How novel structures inform understanding of complement function

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Summary

During the last decade, the complement field has experienced outstanding advancements in the mechanistic understanding of how complement activators are recognized, what C3 activation means, how protein complexes like the C3 convertases and the membrane attack complex are assembled, and how positive and negative complement regulators perform their function. All of this has been made possible mostly because of the contributions of structural biology to the study of the complement components. The wealth of novel structural data has frequently provided support to previously held knowledge, but often has added alternative and unexpected insights into complement function. Here we will review some of these findings focusing in the alternative and terminal complement pathways.

Keywords: Complement, structural biology, C3\C5 convertase, complement regulators, factor H, membrane attack complex.

1. A brief overview of a golden decade in complement structural biology.

The determination of the structures of both intact C3 [1] and its activation fragment, C3b [2], were outstanding contributions by Piet Gros' laboratory that set the ground for a comprehensive description of C3 activation and regulation. Crucial to this were also the resolution of the structures of both C3c and C3d [3] as well as Factor B (FB) [4], the determination of the crystal structures of the complexes formed by cobra venom factor (CVF) with FB [5], the electron microscopy (EM) analysis of the alternative pathway (AP) C3 pro-convertase (C3bB) [6], the crystal structure of the AP C3 convertase (C3bBb) stabilized by the staphylococcal complement inhibitor (SCIN) [7] and the structures of the AP C3 pro-convertase (C3bB) and C3 convertase (C3bBb) in complex with Factor D (FD) [8]. All these contributions revealed in striking detail the different steps and conformational changes required for the assembly of the AP C3 convertase complex. The structural determination of the AP C3 convertase paved the way to understand the modulation of its activity by the complement regulatory proteins. The determination of the structure formed by C3b with the first four domains (SCR1-4) of Factor H (FH), the main fluid phase complement regulator, shed light on how FH binding to the convertase led to the release of the Bb fragment from the AP C3 convertase (decay activity) [9] and how FH facilitates the Factor I (FI) [10] directed proteolytic inactivation of C3b to iC3b (cofactor activity). The structure of C3b and C3d in complex with the last two domains (SCR19-20) of FH, as well as the ternary structure of FH (SCR-19-20) with C3d and a sialic acid molecule, helped uncover the nature of the specificity of FH for self surfaces where complement deposition has occurred [11,12]. In addition, the structures of other membrane bound complement regulators like membrane cofactor protein (MCP; CD46), decay acceleration factor (DAF; CD55) and Complement receptor 1 (CR1; CD35) bound to C3b confirmed a shared regulatory mechanism with FH, [13], which was expected based on their common evolutionary origin. The study of the regulatory protein repertoire of the alternative pathway has been recently complemented by the EM and X-ray

crystallography studies of the AP C3 convertase complexes with properdin, the only known physiological C3 convertase positive regulator [14,15].

The contribution of structural techniques to understand the activation of the terminal pathway, leading to the formation of the membrane attack complex have also been superlative. Early studies using EM determined the existence of pore lesions on the complement targeted membranes, that were later ascribed to the formation of the so-called Membrane Attack Complex (MAC) and presumed to work as the complement lytic component [16]. In the last few years most individual components of the MAC have been crystallized and their structures solved by X-ray crystallography, [17-20], but only with the new technological breakthroughs in cryoelectron microscopy and image processing has it become possible to determine the structure of the full MAC at high resolution. This in turn has finally allowed the complete topological description of the complex and provided deep insights into its assembly process [21].

2. The assembly of the AP C3 convertase.

The central event in the assembly and regulation of the AP C3 convertase is the activation of C3 to C3b. This process is characterized by a dramatic conformational change in which the thioester-containing (TED) domain in C3 is relocated to a distant position close to the macroglobulin domain 1 (MG-1), opening the C3 structure and exposing an extensive and previously inaccessible molecular surface [2,22]. This conformational change, which in C3 is triggered by the so-called “tick-over” mechanism or by proteolytic cleavage of the α' NT domain, is also shared by the C3 homologs, C4 and C5. Early in complement structural research, it became clear that it was precisely this rearrangement in the C3 molecule that was responsible for generating a novel surface area, the location of the FB binding site.

