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The Making of a Macromolecular Machine: Assembly of the Membrane Attack Complex

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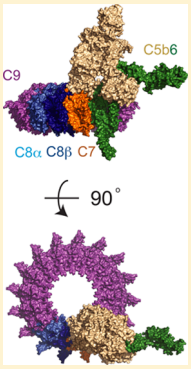
ABSTRACT: The complement terminal pathway clears pathogens by generating cytotoxic membrane attack complex (MAC) pores on target cells. For more than 40 years, biochemical and cellular assays have been used to characterize the lytic nature of the MAC and to define its protein composition. Although models for pore formation have been inferred from structures of bacterial cytolysins, it was only recently that we were able to visualize how complement components come together during MAC assembly. This review highlights structural analyses of terminal pathway complexes to explore molecular mechanisms underlying MAC formation.



■ ASSEMBLY OF THE MAC FROM SOLUBLE COMPONENTS

Activation of the complement terminal pathway results in formation of the MAC, a lytic immune pore that disrupts lipid bilayers. More than a megadalton in size, the MAC pore measures approximately 10 nm in diameter[1] and is assembled from five individual complement proteins: C5b, C6, C7, C8, and C9.[2] MAC formation is an obligate sequential reaction characterized by a series of stable and metastable assembly precursors (Figure 1). The process is initiated by the irreversible proteolytic cleavage of C5 into two components (C5a and C5b) by the C5 convertase.[3] C5a acts as a proinflammatory anaphylatoxin, while C5b serves as a scaffold for generating the MAC.[4−6] C6 binds C5b to produce C5b6, a soluble and stable intermediate.[7,8] Subsequently, C7 binding renders the assembly lipophilic.[9,10] C8 binds the membrane-associated MAC precursor and is the first component to traverse the lipid bilayer.[11,12] Finally, between 12 and 18 copies of C9 join the assembly to complete the MAC ring.[1,13]

Terminal pathway components are found in abundance in serum.[14] Primed for MAC assembly, these soluble proteins undergo dramatic conformational changes as the transmembrane pore is formed. Differences in conformational epitopes between individual proteins and the MAC were suggestive of these large-scale rearrangements.[15,16] Recent studies involving X-ray crystallography[17−21] and three-dimensional (3D) electron microscopy[18,22] have now visualized many of these transitions and have led to significant advances in our mechanistic understanding of MAC assembly.

C5 is a large (196 kDa) protein composed of two peptide chains (α, residues 1−674; β, residues 678−1676). Structurally homologous to C3,[23] C5 is organized into 13 discrete domains (Figure 2). In the C5 structure, seven of the α-macroglobulin (MG) domains assemble to form a ringlike scaffold (Figure 3A). Together with C5a, the C-terminal domains "complement C1r/C1s, Uegf, Bmp1" (CUB), thioester-like domain (C5d/TED), MG8, and C345C pack against this scaffold like a coiled spring.[24] Structural analysis of the C5b6 complex shows that release of C5a by the convertase results in dramatic conformational changes,[18,20] similar to the transition from C3 to C3b.[25] Upon cleavage, the CUB and TED domains undergo major relocations and uncoil from the closely packed arrangement found in the native C5 structure (Figure 3B). The newly positioned TED domain then forms the primary binding site for C6.

Structural studies of C6 reveal that it is an elongated molecule composed of 10 distinct domains (Figures 2 and 3C). A flexible linker tethers its C-terminal modules to the core membrane attack complex/perforin-like (MACPF) domain. The linker and complement control protein (CCP) domains wrap around the TED domain in the C5b6 complex and serve as the principal binding site for C5b[18,20] (Figure 3D). Mutagenesis experiments corroborate these structural data, showing the importance of the linker in hemolytic activity.[18] Analyses of more subtle structural changes that occur upon binding suggest a role for other modules in priming C6 for MAC assembly. In the C6 structure, a long helix (residues 478−498), termed the "linchpin helix", together with a Y-shaped structural region comprised of the thrombospondin (TSP), epidermal growth factor (EGF), and low-density lipoprotein receptor class A (LDLRa) domains, pack against the MACPF and were hypothesized to regulate structural changes that initiate the MAC.[21]

