CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

ORCA – Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:https://orca.cardiff.ac.uk/id/eprint/102393/

This is the author's version of a work that was submitted to / accepted for publication.

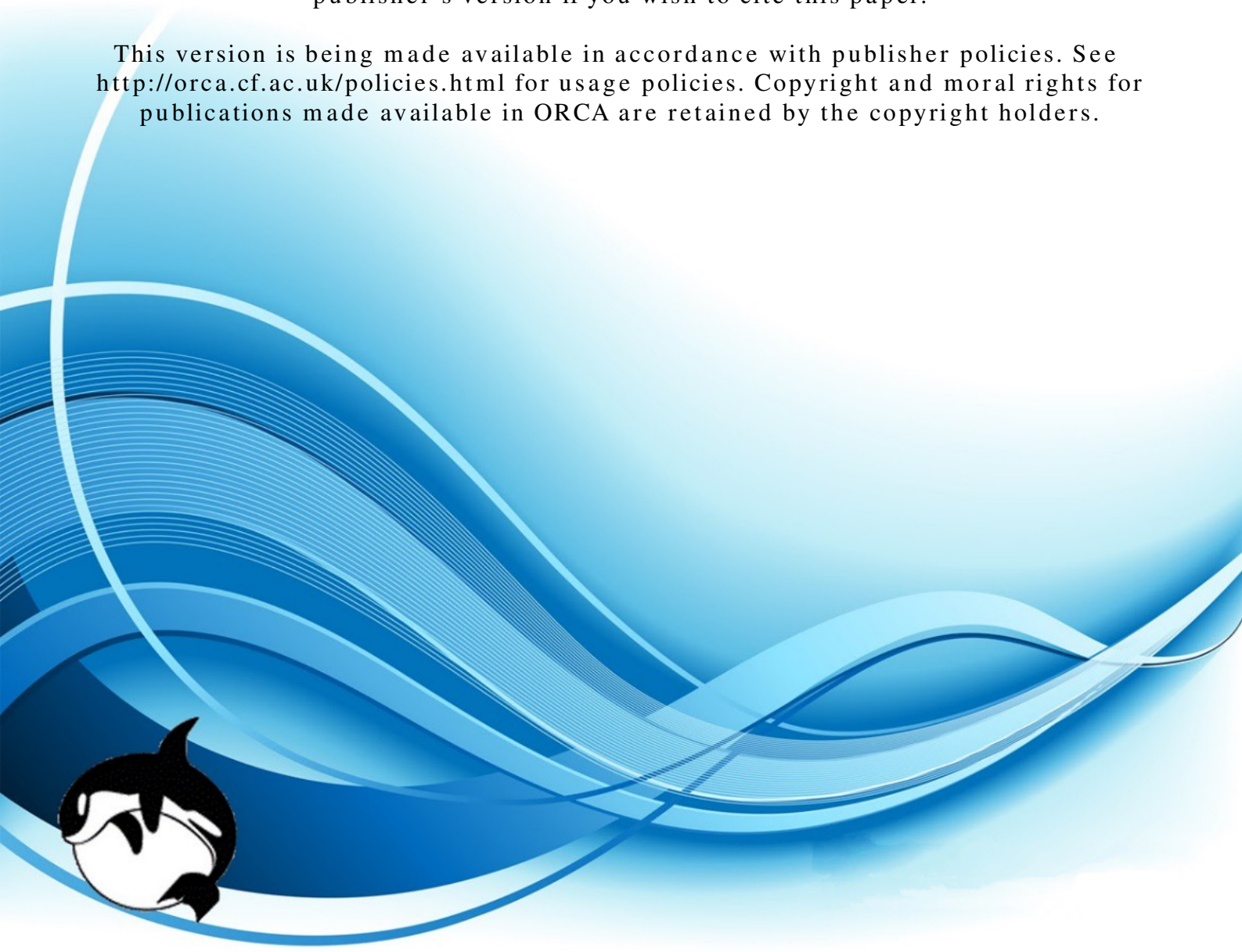
Citation for final published version:

Morgan, Bryan Paul , Boyd, Courtney and Bubeck, Doryen 2017. Molecular cell biology of complement membrane attack. Seminars in Cell and Developmental Biology

Publishers page: https://doi.org/10.1016/j.semcdb.2017.06.009

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Molecular cell biology of complement membrane attack.

