Chapter 7
How Structures of Complement Complexes Guide Therapeutic Design

Jasmine K. Bickel, Tomas B. Voisin, Edward W. Tate, and Doryen Bubeck

Abstract The complement system is essential for immune defence against infection and modulation of proinflammatory responses. Activation of the terminal pathway of complement triggers formation of the membrane attack complex (MAC), a multi-protein pore that punctures membranes. Recent advances in structural biology, specifically cryo-electron microscopy (cryoEM), have provided atomic resolution snapshots along the pore formation pathway. These structures have revealed dramatic conformational rearrangements that enable assembly and membrane rupture. Here we review the structural basis for MAC formation and show how soluble proteins transition into a giant β-barrel pore. We also discuss regulatory complexes of the terminal pathway and their impact on structure-guided drug discovery of complement therapeutics.

Keywords Membrane attack complex · Complement therapeutics · Structure-guided drug discovery · Cryo electron microscopy · MACPF · C5

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Complement

Complement is a protein-based network that is a fundamental component of immune defence. This complex system involves over 30 plasma and membrane-associated serum proteins. Complement proteins are involved in all stages of immune clearance from initial pathogen detection to elimination of infection (Fig. 7.1) (Ricklin et al. 2010). A complement response can be initiated either by recognizing the surface-bound sugars of pathogens, through the lectin pathway, or the antigens presented on cell surfaces, triggering the classical pathway. By contrast, the alternative pathway of complement is initiated by spontaneous hydrolysis of the serum protein C3 to form C3b, a process referred to as tick-over (Pangburn et al. 1981; Bexborn et al. 2008). Broadly, complement activation triggers proteolytic cleavage events that irreversibly propagate a series of cascades. If left unchecked, these cascades cause proinflammatory responses, phagocytosis of microbes, and lysis of targeted cells (Ricklin et al. 2016). Complement also modulates trafficking of foreign antigens for clearance by T- and B-cell lymphocytes, providing a functional bridge between adaptive and innate immune responses (Carroll 2004; Dunkelberger and Song 2010).

The terminal pathway is the direct killing arm of complement and can be activated by any of the upstream cascades (Fig. 7.1). The lectin, classical, and alternative pathways generate a complex that cleaves complement protein C5, referred to as the C5

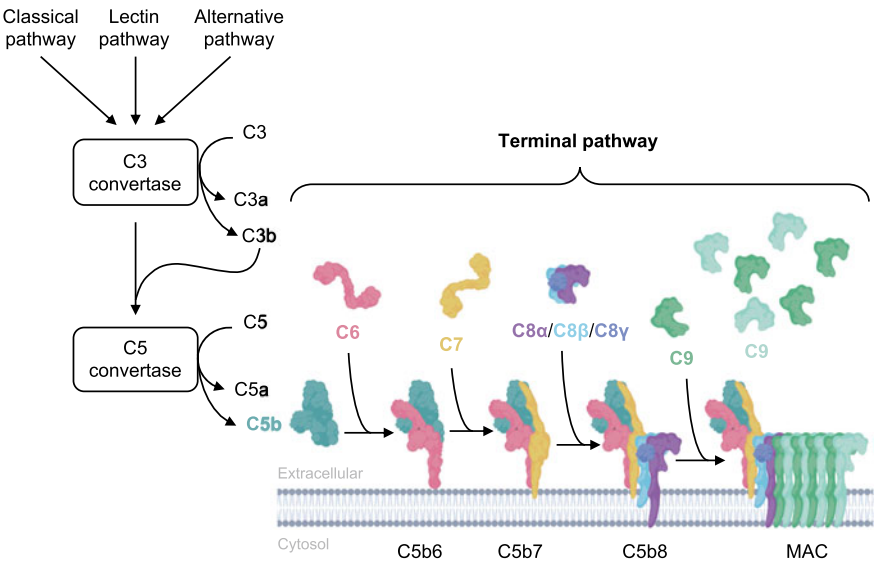

Fig. 7.1 Schematic outlining how the three upstream complement cascades (classical pathway, lectin pathway, and alternative pathway) activate the terminal pathway to generate the MAC pore. MAC is formed on target cell membranes by the sequential assembly of C5b, C6, C7, C8 and several copies of C9. Intermediate subcomplexes (C5b6, C5b7, C5b8) and a cross-section through the final pore are shown

convertase. The proteolytic activity of the C5 convertase is responsible for the irreversible assembly of the membrane attack complex (MAC), a multi-protein immune pore that ruptures membranes (Bubeck 2014; Bayly-Jones et al. 2017). MAC directly lyses and kills a multitude of pathogens, including enveloped viruses, parasites, and Gram-negative bacteria (Hoover et al. 1984; Tomlinson et al. 1989; Nakamura et al. 1996). Furthermore, the pore is essential for defence against *Neisseria meningitidis*, with genetic deficiencies in terminal complement proteins leading to recurrent meningococcal and gonococcal infections (Harriman et al. 1981; Schneider et al. 2007; Botto et al. 2009).

Beyond lysis, deposition of MAC pores on the surface of self-cells impacts diverse cellular processes. These pores, referred to as sub-lytic, cause an influx of calcium ions (Ca^{2+}) into the cell and activate downstream effector functions ranging from proliferation to apoptosis (Morgan 1989; Xie et al. 2020). Ion influx can also induce proinflammatory responses (Morgan 2016), resulting in secretion of cytokines such as interleukin (IL)-6, -8, and the vascular endothelial growth factor (VEGF) (Lueck et al. 2011). The effects of sub-lytic MAC include further downstream inflammatory effectors and initiate NLRP3 inflammasome assembly (Triantafilou et al. 2013). While many of these interaction partners have yet to be identified, a signalling complex within Rab5(+) endosomes has been shown to activate non-canonical NF-κB in endothelial cells (Jane-wit et al. 2015; Fang et al. 2019).

The balance between activation and regulation of complement is essential for tissue homeostasis during an immune response. Understanding the assembly and regulation of MAC at a molecular level will likely underpin the development of new therapeutics that tune complement activity. In this chapter we will review individual terminal pathway proteins and how they assemble into a lytic pore on target cells. We will also investigate the structural basis for how this process is controlled, starting from initiation of the terminal pathway by the C5 convertase to catching MAC pores on the membrane at the final step in the cascade. Finally, we will discuss how structural studies of complement complexes can support drug discovery.