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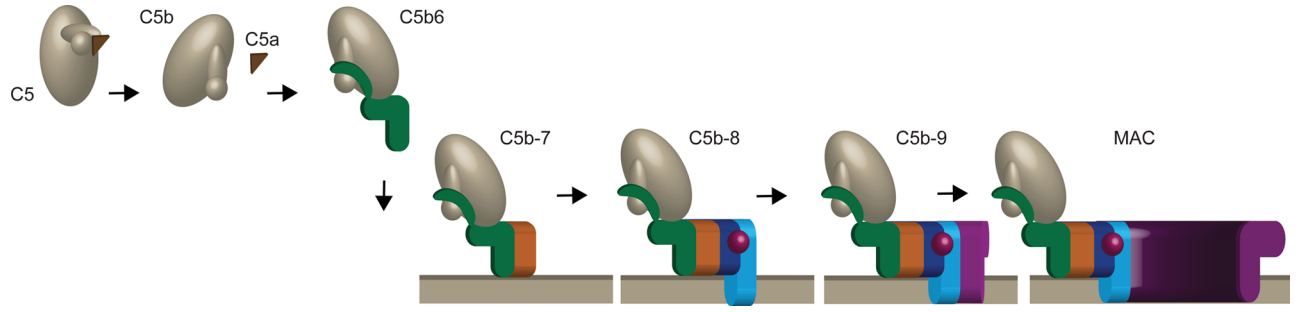

Figure 1. Cartoon diagram depicting MAC assembly. MAC formation is initiated by the proteolytic cleavage of C5 into C5a (brown triangle) and C5b (tan). C6 (green) captures a labile binding site exposed on C5b. C7 (orange) binds the nascent C5b6 complex to generate a lipophilic C5b-7 complex. C8 (C8α, light blue; C8β, dark blue; C8γ, red sphere) joins the assembly and penetrates the lipid bilayer, resulting in C5b-8. Finally, C9 (purple) is incorporated, and its oligomerization completes the MAC ring, a cross section of which is depicted.