B. Paul Morgan[1], Courtney Boyd[2] and Doryen Bubeck[2]

Authors' addresses:

[1] Systems Immunity University Research Institute, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff CF144XN, UK.

[2] Faculty of Natural Sciences, Department of Life Sciences, Imperial College, 506 Sir Ernst Chain Building, London SW7 2AZ, UK.

Correspondence to:

B. Paul Morgan,

Systems Immunity University Research Institute,

Division of Infection and Immunity,

School of Medicine,

Cardiff University,

Cardiff CF144XN, UK.

Tel: +44 2920 687096

Email: morganbp@cardiff.ac.uk

Running title

Biology of membrane attack.

Summary

The membrane attack complex (MAC) is the pore-forming toxin of the complement system, a relatively early evolutionary acquisition that confers upon complement the capacity to directly kill pathogens. The MAC is more than just a bactericidal missile, having the capacity when formed on self-cells to initiate a host of cell activation events that can have profound consequences for tissue homeostasis in the face of infection or injury. Although the capacity of complement to directly kill pathogens has been recognised for over a century, and the pore-forming killing mechanism for at least 50 years, there remains considerable uncertainty regarding precisely how MAC mediates its killing and cell activation activities. A recent burst of new information on MAC structure provides context and opportunity to re-assess the ways in which MAC kills bacteria and modulates cell functions. In this brief review we will describe key aspects of MAC evolution, function and structure and seek to use the new structural information to better explain how the MAC works.

Introduction

Complement is a critical component of innate immunity and modulator of adaptive immunity, particularly important for defence against bacterial infections and the efficient removal of debris. These physiological functions of complement are mediated by a number of products, fragments and complexes generated during activation. Larger fragments of the abundant complement proteins C3 and C4 coat pathogen surfaces to mark them for phagocytic clearance, while smaller fragments of these proteins (and of C5) recruit the phagocytes to the site of injury, activating them for enhanced bacterial killing. Activation of the complement terminal pathway initiates the assembly on bacteria and other targets of the multi-component membrane attack complex (MAC) [1,2]. MAC assembles from its components in membranes to create a transmembrane pore that causes prompt osmotic lysis of metabolically inert targets such as aged erythrocytes. Water and ions drawn into the target through the pore cause swelling and eventual lysis. Nucleated cells targeted by MAC resist lytic killing by virtue of multiple layers of defence; however, MAC can have profound effects on nucleated cell functions that likely contribute both to homeostasis and pathology [3,4].

Recent structural studies of the MAC and its precursors have provided some surprises, revealing structural features that might help explain how the MAC forms and how it has different effects on different targets [5-7]. In this brief review we will provide some historical and phylogenetic background to introduce the MAC as an ancient and important component of immune defence; we will then introduce the new insights into MAC structure and speculate on how the improved understanding of structure might explain aspects of MAC function that have hitherto escaped explanation.

Historical perspective

The very earliest reports of the plasma activity that became known as complement described a heat-labile plasma component that could cause lysis of target bacteria or erythrocytes – so from a functional perspective, the MAC has been recognised since the 19th century! Early theories around

complement lysis suggested that the activity was a detergent-like property of complement, dissolving the membranes of targets [8]. It was therefore something of a surprise when electron microscopy of complement-lysed erythrocytes revealed the presence of numerous ring lesions creating pores in the membranes [9,10]. These images, together with a growing body of biochemical evidence, led to the recognition that the MAC was a protein-lined transmembrane pore made up of the five terminal complement proteins C5b, C6, C7, C8 and C9 [2,11].

The availability of pure proteins and depleted sera allowed more definitive structure-function studies. It was shown that C5b, still attached to the convertase, binds C6 followed by C7; the tri-molecular C5b-7 complex is released and becomes associated with adjacent membrane [1,11]. C8 and then C9 are recruited to complete the lesion which contained one molecule each of C5b, C6, C7, C8 and several C9 molecules, the precise number varying between studies [11,12]. Despite this biochemical clarity and the microscopy evidence, a debate continued as to whether the MAC was truly a rigid, protein-lined pore or a lipid-disrupting leaky patch generator [13]. The demonstration that purified C9 could be induced to polymerise in vitro to form a fluid-phase pore-like structure resembling the MAC went some way towards resolving the debate, strongly supporting the champions of the rigid pore [14,15]. Recent structural studies have brought a degree of clarity as well as rasing further questions; these are described in a later section after we look back at the evolution of the MAC.