Understanding MAC at the Molecular Level

The "Resolution Revolution" of cryo-electron microscopy (cryoEM) has swept across the complement field, providing scientists and clinicians an unprecedented view into how these complex machines function. The first electron microscopy images of the MAC were taken in the early 1980s and in agreement with electrophysiology experiments, these images revealed the formation of a large ring-shaped pore that punched holes in target membranes (Podack et al. 1980). However, crucial details about how individual blood-based proteins convert to form a transmembrane pore remained unanswered. In the last few years, cryoEM has transformed the field of structural biology, with the biggest step change coming from improvements in image detection that allow direct recording of electrons (Faruqi and Henderson 2007; Li et al. 2013; Wu et al. 2016). New computer algorithms have exploited the better

signal-to-noise ratio of the collected images, dramatically improving the accuracy of aligning individual single particles (Punjani et al. 2017; Zivanov et al. 2018). As a result, multiple conformations of complexes can be disentangled to provide not just one structure, but a portfolio of snapshots illustrating machines in action. This type of in silico purification is essential when trying to visualise complexes where further biochemical purification is simply not possible. Further adding to the complexity of preparing homogeneous samples for structural studies, large macromolecular complexes are often extremely flexible. The inherent mobility of these machines limits their ability to be captured within the restraints of a crystallographic lattice, an obligate requirement for X-ray diffraction experiments. With its ability to visualise large heterogeneous complexes at atomic resolution, cryoEM has revealed key insights into both the assembly and regulation of the complement MAC pore (Dudkina et al. 2016; Sharp et al. 2016; Serna et al. 2016; Menny et al. 2018; Spicer et al. 2018).

Molecular Assembly of MAC

Activation of the terminal pathway initiates an irreversible signalling cascade that ends in the formation of MAC (Fig. 7.1). The C5 convertase triggers MAC assembly on target cells by proteolytically cleaving C5 into two fragments: C5a and C5b (DiScipio et al. 1983). While C5a is a small anaphylatoxin that goes on to activate inflammatory pathways through GPCR signalling (Gerard and Gerard 1991; Guo and Ward 2005), C5b serves as a platform for assembly of complement proteins C6, C7, C8 and C9 (Podack 1984). The proteolytic cleavage of C5 causes a dramatic conformational re-arrangement of its domain architecture (Fig. 7.2a, b) (Hadders et al. 2012). The thioester domain (TED) of C5 is released like a coiled spring to extend half-way along a scaffold of macroglobulin (MG) domains. C6 captures the newly cleaved C5b to form the C5b6 complex (Fig. 7.2c) (Cooper and Müller-Eberhard 1970; Hadders et al. 2012). In the absence of C6, this intermediate C5b conformation is highly labile and will decay to a state unable to continue the cascade (Cooper and Müller-Eberhard 1970). In plasma, C6 is an elongated molecule whereby its core Membrane Attack Complex Perforin Fold (MACPF) domain is sandwiched by a series of smaller auxiliary domains tethered by flexible linkers (Aleshin et al. 2012). The crystal structure of C5b6 revealed that the C-terminal domains of C6, together with a flexible linker, trap the transient position of the C5 TED domain and are essential for initiating MAC formation (Hadders et al. 2012). C7 is the next protein to join the growing MAC precursor. Although there is currently no structural information for soluble C7, the protein is thought to undergo dramatic conformational rearrangements upon membrane insertion. C7 displaces the assembly precursor from the convertase and results in irreversible interactions with the membrane (Preissner et al. 1985; DiScipio et al. 1988). The hetero-trimeric protein C8, comprised of C8α C8β and C8γ, is subsequently recruited. Large-scale conformational changes in C8 induced by binding the MAC precursor enable C8β to associate with the newly

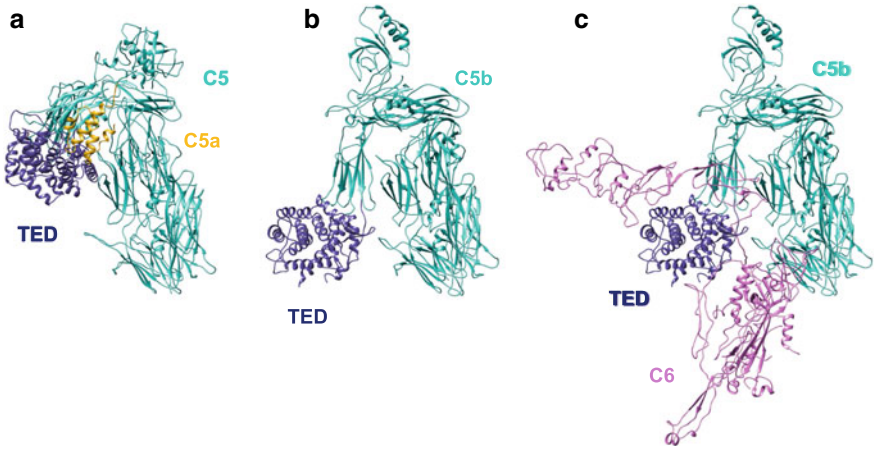

Fig. 7.2 Conformational changes of C5 initiate MAC assembly. **a** Ribbon diagram of the C5 crystal structure (PDB 3CU7). C5a is shown in gold, the TED domain in dark blue and the remainder of the structure in turquoise. **b** Ribbon diagram showing the conformation of C5b within the C5b6 complex (PDB 4A5W). Following cleavage of C5a, the C5b TED domain (dark blue) moves along the MG scaffold. **c** Structure of the C5b6 complex. Binding of C6 (magenta) holds the C5b TED domain (dark blue) in position. Remainder of C5b is in turquoise

exposed face of C7 (Stewart et al. 1987; Brannen and Sodetz 2007). In addition, C8γ rotates from its position in the soluble complex to reveal the nascent C9 binding site on C8α (Serna et al. 2016). C9 is the last component of the cascade and homo-oligomerizes to complete the final pore (Podack et al. 1982; Tschopp et al. 1985; Parsons et al. 2019).