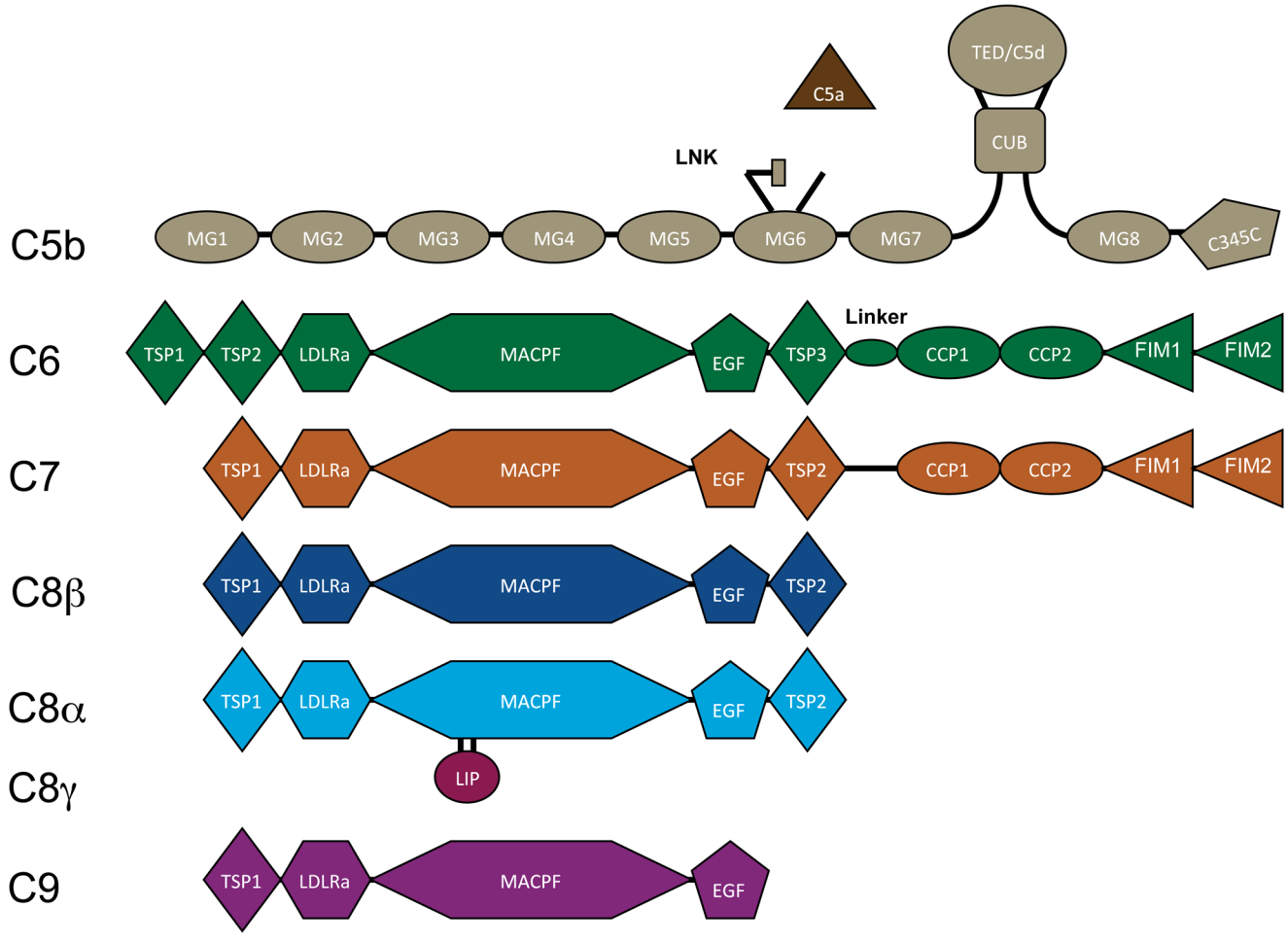

Figure 2. Schematic domain organization of MAC components. Thioester-like (TED), α-macroglobulin (MG), complement C1r/C1s, Uegf, Bmp1 (CUB), C345C, thrombospondin (TSP), epidermal growth factor (EGF), low-density lipoprotein receptor class A (LDLRa), factor I/MAC (FIM), complement control protein (CCP), the membrane attack complex/perforin-like (MACPF), and lipocalin (Lip) domains are shown. Coloring is the same as in Figure 1.

In contrast to the elongated architecture of C6, the crystal structure of C8 reveals a compact globular complex consisting of subunits C8α, C8β, and C8γ.[19,22] C8α and C8β are highly homologous. Both possess a core MACPF domain and four smaller modules that wrap around the periphery of the molecule (Figures 2 and 4A). The MACPF domains of C8α and C8β have essential yet unique roles in MAC formation.[26,27] The C8α MACPF domain binds to C9[26,28] and is the first component to penetrate the lipid bilayer.[11,12] The C8β MACPF domain is responsible for integrating the complex into the MAC precursor (C5b-7).[27] The four smaller modules of C8α and C8β, while not strictly required for pore formation, do impact lysis.[29,30] These domains may therefore play a secondary role in facilitating the MAC. The function of the third subunit, C8γ, is less clear. Covalently linked to a hairpin extension of the C8α MACPF domain via a disulfide bond,[31,32] C8γ at first glance seems superfluous to MAC formation. Its presence augments but is not required for lysis.[33] Unlike any other

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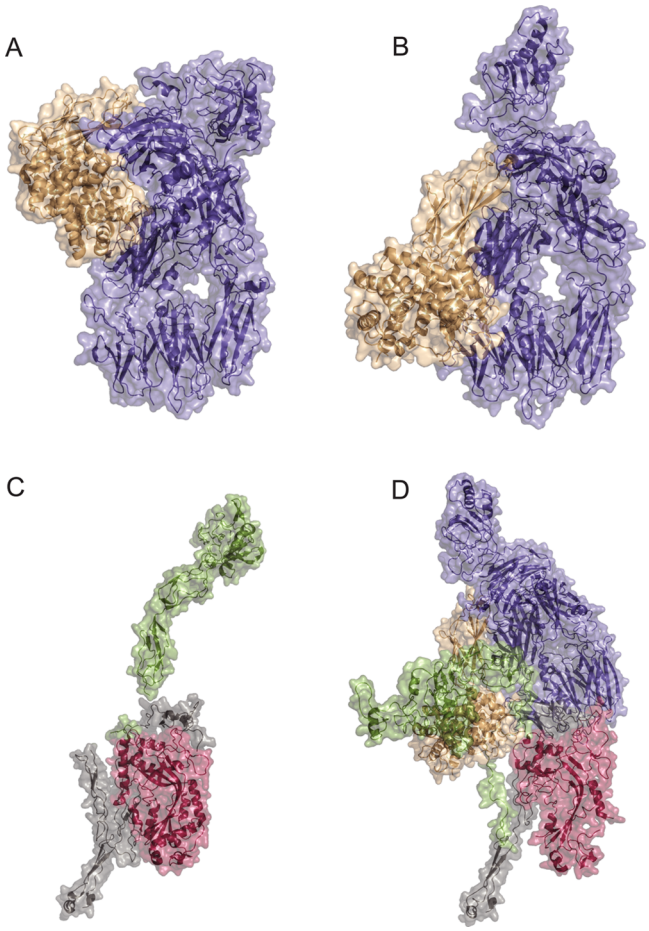