Phylogeny of complement membrane attack

Complement is an ancient immune defence system but membrane attack is, comparatively, a more recent innovation. Invertebrates express a complement system capable of recognition (lectin-like) and opsonisation (alternative pathway-like) of pathogens [16]. The phylogenetically earliest suggestion of a complement terminal pathway is the demonstration that the genome of the ascidian (sea squirt) *Ciona intestinalis* encodes C6-like molecules with the prototypical MAC/perforin (MACPF) domain; demonstration of lytic function of these molecules is lacking [17]. In teleosts

(most fish species), multiple terminal complement proteins are present in the genome and expressed; for example, rainbow trout express at least C6, C7 (duplicated), C8β, C8γ and C9 [18], and trout serum forms MAC-like lytic lesions in targets [19]. Nurse shark complement also generates obvious pores in targets and the shark membrane attack pathway has been explored. Shark MAC comprises at least C5b-like, C8-like and C9-like molecules; the latter two can substitute for human C8 and C9 for lysis of human C5b-7-coated targets [20].

The order in which the multiple homologous proteins that comprise the human MAC have emerged has been the subject of debate. One side contended that C9 came first, evolving out of a common C9/perforin precursor with the terminal pathway components arising from this by gene reduplication [21]; a complication here is that C9 is the simplest of the terminal pathway components, requiring C8α, C8β, C7 and C6 to acquire modules as they evolved. The other side suggested that the more complex C6 and/or C7 evolved first, with gene reduplication and loss of domains generating the later components [22]. Phylogenetic analyses across species support the latter contention. Whether C6/C7 alone in these early terminal pathways were capable of generating a lytic lesion is unclear, although it is difficult to conceive of any alternative role for these primitive "MAC" proteins. The reasons for the evolution of increased complexity in MAC design are also uncertain – does the addition of more components increase MAC efficiency of killing? Does it enable regulation on self-cells? These questions may be addressed by better understanding of MAC structure.

Regulating the MAC

The MAC, a pore-forming toxin, represents a threat not only to pathogens but also to self-cells. A number of defence strategies have evolved to limit terminal pathway activation on host cells and protect against MAC-mediated damage. MAC distribution is restricted to the immediate vicinity of the forming convertase because the membrane binding site in the C5b-7 intermediate is extremely labile and prone to be masked by binding chaperones clusterin or S protein (or indeed, C8 while in

the fluid phase); hence, relatively few C5b-7 attach to the membrane to seed MAC formation [4,23]. The second line of defence on host cells is CD59, a broadly expressed glycolipid-anchored inhibitor of MAC assembly; CD59 locks into the C5b-8 complex and prevents recruitment of C9 – and hence, MAC formation [24]. The final defence for MAC that escape CD59 surveillance involves active recovery processes that efficiently remove MAC from the membrane [25]; signalling of these processes will be discussed below.

CD59 is a member of the Ly6/uPAR superfamily of GPI-anchored, cysteine-rich proteins that share a conserved disulfide bond pattern that defines the three-fingered Ly6/uPAR domain [26]. It is ubiquitous in mammals, broadly expressed and functional in MAC inhibition in all mammals tested [27-30]. Structural "homologues" of CD59 have been described in diverse species, including cyclostome and teleost fish and flatworms [31-34]. The Zebrafish CD59 homologue was reported to have bacterial binding and growth inhibition properties [35]; however, evidence of MAC-inhibitory function is lacking.

CD59 binding into the forming MAC raises several interesting structure-function questions. CD59 does not bind to C5b-7 but binds tightly to C5b-8 and also to C5b-8 complexes that have bound a first C9 [24], suggesting that binding sites exist in both C8 and C9. Once bound, CD59 hinders further C9 recruitment. The sites on CD59 that mediate MAC inhibition have been identified by mutagenesis and map to a surface-exposed hydrophobic groove in the CD59 crystal structure [36]. Homologous sites in C8α and C9 have been implicated in binding CD59 [37,38]; as CD59 does not bind either native C8 or C9, it is likely that these sites are exposed only during MAC formation. No structure for CD59 in complex with a MAC precursor has been described and is now overdue.