Interactions with the Membrane

Complement proteins interact with the target membrane at each stage of MAC assembly. The C5b6 complex, though soluble, can bind reversibly to lipid bilayers (Silversmith and Nelsestuen 1986; Parsons et al. 2019). Biophysical experiments have shown that C5b6 preferentially binds negatively charged lipids (Yorulmaz et al. 2015; Parsons et al. 2019). This interaction reduces the energy required to bend the lipid bilayer and primes the membrane for rupture (Menny et al. 2018). Based on current structural data, it remains unclear which residues of C5b6 are initially responsible for membrane binding, however, an α-helix with amphipathic characteristics in the C6 thrombospondin domain 1 (TS1) is a potential candidate (Aleshin et al. 2012; Hadders et al. 2012). Binding of C7 to membrane-bound C5b6 triggers the irreversible association of the complex with lipids, while C8 is the first component to pierce the bilayer (Podack et al. 1982; Steckel et al. 1983). Incorporation of C8 renders the membrane permeable to Ca^{2+} ions (Campbell et al. 1981; Morgan 1989),

which in turn causes the membrane to stiffen (Menny et al. 2018). The addition of C9 into the assembling MAC results in further rigidification of the bilayer as the pore widens (Menny et al. 2018).

MAC is comprised of an asymmetric and irregular β-barrel pore (Sharp et al. 2016; Serna et al. 2016; Menny et al. 2018). Unlike related homo-oligomeric pores, MAC is a hetero-oligomeric complex whereby each complement protein contributes two β-hairpins (Menny et al. 2018). The length and sequence of these contributing β-hairpins differ across the complex. Hairpins of early assembly precursors skate across the outer leaflet of the lipid bilayer and are anchored by aromatic residues at the tips. A positively charged patch above the hairpin tips likely interacts with lipid headgroups and distorts the planarity of the bilayer. By contrast, contributing hairpins of C8 and C9 are longer and lack the nonplanar charged patch exhibited by earlier components of the assembly (Menny et al. 2018). Residues of C8 and C9 that face the lipidic edge form a hydrophobic band the width of a bilayer, consistent with a transmembrane pore.

The principal pore-forming component of MAC lies within its MACPF domain. With the exception of C5b and C8γ, all MAC proteins possess a MACPF domain (Hadders et al. 2007; Lovelace et al. 2011; Aleshin et al. 2012; Spicer et al. 2018). The fold is characterized by a central kinked β-sheet, sandwiched by two helical clusters (Fig. 7.3) (Hadders et al. 2007; Rosado et al. 2007). Early crystallographic studies of complement proteins and bacterial pore-forming toxins of the cholesterol-dependent cytolysin (CDC) superfamily revealed that despite no obvious sequence similarity, their structures were highly conserved (Fig. 7.3) (Rossjohn et al. 1997; Polekhina et al. 2005; Hadders et al. 2007; Rosado et al. 2007; Law et al. 2010; Lovelace et al. 2011; Aleshin et al. 2012). During pore formation, the MACPF domain of complement proteins undergoes dramatic changes in secondary structure. Residues comprising one of the helical clusters unfurl to form two transmembrane hairpins (TMH) (Shepard et al. 1998; Shatursky et al. 1999; Rosado et al. 2007). Oligomerization of complement proteins during MAC assembly results in the alignment of these hairpins to form a large β-barrel pore (Fig. 7.4) (Hadders et al. 2012; Serna et al. 2016; Menny et al. 2018).

Structure of MAC

MAC is a multiprotein hetero-oligomeric pore formed from the assembly of MACPF domains. Taking advantage of recent advances in cryoEM, the MAC structure revealed the composition of the oligomer for the first time (Serna et al. 2016). The structure showed that the assembly precursor is comprised of a single copy of C5b6, C7 and C8, forming an integral component of the pore. The pore is completed by the addition of 18 copies of C9, which homo-oligomerize in the membrane (Fig. 7.4). Unlike other previously reported homo-oligomeric pores (Law et al. 2010; Dudkina et al. 2016), the MAC β-barrel is not symmetric (Sharp et al. 2016; Serna et al. 2016).