Figure 3. Structures of complement components. Cartoon and surface representations of C5 (PDB entry 3CU7) (A) and C5b from the C5b6 complex (PDB entry 4A5W) (B). MG and C345C domains are colored light blue. CUB and TED domains are colored tan. (C) Cartoon and surface representation of C6 (PDB entry 3T5O). The C6 MACPF domain is colored red. N-Terminal TSP and LDLRa domains of C6 are colored gray; C-terminal EGF, TSP, CCP, and FIM domains are colored green. (D) Cartoon and surface representation of C5b6. C5b is colored as in panel B and C6 as in panel C. Images were generated with PyMol.[82]

terminal pathway protein domain, C8γ has a lipocalin fold.[32] Although this family of proteins traditionally transports small molecules, there is no experimental evidence so far suggesting this function for C8γ. A number of structures of complexes containing the C8α-γ heterodimer have been determined by both X-ray crystallography[19,34] and 3D electron microscopy.[18,22] A comparison between them reveals a wide range of conformations allowed by a flexible hinge in the C8α hairpin extension and may suggest a role of C8γ in stabilizing MACPF−MACPF interactions during ring assembly.

While NMR data for the CCP and factor I/MAC (FIM) domains of C7 have provided some insight into their function in the MAC,[35,36] there is currently no structural information for intact C7 or C9 molecules. Modeling data suggest that C7 has an architecture similar to that of C6,[21] while C9 is predicted to resemble C8α.[17] Both components form a key part of the MAC, with multiple copies of C9 polymerizing to generate the pore.[1]

MACPF PORE FORMATION AND LESSONS FROM A BACTERIAL MODEL SYSTEM

Four of the five complement proteins that make up the MAC possess a MACPF fold (Figure 2), a domain characterized by a kinked β-sheet and two flanking helical subregions (Figure 4A,B). A comparison with bacterial toxins of the cholesterol-dependent cytolysin (CDC) superfamily revealed structural homology between the combined topology of CDC domains 1 and 3 and the MACPF fold. Therefore, this noncontiguous region within a CDC can be defined as the CDC/MACPF domain. Structural similarity between the MACPF and CDC/MACPF domains has led to the hypothesis that the pore-forming mechanism is conserved across the two families.[17−19,21,37−41] Studies using this bacterial model system revealed a dramatic conformational rearrangement in the structure of water-soluble monomers to generate an oligomeric β-barrel pore. Broadly, two helical segments within the CDC/MACPF domain unfurl to form transmembrane β-hairpins (TMH1 and TMH2),[42,43] which then generate the membrane-inserted part of the pore. Sequence analysis suggests these segments are larger in complement MACPF domains than in CDC/MACPF domains.[17] While the predicted C6 and C7 hairpins are only long enough for their tips to perturb the membrane,[21] those for C8 are amphipathic, and their lengths are consistent with penetration of the bilayer. The segments in C9 are longer still and possess a hydrophilic loop at the tip, which may interact with intracellular proteins or anchor the pore to the cytoplasm. Recent experiments involving chimeric complement proteins provide further experimental evidence that the predicted TMH segments of C8 and C9 are directly involved in membrane penetration and pore formation.[44]