The structure of the MAC

Defining the MAC. In this review we use the term MAC to refer to the complete membrane-associated complex comprising C5b, C6, C7, C8 and multiple copies of C9. Although various functions have been ascribed to "incomplete" MAC precursors comprising C5b6, C5b67 or C5b678, evidence

that they do anything to a target is sketchy and mechanism lacking. Further, in the *in vivo* context where all terminal components are present and in excess, "incomplete" precursor complexes will rapidly progress to completion. Hence, although the precursors are essential intermediates in MAC formation, only the complete MAC is of biological relevance.

Evolving complexity of MAC structure. Imaging studies in the 1960s established the concept of the MAC as a doughnut-like protein-lined pore in the membrane (Figure 1A) [9-11]. The exact composition of the doughnut was hotly debated, some contending that size dictated that it must be a $(C5b\text{-}9)_2$ dimer [39], while others suggested that the pore grew by recruiting more C9 [40]. The demonstration in the 1980s that purified C9 self-polymerised when incubated for prolonged periods at 37^0C was a major step in resolving the debate [14,15]. Electron microscopy (EM) revealed that these polymers (poly-C9) were ring structures with dimensions and architecture closely resembling the MAC (Figure 1C). Biophysical analyses showed that the poly-C9 complex comprised between 12 and 18 C9 monomers in a closed ring. These findings strongly supported a model of the MAC where the bulk of the ring was formed by (poly) C9 with the other components either forming a minor part of the ring or even excluded to the periphery. This model gained general acceptance and stood for thirty years.

Structures of individual MAC components. The publication of a crystal structure for C8 in 2011 represented the first detailed structure of an intact MAC component and a first picture of how C8 and C9 might assemble into the MAC pore [41]. Crystal structures of C6 and the C5b6 complex provided insight into the first steps of MAC assembly [42,43,5]. Docking the C5b6 crystal structure into an electron cryo-microscopy structure of the soluble sC5b-9 complex added further clarity on direction of assembly of the MAC components [5].

Electron cryo-microscopy adds detail and complexity. Technical advances in imaging over the last thirty years have made possible much greater resolution for complex structures, encouraging us and others to re-address the structure of the MAC in order to better understand its function. Using highly purified terminal pathway components in a reactive lysis system, we generated MAC on

liposomes in the absence of "noise" from other proteins [6]. The formed MAC were detergent-extracted, polished by density gradient centrifugation and imaged by electron cryo-microscopy (cryo-EM) (Figure 1D). Multiple images of MAC particles in various orientations were captured and averaged to obtain a model that was refined to a final resolution of 8.5 Å (Figure 1E). At first glance the structure obtained, a regular cylinder with a single stalk, closely resembled that described thirty years earlier; however, as more detail emerged, so did several novel features (Figure 2). First, it was clear that the cylinder was homogeneous with regard to numbers of protein "staves", 22 in total comprising one stave each from C6, C7, C8β and C8α and 18 staves from individual copies of C9. Second, the first four staves, provided by C6, C7, C8β and C8α were asymmetric, oriented differently to the 18 C9 staves and too short to fully penetrate the membrane. Third, the structure was not a closed cylinder – the final C9 was not fixed back to the initial C6, giving a "split-washer" appearance to the cylinder and providing a way in which the diameter of the cylinder pore could vary. The "pitch" of the forming cylinder was set by reorientation and alignment of the CDC/MACPF domains of C6, C7, C8β and C8α, and this, together with a reorientation of C8γ on the inner rim of the pore, exposed binding sites on C8α for the first C9.