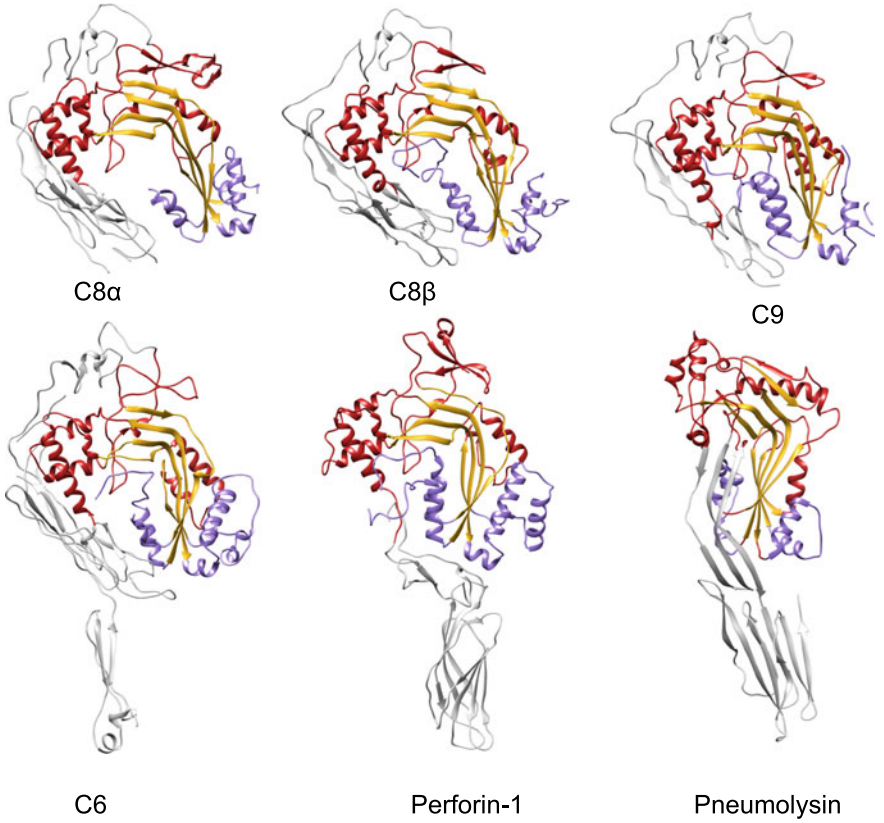

Fig. 7.3 Structural similarities between MACPF/CDC-containing proteins: C8α (PDB 3OJY), C8β (PDB 3OJY), C9 (PDB 6CXO), C6 (PDB 3T5O), Perforin-1 (PDB 3NSJ), and Pneumolysin (PDB 5AOD). MACPF/CDC domain (coloured) contains a bent β-sheet (gold) surrounded by helical clusters (red, purple). Residues that unfurl into transmembrane β-hairpins are purple. All other domains are in grey. Complement control protein (CCP)1, CCP2, TS2, TS3, Factor I membrane attack complex (FM)1 and FM2 domains of C6 are not shown for clarity

Instead, complement proteins are arranged in an asymmetric 'split-washer' configuration whereby the seam of the oligomer is not fully closed (Fig. 7.4b) (Serna et al. 2016). The unusual 'split-washer' architecture observed for detergent-solubilized MAC pores was confirmed by cryo-electron tomography studies of MAC embedded in a lipid model membrane (Sharp et al. 2016).

The interfaces between MACPF domains allow for a flexible assembly of proteins. This conformational variability of MAC accommodates variations in curvature and limited the resolution of these early structures. Using computational approaches that separated stoichiometrically equivalent states, the recent cryoEM MAC structure identified two distinct conformations, 'open' and 'closed' (Fig. 7.4) (Menny et al. 2018). Both states exhibit the characteristic 'split-washer' configuration of MAC,

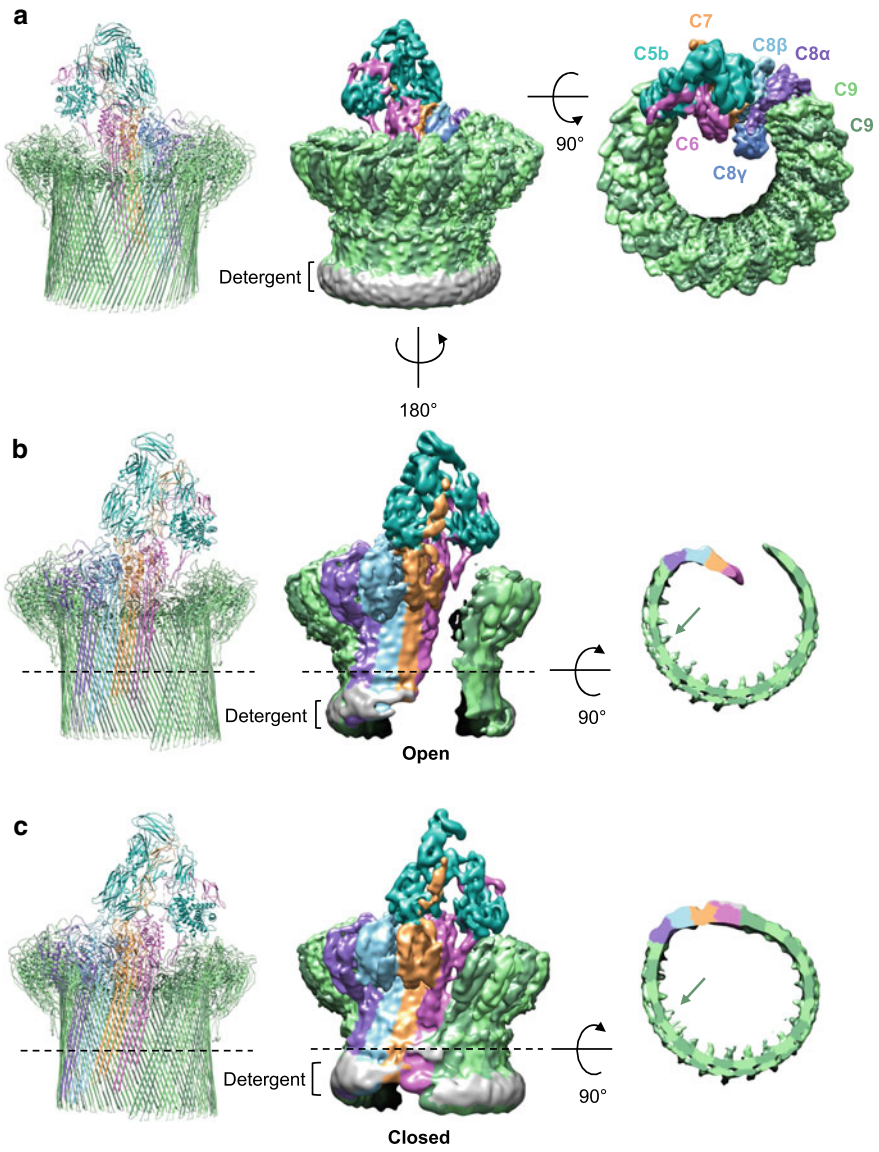

Fig. 7.4 CryoEM structure of MAC. **a**–**b** Ribbon diagram representation (PDB 6H03) and cryoEM reconstruction (EMD-0106) of the MAC 'open' conformation. Map and models in panels **a** and **b** are related by a 180° rotation. **c** Ribbon diagram (PDB 6H04) and cryoEM density map (EMD-0107) of the 'closed' conformation, orientation as in **b**. Dashed line in **b** and **c** indicate the position of the cross-section shown in the far-right panels. Green arrows indicate density for a C9 glycan. Maps and models are coloured according to protein composition, with the detergent belt indicated

however the central β-barrel is sealed in only the closed form (Fig. 7.4b, c). Further investigation is required to understand if these states are specific endpoints or exist in a dynamic equilibrium. The inherent flexibility of the MAC may be important for its ability to navigate complex target membrane environments. The surfaces of Gram-negative bacteria are coated with lipopolysaccharides and their outer membranes are densely packed with porins (Konovalova et al. 2017). An assembly that can adopt flexible curvatures could increase its efficiency for making large lesions; the MAC pore is over 100 Å in diameter (Serna et al. 2016; Menny et al. 2018). Furthermore, interconversion between the open and closed forms may add additional mechanical strain on the lipid bilayer, contributing to membrane destabilization.