The assembly of the AP C3-convertase, C3bBb, is initiated by the Mg^{2+} -dependent interaction between C3b (or the C3b-like molecule C3(H₂O)) and FB, which circulates as an inactive pro-enzyme in plasma. This interaction involves both the N-

terminal Ba and C-terminal Bb fragments of FB and induces a conformational change to an open state FB that can in turn be recognized by the inactive pro-enzyme form of the protease FD. Notably, this interaction activates FD through the release of its auto-inhibitory loop and the active FD then proceeds to cleave FB. After the specific proteolysis of FB, the Ba fragment is released from the complex, and the active C3bBb convertase is formed [8]. The C3bBb convertase is an unstable bimolecular complex with a short half-life, measured at 90 seconds [23]. The extremely low affinity of the Bb fragment for C3b ensures that the C3 convertase disassembly is irreversible.

Later on, it was shown that the novel surface area exposed during the activation of C3 and generation of the C3b molecule is also the site where the complement regulators bind to C3b to control the decay of the AP C3 convertase and to mediate the proteolytic inactivation of C3b.

3. A structural view to the functional differences between complement regulators

Regulation of the AP C3 convertase is designed either to provide increased stability to the C3bBb complex thereby enhancing activity, or to accelerate its decay. Structural studies have shown that activity of the “negative” regulators FH, DAF, MCP or CR1 involves interaction with a surface area in C3b that overlaps the surface that mediates the interaction with FB. In contrast, the activity of properdin stabilizing the C3bBb complex requires its interaction with a different C3b region, involving the C345C domain (discussed in more detail in the next subheading).

The crystal structure of C3b complexed with the first four domains (SCR1-4) of FH provided the molecular basis for understanding the two regulatory mechanisms, cofactor activity and decay-accelerating activity (DAA), by which the complement regulators inactivate complement activation and protect host cells [9]. The C3b-FH (SCR1-4) structure revealed a large and discontinuous interface with four contact regions throughout SCR domains 1 to 4, with each domain establishing contacts with the C3b molecule. Based on the structure comparison of C3b-FH (SCR1-4) and the AP

C3 convertase, DAA seems to be mainly mediated by the two N-terminal domains of FH (SCR1-2). These, through binding of the α' NT, MG2 and MG6-7 domains of C3b, likely displace the Bb fragment from the convertase in a process mediated by electrostatic repulsions and steric hindrance [9,7]. The C3b-FH (SCR1-4) crystal structure also revealed that FH acts as the binding platform for the serine protease FI and enables the sequential cleavage of C3b via stabilization of the TED-CUB positioning with respect to the core of the C3b molecule [9]; cleavage inactivates the C3b because the fragments cannot form a convertase. Notably, the interaction between FH SCR1 and C3b seems to be particularly important for the cofactor activity as was demonstrated by the different cofactor activities of FH variants Val62Ile (located in SCR1) [24].

Several crystal structures of complement regulators other than FH display a similar binding interface with C3b, although the SCRs involved vary depending on the regulator [13,25,26]. DAF requires SCR2-4 for DAA and MCP requires SCR3-4 for cofactor activity [13,27,28]. Three different SCR clusters (SCR1-3, SCR8-10 and SCR15-17) are involved in the DAA and cofactor activity of CR1 [29]. The subtle differences found in the C3b regions involved in the interactions with the regulators explain the different abilities of these proteins as regulators. For instance, in contrast to FH, DAF and CR1, MCP does not interact with the α' NT region of C3b, which explains its lack of DAA [13]. The cofactor activity of MCP, on the other hand, may be mediated in a similar way to that of FH, as indicated by the adoption of the same rearrangement of C3b domains MG2, CUB and TED that is critical for the strong cofactor activity of these regulators [30].

The shared C3b binding patterns of the different regulators suggests that the two inhibitory mechanisms (DAA and cofactor activities) have evolved from a common C3b-regulator binding origin [13]. This is not unexpected given the common evolutionary origin of these complement regulatory proteins. Notably, it has also been suggested that regulators of the classical pathway of complement activation likely bind

C4b through the same binding mode [31]. In fact, the three SCR clusters in CR1 involved in the binding to C3b are also implicated in the binding to C4b, although displaying differential C3b/C4b binding affinities and associated regulatory activities [32].

Pathogens have developed a wide range of evasion mechanisms to escape complement attack, and some of these mechanisms, resemble the way complement components interact in the host. Interestingly, microbial mimics of complement regulators such as the smallpox virus homolog (SPICE) and Kaposi's sarcoma-associated herpesvirus RCA homolog (Kaposica) bind in the same orientation and extended arrangement as the regulators at the binding platform on C3b [13,30].