The role of the lipocalin-like molecule C8γ in MAC assembly has long been a mystery [44]. C8γ is disulphide-linked to C8α and the complex is non-covalently associated with C8β, a relationship that is conserved in all species where C8 subunit structure has been studied. It was known that C8γ was not essential for MAC formation; indeed, when C8αγ was biochemically stripped of the γ-subunit, C8α readily bound C8β and the resultant complex was capable of forming a hemolytic MAC [45]. Structures of the soluble C8 heterotrimer and C8αMACPF/C8γ complex [41,46], together with analysis of C8γ within the context of the MAC [6] provide some clues as to its role. In each structure, C8γ is disulphide linked to the same hairpin extension from the MACPF domain of C8α, in agreement with previous biochemical evidence [47]. However, this hairpin rotates with respect to the core MACPF domain of C8α and adopts a different orientation in each of the three complexes. In the absence of C8β, the crystal structure traps an extended conformation of C8γ, while the intact

heterotrimer shows C8γ curled back on the otherwise exposed C9-binding interface on C8αMACPF. This is consistent with biochemical experiments that showed in the absence of C8β, the C8αγ complex can reversibly bind C9 [48]. While these crystal structures may trap snapshots along a continuum of C8γ orientations, it remains plausible that the presence of C8β may restrict mobility of the hairpin. Furthermore, the published MAC structure shows that C8γ projects into the lumen of the β-barrel and is displaced from its position in the soluble C8 crystal structure [6]. Upon MAC formation, C8αMACPF rotates to align the core β-sheets with C8βMACPF permitting recruitment of C9. Based on these structural data, the role of C8γ would thus appear to be to prevent C8-C9 interactions other than in the forming MAC.

Cryo-electron tomography supports a new MAC model. Another imaging approach to defining MAC structure has recently been published using phase-plate cryo-electron tomography, a method designed to improve contrast in electron microscopy images [7]. MAC lesions were formed on lipid bilayers, either through classical pathway activation or by reactive lysis using purified proteins and imaged in situ in the membrane. Tomograms were reconstructed from multiple images and models fitted using known structures. MAC lesions generated in this manner were heterogeneous in comparison with the "extracted" MAC described above; more than half of the observed lesions were single ring structures with a single C5b-8 stalk, closely resembling the extracted MAC structure but a minority of lesions appeared to be aggregates containing several C5b-8 stalks in various configurations. Averaging of selected single ring lesions and fitting of known component structures yielded a ring structure (23 Å resolution) with a stalk derived from C5b6 and a ring diameter that is consistent with a 22-component complex, with one "stave" each from C6, C7, C8β and C8α and the rest from C9. Despite being the same diameter as the detergent extracted MAC, only 16 C9 units are modelled in this structure. This may be a result of unresolved density at the lower resolution ring seam, or indeed may indicate a level of heterogeneity in the composition of the MAC. The ring was incomplete, a split washer, and the precursor complex staves did not traverse the membrane, precisely as described for the extracted MAC.

The similarities between these structures, derived independently and in different ways, is remarkable and gives confidence that they represent the complete MAC; the differences relate predominantly to heterogeneity and an apparent predilection for precursor complexes to associate and aggregate in the membrane.

Poly-C9 structures reveal similarities and important differences. Yet more evidence supporting the 22-fold composition of the MAC pore is provided by a new structure of poly-C9 [49]. Whereas earlier analyses based on low resolution EM and biochemical studies had suggested that the poly-C9 ring was composed of between 12 and 18 C9 molecules [50], the new cryo-EM structure at 8 Å resolution clearly demonstrate that poly-C9 is assembled from 22 C9 monomers in a closed ring. The closed ring structure illustrates an important difference to the MAC – in poly-C9 the final C9 can intercalate with the first to complete the ring, while in the MAC the final C9 cannot form a stable link with the initiating C6. The poly-C9 structure demonstrates an important contribution of the thrombospondin-1 (TSP1) domains to C9-C9 oligomerisation.

Emergence of a consensus structure. The deluge of structural information provides both clarity and clues to the process of MAC formation and the structure of the complete MAC. The structures confirm the order and clockwise direction of assembly from precursors through to pore as proposed in structures extrapolated from the soluble C5b-9 complex [5]. They demonstrate that the obvious stalk protrusion is predominantly C5b-derived. They unequivocally define the stoichiometry of the complex and show that this is dictated by the rotation of precursors to an orientation that sets the "pitch" of the forming pore. They identify interaction interfaces that are key to the MAC sequential assembly mechanism. Surprisingly, the physiological pore is not a symmetric ring as modelled in previous structures referenced above, but rather has a unique split-washer configuration, which likely impacts biophysical properties of its lipid environment.