Amidst this flexibility, MAC maintains its giant β-barrel through a number of core structural features. The central MACPF domains of complement proteins provide a major interaction interface for the complex and a sound foundation for the assembly (Hadders et al. 2012). However, the most extensive interfaces are located nearly 100 Å above the lipid bilayer. Along the barrel there are three key elements that prevent collapse of the β-barrel in the absence of large buried surface area (Menny et al. 2018). Firstly, The C-terminus of C9 wraps across the newly extended hairpins of an adjacent monomer. The collective C-termini then form a belt around the outer surface of the barrel. Juxtaposing the belt on the lumen of the barrel are concentric rings of glycans that provide shape and stability to an otherwise unsupported structure (Fig. 7.4c). Finally, disulfide bonds within β-hairpins further ensure that elongated strands maintain their secondary structure.

Converting Soluble Proteins into a Transmembrane Pore

MAC assembly requires large scale structural rearrangements of soluble proteins to form a transmembrane pore. In addition to the core pore-forming MACPF domain, complement proteins possess a number of auxiliary domains that ensure activation happens at the right place and time. Conserved across all MACPF-containing MAC proteins (C6, C7, C8α, C8β and C9), are core regulatory modules that include epidermal growth factor (EGF) and thrombospondin (TS) domains (Fig. 7.5) (Menny et al. 2018). Upon incorporation into MAC, EGF domains of complement proteins shift away from the helical bundles responsible for forming transmembrane hairpins (Menny et al. 2018). Conformational changes in both EGF and TS domains expose interfaces primed for binding the next component of the assembly. Likewise, a helix-turn-helix (HTH) in the MACPF which does not have a direct role in membrane insertion is displaced by adjacent monomers (Menny et al. 2018). C8γ has a similar role in the heterotrimeric C8 complex. Compared to its soluble form, C8γ rotates about an extended hairpin to expose the C9 binding site (Serna et al. 2016; Menny et al. 2018). While not essential for pore formation, C8γ enhances the efficiency of MAC assembly (Parker and Sodetz 2002), consistent with its role in regulating accessibility for C9. Conformational changes of auxiliary domains reveal

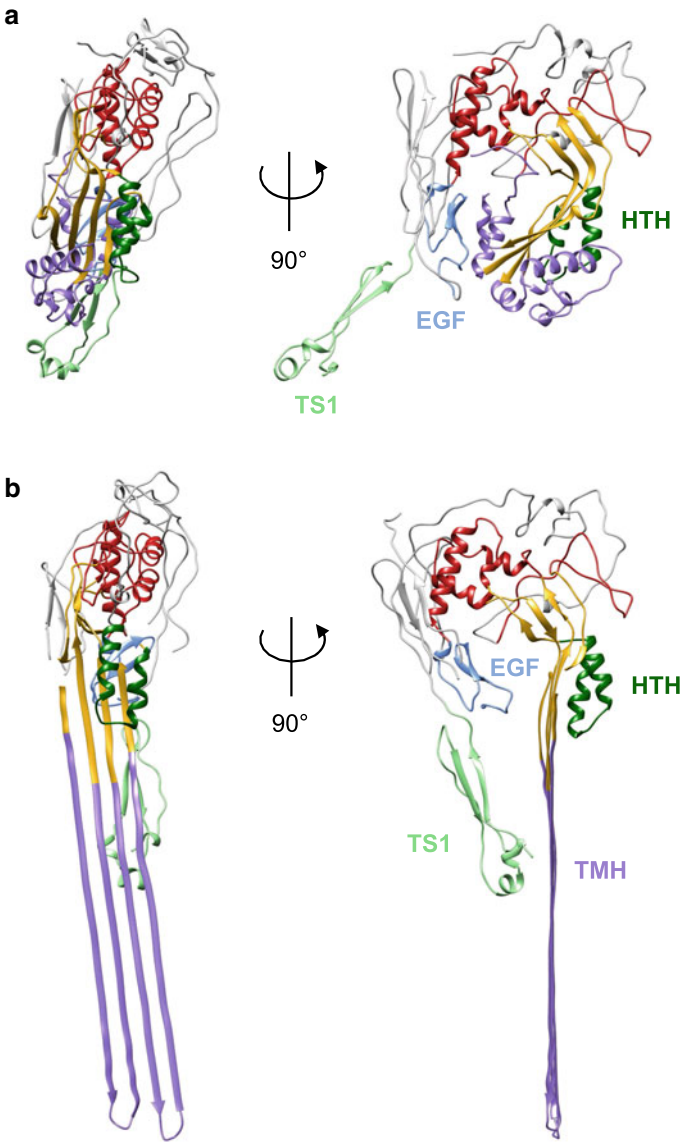

Fig. 7.5 Conformational changes in C6 upon MAC formation. **a** Ribbon diagram of soluble C6 (PDB 3T5O). **b** Ribbon diagram showing the conformation of C6 within the MAC 'open' conformation (PDB 6H03). Conformational rearrangements of the thrombospondin (TS) 1; green, epidermal growth factor (EFG; blue) and MACPF domains are highlighted. Within the MACPF: central β-sheet is gold, helical cluster is red, helix-turn-helix motif (HTH) is dark green, residues that form transmembrane hairpins (TMH) are purple. Low-density lipoprotein receptor class A, TS2 and TS3 are grey. All other domains have been removed for clarity

specific and highly charged surfaces on the leading MACPF edge. Oligomerization of MAC is driven largely by complementarity of electrostatic surface potentials between the newly exposed leading edge and the already accessible face of the next soluble complement protein (Menny et al. 2018). While there are currently no structures available of complexes stalled during assembly, C9 mutants that prevent the helix-to-hairpin transition of transmembrane residues also prevent further C9 oligomerization (Spicer et al. 2018).