4. Conformational flexibility of C3 in the assembly and regulation of the AP C3 convertase.

This mechanistic view has provided an explanation for most observations concerning AP C3 convertase assembly and regulation, but structural evidence beyond crystallography postulates a more complex scenario. Time-resolved FRET measurements using a fluorophore attached to the TED domain, structural studies of C3b using X-ray and neutron scattering and EM analyses have confirmed that C3b coexists in several conformations, and the positioning of the TED is not always adjacent to the MG ring, as it appears in the crystal structures (Fig. 1). A wealth of evidence suggests that such conformational flexibility can have a functional impact on the assembly and regulation of the AP C3 convertase.

Structural analysis combining EM and computational modeling has recently confirmed that the TED domain in C3b is ionically tethered to the macroglobulin (MG) ring [33]. Thus, ionic strength modulates the magnitude of the electrostatic forces anchoring the TED domain to the rest of the C3b molecule. These findings are consistent with previous data demonstrating the importance of the R102-E1032 salt

bridge to maintain the interaction between the MG ring and the TED domain in C3b [34].

It seems apparent that the conformational variability of the TED in C3b is higher than previously thought, providing a structural explanation for the functional differences between two variants of C3b in the common R102G polymorphism (rs2230199) [35]. Residue R102 (C3/S) is the most common C3 allele, whereas G102 (C3/F) is a risk variant for developing age-related macular degeneration (AMD). In C3b/F, G102 does not support an ionic interaction with residue E1032 and therefore is more prone to a displacement of the TED domain than in C3b/S (R102). The larger flexibility of C3b (G102) would rationalize why this allele binds the complement regulator FH less efficiently than the R102 variant, causing decreased FI cofactor activity and extended C3 convertase lifetime. Several mutations in C3 have been recently described and characterized where the pathogenicity associated with these genetic variants correlates with the conformational stability of the TED domain in C3b. These facts support the idea that the relative position of the TED is a critical structural element that modulates the interaction of C3b with complement regulators [36].

The issue of the conformational flexibility of the TED domain has been especially controversial in the case of iC3b, the first product of the FI-mediated proteolytic cleavage of C3b. Whereas iC3b cannot assemble a C3 convertase, it plays important effector and signaling functions through its interaction with the complement receptors CR1, CR2 and CR3. iC3b can be further cleaved to C3dg by FI in the presence of CR1. Despite its functional relevance, there is no high-resolution structure of iC3b yet. Several electron microscopy reports described iC3b as a flexible extended molecule [37,38] (Fig. 1). On the other hand, Alcorlo et al. found that iC3b appeared in a compact conformation with the TED domain placed in a position closely resembling that found in C3 [39]. Although these latter observations correlated well with previous spectroscopic data, structural studies using hydrogen-deuterium exchange mass spectrometry strongly support a disordered CUB domain and presents iC3b mainly as

a distorted extended molecule [40]. Hypothetically, the compact conformation of iC3b likely represents one of a multitude of possible conformations that may be enriched under certain experimental conditions. What the conformational flexibility of the TED would mean for iC3b function *in vivo* is still a matter of speculation, but one study has described two distinct conformations of iC3b on surfaces with differential binding to complement receptors [41].

The stability of the C3 convertase on complement-activating surfaces must be enhanced to allow complement to efficiently perform its functions *in vivo*. This is the role of properdin, the only positive complement regulator described to date. Initial EM studies of the properdin-C3bBb complex provided insights into the mechanisms by which properdin increases the stability of the C3b-Bb interaction.

Each properdin monomer is an elongated molecule that interacts with another properdin monomer forming a vertex, and it is this vertex structure that interacts with and stabilizes C3bBb. The EM images suggested that the properdin vertex contacted the C345C domain in C3b in the proximity of the Von Willebrand factor A (VWA) domain in the Bb fragment of FB. Hence it seems that properdin physically grabs and holds together both the C3b and FB components of the C3-convertase, increasing the stability of the complex. Recent, and much more detailed, crystallographic data of the properdin-C3bBb complex fully supports the EM data [15], but the higher resolution of the crystal structure shows that properdin contacts mostly with the C345C domain and that potential contacts with the vWA will be probably much less extensive than expected.