All three newly resolved MAC/polyC9 structures are consistent with the MAC forming a giant β-barrel pore. All components, except C5b, contain a central MACPF/CDC fold, found in a number of

pore-forming proteins such as the cholesterol-sensing bacterial toxins [51]. A series of biochemical studies on members of the CDC family have shed light on how this domain facilitates pore formation. The MAC/CDC domain is formed of a central 4-stranded kinked β-sheet, conferring an 'L' shape to the domain, flanked by a series of 3 helical bundles that are critical to membrane insertion. During pore formation, transmembrane segments (TMS) 1 and 2 undergo a large conformational rearrangement to form β-hairpins [52]. These hairpins insert into the lipid bilayer, forming the wall of the β-pore. A third helical bundle (CH3) appears to act as a 'latch', swinging towards the center of the pore lumen and triggering TMS unfurling [53,54]. The presence of this highly conserved MACPF/CDC fold within the terminal complement proteins forming the MAC suggests a similar mechanism of pore formation, supported by mutational data and contiguous barrel density observed in the recent EM structural studies [6,7]. This structural model of pore formation is supported by the demonstration that transmembrane segment swaps between C8α or C9 and perfringolysin yielded hybrids that were capable of lytic pore formation [55].

The stages of MAC assembly

A synthesis of the available structures provides insight into how the MAC is initiated. Cleavage of C5 causes major conformational change in C5b, creating a labile conformation that rapidly decays unless stabilised by C6 binding through its short consensus repeat/complement control protein (SCR/CCP) and factor I/membrane attack complex (FIMAC)-like domains. Rotation of the C6 molecule and opening up of its central β-sheet reveals binding sites for C7; the SCR/CCP and FIMAC domains of C7 likely bind into the C5 macroglobulin domains, thereby displacing the C5b67 complex from the convertase [5]. The C5b67 complex possesses a poorly defined hydrophobic site enabling complexes that encounter membrane before the site decays or is captured by fluid-phase inhibitors to bind membrane. Recent cryo-EM tomography images of membrane-bound C5b67 suggest that the complex is buried in the outer lamella of the membrane [7]. Once bound to a membrane, C5b67 is stable and ready to accept C8. The stability of C5b67 is apparent from studies of erythrocyte

intermediates; whereas C5b6 binds weakly and is removed by washing, C5b-7 binds tightly, surviving multiple wash steps and prolonged storage, without impairing the integrity of the membrane. Binding of C5b67 does not cause leakage through the membrane, supporting the imaging evidence that it disrupts only the outer lamella [7]. C8β binds C7 in the tri-molecular complex, both C8β and C8α unfold, unwind and align with C7, the TMS1/TMS2 domains inserting deep into the bilayer, likely disrupting but not fully penetrating the inner lamella. Imaging and some functional studies suggest that the membrane is breached at this stage; however, this is likely dependent on the lipid composition of the membrane and of minor functional consequence in comparison to the MAC pore. Rotation of the C8α chain and displacement of C8γ exposes the binding site for the first C9 as noted above. C9 binds, unfolds and inserts, facilitated by the membrane disruption caused by C5b-8 which lowers the activation energy for C9 binding and insertion [56,57]. Further acquisition of C9 molecules into the complex is then energetically favoured leading to rapid completion of the pore.

The importance of the asymmetric pore

We contend that the most important difference between the newly described MAC structures and previous models (and from the structure of poly-C9) is the observed asymmetry, a consequence of its heterogeneous molecular composition with contributions from each of the five terminal components (C6, C7, C8β, C8α, C9). The β-hairpins of the terminal components comprising the C5b-8 precursor are shorter than those of C9 and hence fail to completely breach the membrane but nevertheless cause disruption of both the outer and inner lamellae of the lipid bilayer. **By analogy to the lipid-binding residues of** cholesterol-dependent bacterial pore-forming cytolysins, we suggest that these partially inserted C5b-8 β-hairpins may confer a degree of lipid selectivity to MAC formation. The concept that MAC preferentially targets certain membrane domains in nucleated cells is an old one linked to the well-documented recovery process of MAC removal [25]. Similarly, MAC targeting to specific lipid environments in the bacterial cell membrane has been suggested to facilitate bacterial killing [58-60].

The second component of asymmetry is the split-washer appearance seen in both the cryo-EM and cryo-tomography structures; this confers an unanticipated degree of flexibility to the pore which is imaged in various degrees of closure. Within the context of a membrane, terminal pathway proteins can form pores with varied stoichiometries [7]. The biological implications of this heterogeneity merits further investigation and may be influenced by the local lipid environment of the target membrane.

