function. Efforts in that sense have already been made for example by fusing regulator-mimicking molecules to targeting modules in order to deliver engineered versions of these inhibitors to sites of complement activation, with the factor H-CR2 fusion protein TT30 as an excellent example [134, 207, 208]. Such targeting approaches may in the future be combined with nanoparticle-based delivery systems for delivery of anti-inflammatory drugs. More thoughts are to be put in this design strategy and organ- or tissue-specific delivering strategies used for other systems should be addressed as well in the complement therapeutics field. Finally, the control of complement activation may become an important issue for the future success of nanomedicine as surfaces considered as “foreign” to the complement system are introduced into the human body and often elicit activation of the system [209] and deeper structural knowledge of the mechanisms at play during complement activation will undoubtedly provide new tools to successfully overcome these challenges.

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Nanomedicine

Howard, K.A.; Vorup-Jensen, Th.; Peer, D. (Eds.)

2016, XVIII, 378 p. 70 illus., 59 illus. in color., Hardcover

ISBN: 978-1-4939-3632-8