Cryo-EM reconstructions of CDC prepore and pore complexes[45] together with fluorescence-based biophysical data[46] have provided key insights into how MACPF domains are arranged in membranes (Figure 4C). For the majority of CDCs, association with cholesterol through domain 4 (lacking in complement components) is allosterically coupled to large conformational changes in the CDC/MACPF domain during pore formation.[47] Experiments involving disulfide-locked mutants have captured two distinct prepore states prior to membrane penetration. Assays using these mutants have shown that a weak and reversible association of membrane-bound monomers is stabilized by displacement of the CDC/MACPF domain's fifth β-strand.[46] This conformational change facilitates the intermolecular interactions of β-strands between CDC monomers, propagating oligomerization. Monomer−monomer interactions within the CDC/MACPF domain then drive further structural transitions in which the two TMH segments undergo a final transition to generate the β-barrel transmembrane pore. A small subclass of CDCs, of which Intermedilysin (ILY) is an archetypal member, requires binding to complement receptor CD59 in addition to cholesterol.[48] Here, structural transitions allowing oligomerization are governed largely by CD59 binding, rather than an interaction with cholesterol.[38,49] In the final stages of pore formation, cholesterol binding helps anchor the prepore as CD59 is released.[47,50] Recently, a crystal structure of the ILY−CD59 complex showed how CD59 coordinates ILY monomers though two binding faces.[51] Intriguingly, although CD59 promotes ILY pore formation, it remains a potent inhibitor of MAC-mediated lysis. Mutagenesis data reveal that the ILY and MAC binding sites on CD59 overlap;[52] therefore, this

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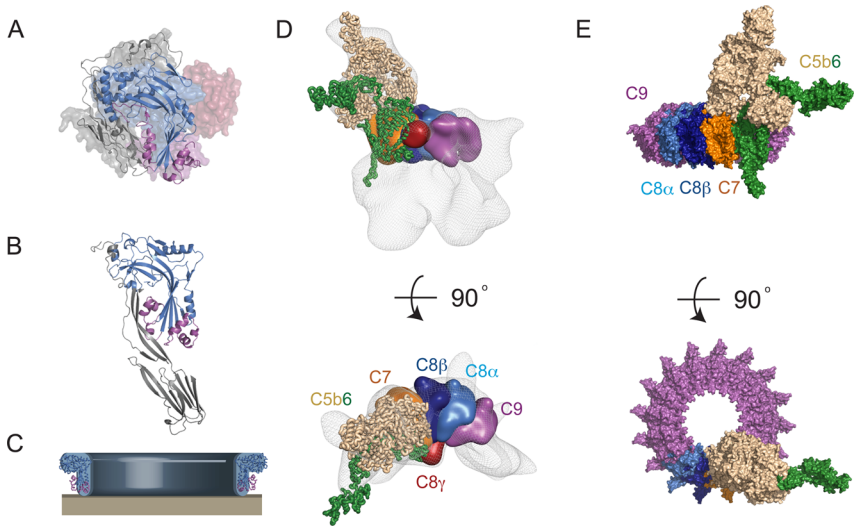

Figure 4. Mechanism of pore formation. (A) Representation of the C8 crystal structure (PDB entry 3OJY). C8β is shown as cartoon ribbons. C8α and C8γ are semitransparent surfaces. MACPF domains of C8α and C8β are colored blue with the predicted TMH segments colored magenta. N-Terminal domains (TSP and LDLRa) and C-terminal modules (TSP and EGF) that wrap around the MACPF domains are colored gray. (B) Cartoon representation of perfringolysin O (PDB entry PFO) highlighting its CDC/MACPF domain (blue) and two helical segments that undergo a transition to transmembrane β-hairpins during pore formation (magenta). Domains 2 and 4, not present in complement proteins, are colored gray. (C) Schematic diagram depicting the orientation of CDC/MACPF and complement MACPF domains (colored as in panels A and B) in the membrane. A cross section of the complete ring is shown. (D and E) The electron microscopy map of the sC5b9 complex (mesh) together with crystal structures of C5b6 (cartoon) and C8 suggests a model for the MAC pore that resembles CDCs. (E) Model for the complete MAC ring based on MACPF–MACPF interactions observed in the C8 crystal structure. Complement components colored as in Figures 1 and 2. Images were generated with PyMol.[82]

host–pathogen structure could also provide clues about how the MAC pore is regulated.