Cryo-tomography images of membranes bearing precursor C5b67 or C5b-8 complexes, and even intact MAC, showed different-sized aggregates of the complexes. Whether MAC/MAC precursor aggregation is of biological relevance is uncertain; however, images of nucleated cells exposed to MAC show obvious aggregation of MAC into tightly packed clusters for elimination from the cell during recovery [25].

Structures inform bacterial killing by MAC

The single essential role of the MAC in immune defence is to kill a subset of gram-negative bacteria, cocci of the phyla *Neisseria*, *Moraxella* and *Haemophilus*, that are inefficiently eliminated by opsonophagocytosis [61-63]. Of these, protection against *Neisseria* is of most clinical relevance as is apparent from the fact that terminal pathway component deficiencies most commonly present with meningitis and/or septicaemia caused by *N. meningitidis* [61]. The Neisserial cell wall, comprising two lipid membranes separated by a layer of peptidoglycan, represents a particular challenge for a pore-forming toxin; how are the outer barriers breached to deliver the lethal hit to the inner membrane? A variety of mechanisms have been suggested, including targeting of MAC formation to areas of adhesion between inner and outer membranes [62], and delivery of terminal complement proteins into the periplasmic space through the MAC pore with subsequent MAC assembly on the inner membrane [63,64]. The first proposed mechanism can be effectively dismissed when considered in light of the MAC structure [65]; the transmembrane region of the MAC is located at

the extreme end of the barrel, with no space for a second insertion site! The second hypothesis requires that C5b6 and each of the terminal components can pass through the MAC pore. This is unlikely given a pore size of ~10nm, too small for many of the MAC proteins, though the pore size does not preclude transport of lysozyme. A third model, based on recent structural data for the MAC [6], suggests a role for local membrane distortion in bacterial killing.

Structures inform cell response to MAC

The classical view of the MAC is that of a rigid pore penetrating the membrane through which water and ions transit unhindered into the cell to cause lysis; however, it has long been clear that the situation is much more complex, particularly when MAC is activated on nucleated cell targets or on bacteria. Nucleated cells express multiple resistance mechanisms to avoid MAC-mediated killing but respond to MAC in a variety of ways, including the rapid removal of MAC lesions from the cell surface, a process termed recovery [3,4,25]. Imaging of MAC-exposed cells and shed membrane vesicles shows that MAC lesions are aggregated into densely packed clusters on the cell and vesicle membrane; precisely how these aggregates form within minutes of attack has been a puzzle. The structures indicate a tendency for MAC precursors to aggregate which might contribute to the observed clustering; however, no such efficient clustering is seen on inert targets such as erythrocytes. The suggestion that MAC pore asymmetry confers selectivity for specific lipid microdomains raises the possibility that MAC selectively deposit *en masse* and at high density in these microdomains. Indeed, it has been shown that shed MAC-decorated vesicles differ in protein and lipid content from bulk membrane in [66,67], and a recent analysis of microvesicles released from tumor cells in response to MAC showed that they were enriched in lipid raft markers and GPI-anchored proteins [68]. If a tendency to aggregate and to deposit in lipid raft microdomains explains the clustering, what then explains the efficient elimination from the membrane? Again, the structures suggest an answer; the lipid perturbation induced by pore asymmetry is likely most marked at the edges of MAC clusters, destabilising the membrane and leading to shedding or

internalisation. A final puzzle related to recovery concerns the shed MAC-decorated vesicles – despite the abundance of MAC lesions they don't leak entrapped dye [4,23]! Others have suggested that the pore is "plugged" in some manner [69]. The recent structures allow us to speculatively suggest a mechanism for pore "plugging" involving loops of C9 that in the poly C9 structure are modelled as folded back on the transmembrane regions, spanning approximately 50 Å. If these loops are reoriented to lay flat across the missing membrane of shed vesicles they could span the entire diameter of the MAC pore (110 Å).