Another important observation is that binding of properdin to C3bBb increases the ratio of C3b molecules where the TED domain is repositioned. These observations have been interpreted as an additional mechanism to extend the lifetime of the C3 convertase by removing structural determinants needed for the interaction with the complement regulators FH, DAF and CR1 [14]. Overall, the mobility of the TED domain in C3b adds a new layer of complexity to complement regulation, which, inevitably,

seems to have been exploited by some pathogens to escape the host complement system. *Staphylococcus aureus* produces a protein, the extracellular fibrinogen-binding protein (Efb-C), which induces conformational changes in C3b that propagate through the molecule, displacing the TED domain to a position that impairs FB binding and C3 convertase assembly [42].

5. Complement regulation differentiates self from pathogens and altered self.

Because the continuous, low-level, spontaneous activation of C3 can trigger the AP everywhere, complement activation requires regulatory mechanisms that limit its action to the activating surface avoiding non-specific damage to host cells and preventing the consumption of its components. These regulatory mechanisms allow discrimination between pathogens, host cells and altered host cells so that the role of complement in fighting pathogens and removing cellular debris is not compromised. Complement regulation results from the collective activities of a group of plasma and membrane-bound regulatory proteins among which FH plays a leading role. Importantly, FH distinguishes host cells from pathogens by recognizing self-associated molecular patterns (SAMPs), such as glycosaminoglycan (GAG) chains, like heparin sulphates (HS) and sialic acids moieties which are ubiquitously found as part of the glycocalyx structures on mammalian tissues [43,44]. Variations in the composition and distribution of SAMPs, for which FH shows different affinities, contribute to restrict complement activation to particular sites, protecting other sites from complement damage [45,44].

While the complement regulatory activities of FH span SCR1-4 (FH₁₋₄), the ability of FH to discriminate complement-activating surfaces resides in the regions comprising SCR6-7 (FH₆₋₇) and SCR19-20 (FH₁₉₋₂₀). Both regions can bind to GAGs, but only SCR20 contains a sialic acid binding site [45]. In addition, the FH₁₉₋₂₀ region contains a C3b binding site that is required to target the FH regulatory activity to the complement-activating surface. A full-length high-resolution structure of native FH is

not available yet, nevertheless, recombinant fragments of different FH regions spanning the full length of the FH molecule have been investigated and their structures either alone or in complex with ligands have been determined (reviewed in [46,47]). All together, these data suggest that FH adopts a hinge-like conformation toward the middle of the molecule. This conformation is facilitated by the compact arrangement of the central SCR10-15 domains bringing together the N- and C-terminal regions and allowing them to interact with distinct sites over the same C3b molecule. The proposed bend-back architecture of FH would also facilitate the simultaneous interaction of FH with additional glycans via SCR6-8, increasing the capacity of FH to discriminate between complement-activating surfaces [48,47]. The crystal structure of C3d-FH₁₉₋₂₀ complexes has revealed that the interaction of FH₁₉₋₂₀ with C3d mostly involves SCR19 [49,12], while SCR20 may contribute with an additional anchor point to a secondary C3b molecule [49]. Most importantly, the site in the TED domain of C3b recognized by FH₁₉₋₂₀ is different from that recognized by the FH₁₋₄ region, which further support the possibility that both N-terminal and C-terminal regions of FH bind simultaneously to the same C3b molecule.

Interestingly, the Y402H polymorphism (rs1061170), strongly associated with the development of AMD, lies in SCR7 of FH, within the cluster of positively charged amino acids implicated in the binding of GAGs. Structural studies of a FH fragment including SCR6-8 (FH₆₋₈) have demonstrated the presence of multiple GAG-binding sites in this FH region, which is consistent with a single bound GAG molecule spanning these three SCRs [50]. These data also showed that the AMD-associated amino acid H402 residue is directly involved in GAG binding and suggest that switching between histidine and tyrosine at this position will change the GAG specificity. These findings, in the context of age-related alterations of retinal GAG composition help explain the association of this FH polymorphism with disease.