As the MAC is a multiprotein complex with no dependence on cholesterol, a number of more subtle questions arise when thinking about its assembly. Is the orientation in the membrane of bacterial and complement MACPF domains conserved? In what direction does the arc extend to close the ring? When do the TMH segments unfurl? While these questions will ultimately be answered by a high-resolution view of the membrane-inserted MAC, technical challenges arising from large heterogeneous assemblies make this difficult to achieve. Recent cryo-EM data of a soluble form of the MAC (sC5b-9)[18] together with crystallographic structures of C5b6[18,20] and C8[19] have, however, allowed progress to be made in answering some of these questions.

A number of lines of evidence support a model for the MAC in which the orientation of MACPF domains resembles that of CDC pores (Figure 4C). The crystal structure of C8 showed, for the first time at high resolution, how MACPF domains pack together[19] (Figure 4A). The two MACPF domains of C8 are related by a 22° rotation in the heterodimer and may serve as a template for establishing the curvature of the pore. Repetition of this rotation generates a model for a poly-C9 ring in which the MACPF domains are oriented like the CDCs[17,19] (Figure 4E), in contrast to the perforin pore model.[53] In further agreement with a CDC arrangement, this same curvature and density consistent for the C8γ subunit of C8 on the inner face of the ring were also observed in a cryo-EM reconstruction of a soluble MAC, sC5b-9[18] (Figure 4D).

Although the assembly of complement components into the MAC is a sequential reaction with the order of incorporation firmly established, the direction of arc elongation was less clear. In agreement with a model for the MAC based on the crystal structure of C8,[19] a recent cryo-EM structure of a soluble MAC (sC5b9) combined with crystallographic data for the C5b6 and C8 complexes has led to a model for assembly that arranges the proteins in the following order: C5b6 → C7 → C8 → C9 (C9 then continues to oligomerize until ring closure is achieved)[18] (Figure 4D). Further experimental data involving C6 and C7 mutants or a C5b-7 crystal structure will be necessary to define specific residues responsible for protein binding interfaces.

IMPORTANCE OF TIMING

Unlike the CDCs, there is no MAC-specific receptor targeting the pore assembly. So how does this immune pore so effectively clear pathogens? Following the proteolytic cleavage of C5, MAC formation is largely stochastic with many "dead-end" products. Therefore, being in the right place at the right time is everything. There are data supporting transient preactivation associations,[54] likely to keep components that form irreversible intermediates close at hand. Specifically, the FIM domains of both C6 and C7 are known to reversibly associate with the C345C domain of C5.[55] Once activation occurs, however, MAC assembly is a sequential process in which the half-lives of intermediates vary considerably. C6 captures a labile binding site on C5b exposed upon cleavage by the convertase (half-life of 2 min).[7] The transition observed from C5 to C5b is similar to that seen from C3 to C3b.[18,20,25] However, unlike C3b, the resulting position of the TED domain is captured halfway up the MG ring by C6 (Figure 3B,D). The requirement for this conformation to be trapped and stabilized by C6 may explain the fleeting binding site exposed on C5b. Once formed, C5b6 is a stable soluble complex, primed to bind C7. In the presence of membranes, the addition of C7 drives the pathway toward successful MAC formation. However, if there is no lipid bilayer nearby, the metastable C5b-7 complex degrades to a state incapable of supporting pores.[9] In fact, in the absence of membranes, C8 is the most potent inhibitor of the MAC.[56,57]

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These soluble dead-end products (sC5b-7, sC5b-8, and sC5b-9) are scavenged by clusterin and vitronectin,[58−60] which act as chaperones preventing nonspecific membrane association and aggregation. Recent cryo-EM data for the sC5b-9 complex,[18] supported by labeling experiments[60] and proteolytic digests,[61] suggest a model in which these chaperones function by capping the ends of the C5b-9 MACPF arc and by binding exposed hydrophobic regions (Figure 4D).