Regulating MAC

During an immune response complement activation goes into overdrive. Unlike related bacterial homologues (CDCs), there is no lipid specificity for MAC pore formation. Once the terminal pathway is activated on a cell surface, any lipid bilayer is susceptible to rupture. As such, there are two main checkpoints for blocking MAC. The first prevents initiation of the cascade by inhibiting C5 cleavage. The second blocks membrane insertion at the final stages. Off-target assembly complexes are also scavenged and cleared by blood-based chaperones. Dysfunction in either of these control pathways can have devastating consequences for human health.

Overwhelming intrinsic control mechanisms for upstream complement pathways has knock-on consequences for MAC-mediated cell damage. Atypical haemolytic uremic syndrome (aHUS) is a disease characterized by acute renal failure and haemolytic anaemia (Noris and Remuzzi 2009). The pathogenesis of aHUS is heavily affected by uncontrolled amplification of the complement alternative pathway, resulting in overproduction of the C5 convertase on cell membranes (Noris and Remuzzi 2009; Roumenina et al. 2011). Consequently, MAC inserts into renal capillary endothelial cells causing damage, while C5a triggers proinflammatory signals in nearby immune cells. Similarly, age-related macular degeneration (AMD) is a disease associated with over-activation of complement and increased MAC deposition on retinal pigmented epithelial cells in the macula (McHarg et al. 2015).

Dysregulation of the terminal pathway has been implicated in the pathogenesis of a variety of autoimmune disorders as well as cancer. Paroxysmal nocturnal haemoglobinuria (PNH) is a rare and severe blood disease whereby patients critically lack CD59, the only membrane-bound regulator of MAC (Brodsky 2014). As a result, the red blood cells of these patients are vulnerable to complement attack and MAC-mediated haemolysis. Conversely, overexpression of CD59 has been implicated in the immune evasion mechanism in a variety of cancers, including breast, prostate, lung, ovarian, colorectal, and cervical carcinomas (Bjøge et al. 1994; Bjørge et al. 1997; Jarvis et al. 1997; Varsano et al. 1998; Jurianz et al. 1999; Gelderman et al. 2002; Fishelson et al. 2003; Donin et al. 2003). Overexpression of CD59 on cancer cells contributes to resistance of several monoclonal antibody anti-cancer therapies, including rituximab, a treatment for non-Hodgkin's B-cell lymphoma and chronic lymphocytic leukaemia (Treon et al. 2001; Smith 2003).

Targeting C5 Activation

Complement inhibition is a major focus of pharmaceutical research (Morgan and Harris 2015). Most therapeutic strategies focus on inhibiting the terminal pathway by targeting C5. Specifically blocking C5 reduces potential side-effects by preserving the immunoprotective and immunoregulatory functions of upstream complement cascades. This strategy is also versatile in that MAC is inhibited regardless of the upstream activating pathway. Furthermore, preventing C5 cleavage also suppresses the release of C5a, a potent anaphylatoxin implicated in inflammatory diseases including rheumatoid arthritis and sepsis (Woodruff et al. 2011).

Eculizumab is a potent anti-C5 monoclonal antibody therapeutic that prevents cleavage of C5 by the C5 convertase (Rother et al. 2007). Approved by the Food and Drug Association (FDA), eculizumab is used for the treatment of both PNH and aHUS (Hillmen et al. 2006; Zuber et al. 2012). The structure of C5 in complex with eculizumab revealed that the antibody specifically binds the MG7 domain of C5 and may act by sterically blocking the convertase binding-site (Fig. 7.6a) (Schatz-Jakobsen et al. 2016). Though effective for some, patients with genetic polymorphisms in the MG7 domain of C5 are not responsive to treatment (Nishimura et al. 2014). Eculizumab is also one of the most expensive therapies world-wide, costing nearly $500,000 per patient for one year of treatment (Jayasundara et al. 2019). With the prohibitive cost of therapy and some not responsive to treatment, there is clear need for alternative complement inhibitors that target the terminal pathway.

Structures of C5 in complex with virulence factors provide clues to designing new therapeutic alternatives that target convertase activity. Cobra venom factor (CVF) is a C3b homologue that binds Factor B in the presence of Mg^{2+} ions to mimic convertase activity (Vogel and Fritzinger 2010). Though there are currently no structures of the C5 convertase, the C5-CVF complex suggests that the MG4, MG5, and MG7 domains of C5 likely play a role in binding the endogenous convertase (Fig. 7.6b) (Laursen et al. 2011). While these data are in agreement with the eculizumab binding site, it is important to note that the C5-CVF complex remains only a proxy for investigating endogenous convertase interactions. Tick C5 inhibitors, such as OmCI from *Ornithodoros moubata* and the RaCI protein family from *Rhipicephalus appendiculatus*, block endogenous convertase-derived C5 activation, yet have no effect on CVF-based convertase activity (Fredslund et al. 2008; Jore et al. 2016). OmCI interacts with the C5 TED, CUB and C345C domains, far from both the eculizumab binding and convertase cleavage sites (Fig. 7.6c) (Jore et al. 2016). These structural data combined with competition assays suggest that C5 activation by endogenous convertases may involve an initial priming step that is not required for CVF-derived cleavage. Furthermore, high-resolution cryoEM studies of a novel class of tick inhibitors from the CirpT family reveal another way to block C5 activation by targeting C5 MG4 that could be exploited for drug discovery (Fig. 7.6d) (Reichhardt et al. 2020).