The specific recognition of SAMPs by the FH₁₉₋₂₀ region seems to involve more sophisticated mechanisms. Notably, Barbel *et al.* [11] solved the crystal structure of a

ternary complex formed by FH₁₉₋₂₀, a sialylated trisaccharide and the C3b TED domain, proposing a mechanism for the specific recognition of the main sialic acid variant in humans, Neu5Ac, by the C-terminal region of FH. According to this model, the sialic acid binding site, located in SCR20, only becomes accessible to the sugar after FH interacts with C3b (Fig. 2a). Thus, when FH interacts with the TED domain of C3b, the so-called hypervariable loop in SCR20 adopts an open conformation that allows the accommodation of the sialic acid. By contrast, when FH is unbound, the loop, and in particular the K1188 side-chain, obstructs the access to the sialic binding site (Fig. 2a) [51,11]. These structural analyses identified the relevant amino acids residues in FH₂₀ and the TED domains and showed that the key residues are highly conserved across mammals. These data have provided a structural framework that is proving extremely useful to understand the functional consequences of many disease-associated mutations identified in the genetic testing of patients with pathologies related to complement dysregulation.

The ability of FH to regulate complement activation on self and pathogen surfaces is modulated by the factor H-related proteins (FHRs) [52]. The FHRs are a family of five proteins (FHR-1, -2, -3, -4 and -5) structurally similar to FH. They lack the complement regulatory domains of FH, but have conserved the FH surface recognition domains, which confers the capacity to compete with FH for binding to complement-activating surfaces. This FH antagonistic function of the FHRs is termed complement de-regulation. A remarkable characteristic of FHR-1, FHR-2 and FHR-5 is that the two N-terminal SCRs, which are almost identical in these FHRs, include a dimerization domain [53]. This determines that these FHRs, in contrast to FH, always circulate in plasma as dimers or tetramers [53,54]. This structural organization increases the avidity of FHR-1, FHR-2 and FHR-5 for their ligands (C3b, iC3b, C3dg and polyanions like sialic acids, glycosaminoglycans or heparin), facilitating competition with FH. In addition, oligomerisation helps to generate a combinatorial repertoire of different FHRs,

which may be useful to provide a fine-tuning for identification of opsonised surfaces with different carbohydrate compositions.

A critical issue regarding the postulated competitor role of the FHRs is what prevents them from inhibiting the normal function of FH in protecting the host cell surfaces, thus causing disease. In the case of FHR-1, there are two amino acid differences (S1191, V1197 in FH; L290, A296 in FHR-1) between the C-terminal regions of FH and FHR-1. Interestingly, these differences involve two critical residues that appear sufficient to alter sialic acid recognition [11] (Fig. 2b, 2c), conferring distinct surface binding specificity to FH and FHR-1. In fact, functional analyses suggest that these amino acid substitutions do not inactivate the sialic acid binding site in FHR-1, but determine a change in its specificity [55,56]. As a whole the available data support that these two amino acid differences are sufficient to eliminate the risk of an undesirable competition between FH and FHR-1 from most host tissues. This implies that competition between FH and the FHRs may be limited to surfaces where particular variants of sialic acid are displayed. The presence of the activated C3 fragments iC3b and C3dg, which are better ligands for some FHRs than for FH [49,48], may be crucial to sustain the competition between FH and the FHRs at these surfaces. Additional studies are needed to define the ligands and surface specificities of the FHRs in more detail and also the conditions under which competition with FH occurs. This knowledge will advance our understanding of biological function of the FHRs and provide very relevant insights into the pathogenic mechanisms associated with a number of complement dysregulation disorders.

6. C3 to C5 substrate recognition shift by the AP C3/C5 convertase

In contrast with the precise knowledge that we have of the C3bBb convertase, the structure of the C5 convertase is largely unknown. The simplest form of the C5 convertase has been shown to be the C3 convertase C3bBb, but this so-called

monomeric C5 convertase has a very weak affinity for C5 and under physiological conditions and proteins concentrations will cleave C3 and C5 in approximately a 10000 to 1 ratio [57]. The endogenous, fully active, C5 convertase is found exclusively on surfaces. Work done during the last century showed that the clustering of additional C3b molecules around the C3 convertase increases 100-1000 fold its affinity for C5, switching the convertase substrate specificity from C3 to C5 [58]. The molecular basis for this remains a major challenge in complement structural biology. It was initially thought that formation of active classical pathway and AP C5 convertases involved covalent-linked C3b-C3b and C4b-C3b dimers [59]. Recent experiments using C3b coated beads have shown that the covalent association between C3b molecules is not necessary and instead the critical requirement to develop C5 convertase activity is a sufficiently-dense deposition of C3b with the thioester oriented towards the surface [60]. Whether the additional C3b molecules directly interact with C5 or change the conformation of the C3 convertase subunits to increase affinity for C5 is currently unknown.