INHIBITION OF THE MAC

Pore formation of membrane-bound MAC precursors and subsequent cell lysis are inhibited by CD59,[62,63] a GPI-anchored cell surface receptor that at binds C8 and C9.[64] It specifically acts by blocking the incorporation of C9 into the MAC and prevents membrane perforation of MAC precursors C5b-8 and C5b-9.[63] CD59 is a small (77-amino acid) glycosylated protein, abundant on a variety of cell types, including leukocytes, epithelial cells, and endothelial cells.[65] Its compact structure, rich in disulfide bonds, is composed of a central β-sheet sandwiched by a short α-helix and β-hairpin.[66,67] Mutagenesis studies coupled with direct binding assays of CD59 with C8 and C9 have identified key residues involved in the interaction.[52,68−70] For both complement components, the CD59 binding site maps to the TMH region of their MACPF domains. Interestingly, the crystal structure of C8 shows that these residues (C8α, residues 350−355) are not accessible in its native form.[19] This is consistent with the observation that CD59 cannot bind C8 or C9 unless partially unfolded and may suggest that CD59 binding follows conformational changes induced upon MAC assembly. Rather unusually, the C8 and C9 binding sites map to two faces of CD59 that overlap largely with the interaction interface observed for ILY.[52] Though ILY binds CD59 with a β-hairpin extending from its domain 4 (absent in complement components),[51] CD59 may prevent MAC membrane insertion by similarly engaging the hairpin state of C8 and C9 TMH segments. The two binding modes observed for the ILY−CD59 complex also suggest a mechanism in which CD59 may intercalate between C8 and C9 molecules to lock the MAC in an inhibited conformation.

MEDICAL IMPLICATIONS

The MAC is an important innate immune effector, while dysregulation of the terminal pathway impacts various human diseases. MAC-mediated lysis is particularly effective in combating the Gram-negative bacterium *Neisseria meningitidis*, with genetic deficiencies in MAC components leading to recurrent infections.[71−73] During activation of the terminal pathway, CD59 protects host cells from unwarranted tissue damage. However, deficiency in CD59 can cause paroxysmal nocturnal hemoglobinuria, a disease characterized by hemolytic anemia and thrombosis.[74] In contrast, a number of human cancers overexpress CD59,[75] providing a mechanism for immune evasion.[76] Antibody-based immunotherapies that activate the complement terminal pathway have been found to be effective in treating some cancers. For example, rituximab is an anticancer immunotherapy that works in part by stimulating the MAC to treat stage III or stage IV follicular lymphoma, the most common form of non-Hodgkin's lymphoma (NHL).[77,78] Unfortunately, some patients are not responsive to rituximab treatment, while others develop resistance over time.[79] Recent data have shown the sensitization of rituximab-resistant B-cell NHL *in vitro* and *in vivo* by a CD59 inhibitor derived from ILY.[80,81] These findings reinforce the biomedical relevance of understanding complement terminal pathway regulation and how the interplay between MAC and ILY pore formation affects human disease.

CONCLUSIONS AND FUTURE DIRECTIONS

Assembly and regulation of the MAC remains an important and timely research focus, spanning innate immunity, host−pathogen interactions, and cancer biology. Structural studies of both terminal pathway components and CDC bacterial toxins have been pivotal in the elucidation of a molecular mechanism for pore formation. Recent efforts involving MAC components and protein complexes have led to significant progress in determining the role of auxiliary domains in facilitating the assembly. Further high-resolution structures of MAC precursors combined with studies of MACs in membranes will be necessary to fully understand how terminal complement proteins come together to generate the complete pore. The structure of a soluble form of the MAC reveals how nonspecific membrane association and aggregation might be prevented, and although structures of CD59-bound complement complexes are still lacking, recent crystallographic data for the ILY−CD59 complex suggest a model for how MACs might be inhibited on the cell surface. While the current structures of soluble MAC complexes have provided a much-needed platform on which to integrate decades of complement research, the next leaps forward will likely come from visualizing their membrane-associated forms.

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ABBREVIATIONS

MAC, membrane attack complex; CDC, cholesterol-dependent cytolysins; MACPF, membrane attack complex perforin fold; TMH, transmembrane hairpin; CRM, cholesterol recognition/binding motif; ILY, Intermedilysin; NHL, non-Hodgkin's lymphoma; TED, thioester-like domain; MG, α-macroglobulin; CUB, complement C1r/C1s, Uegf, Bmp1; cryo-EM, cryo-electron microscopy; TSP, thrombospondin; EGF, epidermal growth factor; LDLRa, low-density lipoprotein receptor class A; FIM, factor I/MAC; CCP, complement control protein; PDB, Protein Data Bank.

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