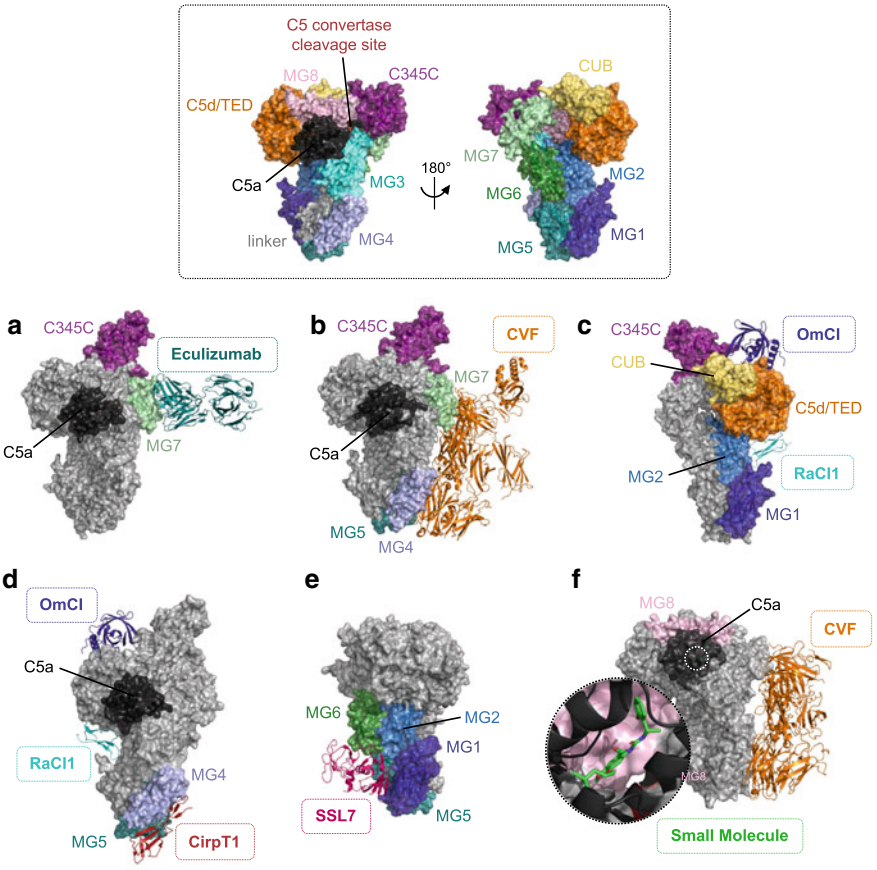

Fig. 7.6 Structural basis of blocking C5 activation. Surface representation of the C5 crystal structure (PDB 3CU7) coloured according to domains is shown above in a dotted box for reference. **a** Crystal structure of C5 (surface representation) in complex with eculizumab (dark teal ribbons) (PDB 5I5K). **b** Crystal structure of C5 (surface representation) in complex with cobra venom factor (CVF; orange ribbons) (PDB 3PVM). **c** Crystal structure of C5 (surface representation) in complex with tick inhibitors OmCI (dark blue ribbons) and RaCI1 (cyan ribbons) (PDB 5HCE). **d** Atomic model derived from the cryoEM structure of C5 (surface representation) in complex with tick inhibitors Cirp T1 (red ribbons), OmCI (dark blue ribbons), and RaCI1 (cyan ribbons) (PDB 6RQJ; EMD-4983). **e** Crystal structure of C5 (surface representation) in complex with Staphylococcal Superantigen-Like protein 7 (SSL7; magenta ribbons) (PDB 3KLS). **f** Atomic model derived from the cryoEM structure of C5 (surface representation) in complex with CVF (orange ribbons) and a small molecule that blocks cleavage of C5 (green sticks) (PDB 6I2X; EMD-4401). Circular inset shows a zoomed in view of the small molecule binding-site. For all panels C5a is in black, domains of C5 that form interaction interfaces are highlighted, and all other C5 domains are grey

Bacterial pathogens have also developed several strategies for regulating MAC to evade complement immune clearance. *Staphylococcus aureus* secretes Staphylococcal Superantigen-Like protein 7 (SSL7), which binds to C5 to block bactericidal activity from the MAC (Langley et al. 2005). A crystal structure of the C5-SSL7 complex revealed that SSL7 binds to C5 more than 70 Å away from the C5a cleavage site (Fig. 7.6e) (Laursen et al. 2010). Proteolytic cleavage of C5 into C5a and C5b may still occur in the presence of SSL7, however, subsequent binding of C6 to C5b to initiate MAC pore formation is inhibited.

Blocking Pore Formation

Once the terminal pathway is activated, there remains only one membrane-bound regulator of MAC: CD59. CD59 is present on all circulating cells and most human tissues, providing protection from autologous MAC attack (Meri et al. 1990, 1991). Comprised of three β-sheets and two α-helices, CD59 is a small 18–20 kDa extracellular glycoprotein (Fletcher et al. 1994; Leath et al. 2007). A complex network of five disulfide bridges maintains the protein's rigid, compact, and discoid structure (Sugita et al. 1993). Extending from the CD59 C-terminus, a glycosylphosphatidylinositol (GPI) lipid anchor tethers the molecule to the outer leaflet of the cell membrane. CD59 binds to the MACPF domains of either C9 or C8α to block subsequent incorporation of C9 molecules and to prevent membrane rupture (Meri et al. 1990; Ninomiya and Sims 1992; Farkas et al. 2002). Plasma-circulating forms of C8 and C9 do not bind CD59, suggesting that the binding sites are only exposed upon MAC formation (Huang et al. 2006). Although there are no structural data for CD59 inhibited MAC complexes, mutational studies have mapped the binding site to a hydrophobic cavity of CD59 (Bodian et al. 1997).

Similar to understanding C5 activation, the interaction of bacterial virulence factors with CD59 can inform new strategies for controlling complement activity. Secreted by over five genera of Gram-positive bacteria, cholesterol dependent cytolysins (CDCs) are pore-forming proteins that are structurally homologous to the MACPF domain of complement proteins (Fig. 7.3) (Tweten 2005; Hadders et al. 2007; Rosado et al. 2007). *Streptococcus intermedius* intermedilysin (ILY), *Gardenerella vaginalis* vaginolysin (VLY), and *Streptococcus mitis* lectinolysin (LLY) comprise a subset of these toxins that hijack CD59 to specifically target human cells (Lawrence et al. 2016). The interaction interface for ILY partially overlaps with the complement binding site on CD59 (Giddings et al. 2004). However, the primary interface lies within a β-hairpin of ILY that extends from domain 4 (D4), a domain that is absent in MAC proteins (Figs. 7.4 and 7.7) (Johnson et al. 2013). While the molecular basis for how CD59 binds C8α and C9 remains to be determined, CD59 may bind the newly extended β-hairpins of these MACPF-containing proteins to block membrane insertion.