Despite these difficulties in understanding the structural organization of the C5 convertase, the structure of the staphylococcal inhibitor SCIN stabilized C3bBb convertase [7] and the C5/CVF complex [61] have provided insights into how the C3 and C5 convertase may recognize their substrates. These data, together with previously available information from disease-associated mutations and complement inhibitors [61,62,58,63,9], have facilitated the structural modelling of the convertase-substrate complexes.

In particular, dimeric complexes observed in crystals of the AP C3 convertase (C3bBb) stabilized with SCIN strongly suggest that the interface between the two C3b monomers in the crystal truly represents the natural binding interface between C3 and C3b molecules during C3 substrate recognition by the AP C3 convertase [7]. This binding interface involves a large surface area of C3 (and C3b) including the domains MG3, MG4-5 and MG6-8. The contribution of these regions to substrate recognition is

further supported by the fact that the binding sites for the inhibitors compstatin [64], CRIg [65] and antibody S77 [66], all of which block C3 binding, map within the same area.

Notably, a deletion of two amino acids (Asp923, Gly924; 923delDG) in the MG7 domain of C3 that does not alter the normal expression and overall structure of C3 has been shown to render it resistant to cleavage by the AP C3 convertase [62]. The deletion displaces amino acid residues on either side from their positions in the wild-type protein structure. Moreover, on the N-terminal side of the deletion a surface-exposed loop (residues Val916 to Ser922) has been postulated as an additional interaction site between C3 and C3b [62]. The mutation data indeed suggest that C3 and C3b would contact at this region and that it is the presence of the SCIN inhibitor that separates them in the crystal structure.

In contrast to C3b, CVF binds with high affinity to C5, which has facilitated the crystallization of the CVF-C5 complex and its structural determination [61]. C5 and CVF are arranged in a head to head orientation with the two molecules aligned in parallel. Two separated regions mediate the interaction between both. The more extensive one involves contacts between the MG5 domains of both proteins. The second area is formed between the C5 MG7 domain and the MG6 and MG7 domains of CVF [61].

As a whole the structure of the dimeric C3bBb complexes stabilized by SCIN, the C5-CVF complex and numerous other experimental observations suggest that C3b and C4b in the C3 and C5 convertases recognize both C3 and C5 in a similar way to how CVF recognizes C5. This recognition involves two separate interfaces, the MG4-MG5 interface and the MG7 interface. Both regions in C3 and C5 are, in this respect, important therapeutic targets for inhibition of C3 and C5 activation. Not surprisingly, binding of most current C3 and C5 convertase inhibitors map to these regions. Among them, eculizumab, a humanized antibody against C5 [67], the first complement inhibitor of the terminal pathway approved to prevent the consequences of complement

dysregulation in patients with paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome, binds to an epitope within the MG7 domain [68] that would cause steric hindrance of C5 binding to C3b in the proposed substrate recognition model [60].

7. Assembly of the MAC generates more than a lytic pore

Proteolytic cleavage of C5 by the C5 convertase initiates the terminal pathway of the complement system, which concludes in the assembly of the immune pore called the membrane attack complex (MAC). The MAC mediates cytotoxicity in a wide range of gram-negative bacteria, in particular *Neisseria* species [69]. Sublytic MAC concentrations are related to a number of cell signaling pathways including inflammasome activation [70] and proliferation [71].

Recent structural studies of the MAC by cryo-electron microscopy (Fig. 3a) [21] and cryo-electron tomography [72] have shed light on the assembly mechanism of the pore. In addition to C5b, this complement effector is formed by a number of soluble proteins, named C6, C7, C8 and C9, which sequentially and irreversibly assemble within cell membranes (Fig. 3b). All of the MAC components except C5b share a common architecture with a central domain called CDC/MACPF (cholesterol-dependent cytolysin/membrane attack complex and perforin) and a number of auxiliary domains. The central CDC/MACPF domain contains two helical clusters responsible for membrane penetration through a major conformational change during complex assembly [73]. Upon C5b formation, C6 recognizes and binds a labile binding site in the former to create the C5b6 complex, constituting the scaffold for MAC assembly [74]. C7 binds to C5b6 and renders the complex lipophilic by unfurling the CDC/MACPF helical clusters into β -hairpins. The binding of C8 trimeric protein sets the curvature of the pore wall and regulates the binding of C9, the last component of the MAC. C9 is the main constituent of the immune pore since it oligomerizes with up to 18 subunits aligning following the geometrical template set by C8. The MAC structure

reveals an important role of the auxiliary domains of the MAC components in establishing specific subunit interactions and holding the complex together [21].

Productive MAC assembly necessarily has to be initiated near the membrane-attached C5 convertase so that MAC precursors can quickly find a nearby membrane [75,74]. This could be facilitated by the existence of transient pre-activation interactions that would keep them nearby so they are immediately accessible for complex assembly [76]. Moreover, it has been proposed that C7 binding to C5b6 could drive the release of the latter from the C5 convertase [77], thus keeping the MAC precursor close to the target membrane until C7 binding allows membrane penetration. Another important factor in MAC assembly dynamics is that this process is also time controlled through the half-lives of some intermediates, which varies significantly. In this way, cleavage of C5 induces a protein domain reorganization exposing the C6 binding site, which is exceedingly labile, with a half-life of about 2 minutes [78,79]. If C6 binding is accomplished within this interval then MAC assembly will proceed [74].

Intriguingly, the absence of any available cell membrane during this process would lead to the formation of MAC-related soluble complexes with relevance in complement activation. In particular, recruitment of the molecular chaperones clusterin and vitronectin to an off-target assembled pore turns it into a soluble complex called sC5b-9 whose membrane penetration capacity is lost [18]. The importance of this complex as a biomarker for complement activation in a number of human diseases has been proved [80-82], whereas its physiological relevance is still under discussion. It has been suggested that the sC5b-9 membrane binding activity is recovered by dissociation of the chaperones [83].

In contrast to other similar immune pores, the MAC is an asymmetric pore that shows a split-washer configuration [21,72]. This asymmetry has important biological consequences and it is notably reflected in the partial membrane penetration of the MAC precursors. Contribution to local membrane distortion by the MAC might have relevant implications in cellular signaling and suggest that the MAC could function as a

lipid composition sensor [21,84]. In fact, there is evidence that MAC lesions are preferentially formed on lipid membrane microdomains enriched in cholesterol and diacyl glycerol [85,84].

The actual MAC-mediated lysis mechanism is still a matter of controversy and newly described structural features further support the concept that the MAC is more than just an immune pore that punches holes in membranes and drives osmotic lysis [58,84]. Interestingly, members of the same CDC/MACPF protein family such as perforin and bacterial CDCs form both complete (ring-pores) and incomplete (arc-pores) homo-oligomeric structures on target membranes that are sufficient for cell lysis [86,87]. Hence, it cannot be excluded that the MAC could mediate cell lysis by forming arc-pores. In fact, classic photolabelling experiments showed that formation of C5b-8 and especially C5b-9 caused effective lysis in liposomes in the absence of classical MAC pores [88], hinting that complete C9 oligomerization might not be essential in that process. In this sense, it has also been found that C9 alone is able to self-oligomerize under certain *in vitro* conditions [89,90] while MAC precursors (i.e. C5b6, C7 and C8) are obligate components for pore formation under physiological conditions. These features point out the importance of the MAC precursors in the initiation and regulation of the complex assembly rather than in membrane penetration.

The only known regulator of the MAC is a GPI-anchored protein named CD59 that constitutes the main defense against host-damage by MAC lesions. A crystal structure of the CDC Intermedilysin (ILY) bound to CD59 [91] has shed light on the CD59-mediated MAC regulation. It has been shown that ILY directly competes for the MAC binding site on CD59 [92] and that CD59 intercalates ILY monomers and coordinates their oligomerization into a membrane pore [91]. Similarly, CD59 would intercalate between C8 and C9 and inhibit MAC formation by limiting the input of C9 [93,94]. Interestingly, viruses such as HIV, human cytomegalovirus and vaccinia incorporate host cell CD59 into their own viral envelopes to evade complement-mediated lysis [95,96]. Even though the regulation of this complement effector assembly has an

impact on a number of important human diseases, many questions still remain to be addressed. On one side and in contrast with the CDCs, there is no identified membrane receptor that triggers MAC assembly on the cell surface. Moreover, upon enzymatic activation of C5, the remaining components proceed by adsorption without requiring additional cofactors.