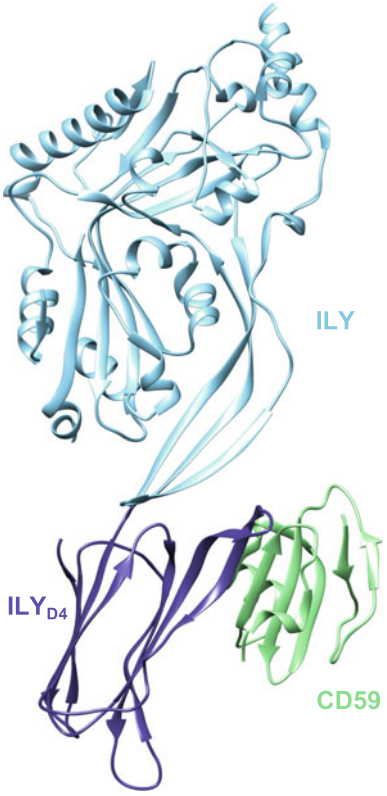

Fig. 7.7 Crystal structure of the ILY-CD59 complex (PDB 4BIK). CD59 is in green; ILY is in blue with domain 4 (D4) highlighted in dark blue

Scavenging By-Products

In the absence of membranes, activated complement components can assemble off-target MAC precursor complexes. This 'soluble MAC' is referred to as sC5b9 and is used as a biomarker for systemic infections and acute meningococcal disease (Mook-Kanamori et al. 2014). Overactivation of complement overwhelms the C5 convertase and may impair its ability to orient MAC efficiently in target membranes (Heesterbeek et al. 2019). Consequently, MAC precursors are released into solution at different stages of assembly, which are then scavenged by blood-based chaperones clusterin and vitronectin to form stable complexes referred to as sC5b7, sC5b8 and sC5b9 (Preissner et al. 1989). Although primarily considered a soluble complex, it is possible that sC5b9 detected in vivo may also be attributed in part to membrane-bound material shed from damaged cells. A low resolution cryoEM structure of sC5b9 suggests that chaperones may act by shielding hydrophobic interactions of otherwise membrane-binding residues (Hadders et al. 2012). Atomic resolution details would

provide new insight into how regulators prevent the helix-to-hairpin transition of pore forming amino acids.

Future of Structure-Based Complement Therapeutics

High-resolution complexes of MAC and upstream regulators set the scene for future development of enhanced therapeutics. The potential for such therapies is highlighted by over a dozen anti-complement drugs currently in hundreds of active clinical trials across a range of drug types including recombinant proteins, peptides, small molecules, antibodies/biologics, and DNA (Morgan and Harris 2015; Mastellos et al. 2019). The potential applications are similarly diverse, ranging from overcoming the treatment failure with eculizumab in PNH and related diseases (Zipfel et al. 2019), to limiting damage from activated complement in bacterial infections (Mastellos et al. 2019; Koelman et al. 2019). Collectively the structures of apo and inhibited C5 provide a platform for development of highly specific complement inhibitors, which may either (i) selectively inhibit MAC pore formation without perturbing the C5a inflammatory signalling network, (ii) selectively impede an inflammation response from C5a, or (iii) halt the entire terminal complement system beyond C5 proteolysis. CryoEM has already started to play an important role in the development of C5 inhibitors, and the enhanced resolution achievable with modern techniques has enabled progress in small molecule drug discovery as well as in biologics (*e.g.* tick protein inhibitor) development. While the use of biologics suffers from substantial challenges of delivery, immunogenicity and pharmacokinetics, which are largely resolved by the use of small molecule drugs, it can be challenging to identify binding modes in large protein complexes by which small molecules could be effective.

A standout example of the small molecule approach is the recent identification of the first small molecule inhibitors of C5 complement protein by researchers at Novartis (Jendza et al. 2019). In this case, the binding site was initially identified by a careful analysis of structures and activities on a range of natural primate polymorphisms and engineered complement mutants. After failure to crystallise the complex for X-ray analysis, the remarkable binding mode of this compound series was only realised by the solution of a 3.35 Å complex with C5 and CVF by cryoEM (Fig. 7.6f). Designing compounds de novo that induce the highly buried conformation that this structure reveals remains challenging from a chemical biology perspective. This structure provides an important starting point for further in silico molecular modelling that may lead to the development of new and improved compounds. This study also highlights the value of cryoEM to understand the impact of polymorphisms by mapping disease-inducing or drug resistant mutations to key interfaces.

Given the complementary limitations of small molecule and protein drugs, constrained peptides provide an interesting middle ground. Peptide-derivative therapeutics limit immunogenicity while maintaining capacity to target large interaction sites including those with drug-resisting polymorphisms. Zilucoplan is a cyclic peptide discovered through mRNA display that allosterically and potently inhibits

C5 cleavage at a site orthogonal to that occupied by eculizumab (Ricardo et al. 2014). This therapeutic peptide has shown marked success in late stage clinical trials for myasthenia gravis in Asian populations, a key demographic of patients with genetic polymorphisms that cause eculizumab treatment failure (Howard et al. 2020). It is likely that cryoEM will play a key role in future structure-guided development of constrained peptide modulators of complement.

While most current therapeutic approaches target upstream regulators, targeting MAC formation itself has the potential to offer a more selective therapy that may be less susceptible to resistance from polymorphisms. With its ability to disentangle heterogeneous and flexible assemblies, cryoEM provides a powerful tool for resolving how CD59 blocks MAC. This information could prove transformational in designing small molecules or peptides which can enhance native CD59 activity, or discovery of agents which mimic CD59 MAC inhibition. A deeper understanding of the role of CD59 may also lead to drugs which can prevent the formation of bacterial lytic pores that hijack CD59 on human cells. Finally, selective activation of MAC on specific cell types is a plausible approach to enhance the efficacy of complement-mediated immunotherapies. Inhibitors of the CD59-MAC interaction may release the brake on complement-mediated lysis imposed by CD59 upregulation in treatment of cancers with antibody drugs such as rituximab. In particular, a more targeted therapy could restrict complement killing to relevant tissue types by conjugation to cell surface targeting motifs (Weiner 2010). To date these approaches have relied on biologics such as siRNA or immunogenic bacterial pore-forming toxin fragments (Geis et al. 2010; You et al. 2011). A cryoEM-enhanced understanding of how MAC is inhibited is likely to lead to knowledge-based design of novel advanced therapeutics that better tune complement activity.

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