8. Conclusions

The processes of complement activation and regulation, how the individual complement proteins that circulate as zymogens get activated and how they interact with other complement components to build the protein complexes that are relevant for complement biological activities have been worked out in great detail during the last decade. This mechanistic understanding has been the result of a multidisciplinary approach in which different structural biology methodologies and biochemical approaches have contributed individual frames assembling into a rather complete tridimensional movie of the whole process. We are still missing frames in that movie. Mainly those dealing with processes that involve elongated, flexible complement components, or processes that happen on the cellular surfaces, which are still difficult to tackle with the current structural biology methodologies, but we will see them solved in the near future. We have discussed here how these structural data have both provided support to previous knowledge and added alternative and unexpected insights into complement function. From different perspective, this knowledge also has an immediate educational benefit that should facilitate accessibility to complement biology by outsiders to the field. In practical terms, understanding the structural basis of complement activation and regulation has a direct impact on the interpretation of complement genetic variability that is revealed by genetic studies; in one way this is because it helps reveal the pathogenic mechanisms underlying a particular disease, in another because this mechanistic understanding of complement has become, together with functional assays, critical to categorize the disease-associated genetic variants

unveiled by genetic testing of patients. Structural knowledge is particularly valuable for those disease-associated gene variants located in functionally relevant regions that do not impact the overall structure of the protein but may cause modifications in their functional activities. Finally, it is expected that all these novel structures will help in the discovery of novel therapeutic targets for the generation of more efficient and specific drugs to control complement dysregulation in a number of pathological situations.

Figures Legends

Fig. 1 Structural flexibility of C3b and iC3b.

Cartoon representing the conformational flexibility of C3b and iC3b and its potential influence in complement regulation. The TED domain can display a range of conformations around the MG ring in C3b. The TED domain attaches to the MG ring in several crystal structures, but other structural methods have characterised alternative positions for the TED domain that are believed to affect the interaction with regulators. Asterisk (*) indicates the conformation of C3b with the TED domain in close proximity to the MG ring that favor the interaction with the complement regulators FH, MCP and CR1 (depicted as a green structure with four SCRs in the figure). iC3b recapitulates some of this conformational flexibility. Adapted from Alcorlo *et al.* [39].

Fig. 2 Structure of the sialic binding site in FH and FHR-1

(a) Superposition of the crystal structures of FH SCR20 in the absence of ligand (green) (PDB ID: 3SW0) or in complex with C3d and sialic acid (red) (PDB ID: 4ONT). Salt bridges with the sialic acid (SA) and residue D1115 in C3b are shown as light blue circles lines. The sialic acid molecule (SA) is shown in grey-white. The displacement of residues K1188 and R1215 in both structures is indicated with an orange curved arrow. (b) Superposition of the crystal structure of SCR5 of FHR-1 in the absence of ligands (yellow) (PDB ID: 4MUC) with the previous FH SCR20 structures. (c) Molecular surface comparison of the ligand binding pocket, FH SCR20 without ligands (red), FH SCR20 in complex with C3d and sialic acid (green) and FHR-1 without ligand (yellow); sialic acid is shown in grey-white and its position maintained in all three structures for comparative purpose. The FH sialic acid binding pocket is partially occluded by the K1188 side-chain and seems to become accessible upon the salt-bridge formation with residue D1115 in C3d. In contrast the FHR-1 ligand-binding pocket seems to be partially accessible even in the absence of ligand.

Fig. 3 Assembly of the membrane attack complex (MAC).

(a) Cryo-EM structure of the membrane attack complex (MAC). Two views of the MAC reconstruction coloured according to complement proteins as indicated in panel B. Scale bar represents 5 nm. (b) Diagram representing the MAC formation. Newly released C5b (pink) initiates the assembly of the complex and sequentially binds C6 (light blue), C7 (green), C8 (orange, red and dark blue for C8 α , C8 β and C8 γ

respectively) and finally C9 (yellow). The latter component oligomerizes to complete the immune pore. sC5b9 is formed by addition of chaperones clusterin and vitronectin to an off-target MAC intermediate. Adapted from Serna *et al.* [21].

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