MAC formation on nucleated cells has been associated with many and diverse effects in different cell types, including altered proliferation, induction/inhibition of apoptosis, changes in cell motility, inflammasome activation and release of inflammatory mediators etc. [70-73] (Figure 3). The many pro-inflammatory effects have established the MAC as an important driver of inflammation; however, the signalling pathways involved remain undefined. In all cells studied, calcium signalling plays a part; calcium ion influx through the MAC pore causes a rapid increase in intracellular free calcium ($[Ca^{2+}]i$) from nanomolar to micromolar levels [25]. Changes in $[Ca^{2+}]i$ impact calcium-sensing signalling systems such as calmodulin to initiate a cascade of downstream effectors, including activation of the core signal hubs AKT and PI3K [74]. These pathways have been implicated in cell responses to MAC in diverse cell types [75,76], and were again highlighted in an unbiased transcriptomics analysis of MAC-triggered signalling pathways [77]. It is, however, clear that calcium is not the only mediator of MAC signals; removal of extracellular calcium by chelation reduced but did not ablate MAC-induced cell activation [25]. Indeed, a rise in $[Ca^{2+}]i$ still occurred, demonstrating MAC-triggered calcium store release. The key trigger for endoplasmic reticulum (ER) calcium store release is inositol 1,4,5-triphosphate (IP3), usually generated at the membrane when a G-protein coupled receptor (GPCR) binds its extracellular ligand; associated G-proteins then activate phospholipase C (PLC) causing release of IP3 into the cytoplasm. It has been reported that both MAC precursors and the MAC associate with G-proteins in the membrane that confer signalling capacity

to the complex. Immunoprecipitates of MAC from attacked cells contained all the component subunits of a pertussis toxin (PTX)-sensitive G protein complex; this finding was given functional significance by demonstrating that PTX inhibited MAC effects on the cell [78,79]. Perhaps the most likely explanation of the reported association with MAC is that the MAC immunoprecipitates contain GPCR and their associated G-proteins. Although there has been no demonstration of direct interaction between MAC and GPCR, it is well known that GPCR localise preferentially to lipid rafts [80], sites of MAC accumulation and multi-functional membrane signalling platforms that sequester numerous receptors and signallers, including GPCRs and GPI-linked proteins. A recent publication suggested that MAC may also signal in intracellular locations; after internalisation and incorporation into Rab5+ endosomes MAC activated non-canonical pathways for NF-kB activation [81]. The cumulative data suggest that MAC signalling requires its location in specialised microenvironments – notably, the signalling-rich lipid raft environment; structural features described above may shed light on why and how MAC prefers such environments and how it interacts (directly or indirectly) with signalling molecules.

Concluding remarks

We here present the MAC as an ancient and critical part of innate immunity that has evolved considerable structural complexity. This evolution from a simple pore to a highly ordered asymmetric oligomer has conferred additional properties such as lipid selectivity and pore flexibility that enable MAC to interact with self-cell protection mechanisms and influence cell function in multiple ways. The pressure to evolve may have come from bacteria that presented membranes difficult to penetrate with a simple pore. The resultant complex is an elegant biological machine that goes far beyond its popular image as a simple pore.

Acknowledgments

We thank Marina Serna for rendering Fig.2A. This work is supported by a CRUK Career Establishment Award (C26409/A16099) to D.B. C.B is funded by a BBSRC Doctoral Training Program grant, Ref: BB/J014575/1.

Figure Legends

Figure 1 Visualising the MAC over the last 3 decades. **(A-C)** Early negatively-stained electron micrographs of both the MAC and poly-C9. **(A)** MAC complexes assembled on rabbit erythrocytes (from reference # 40). **(B)** Side-view of the MAC inserted into liposomes (from reference # 14). **(C)** Poly-C9 complexes showing both top and side views (from reference # 12). **(D-E)** Recent images of the MAC enabled by advanced in both EM hardware and software. **(D)** Cryo-EM image of detergent-solubilised MAC complexes. Scale bar, 50 nm. **(E)** Representative 2D class averages of both a top and side view of the detergent-solubilised MAC.

Figure 2 Comparison of the recent structural insights in both the MAC and poly C9. **(A)** Cryo-EM reconstruction of the complete, detergent solubilised MAC, highlighting the 'split-washer' shape and 22 subunit composition (EMDB: 3134; Reference #6). Components coloured as follows: C5b (purple), C6 (yellow), C7 (green), C8β (orange), C8α (red), C8γ (dark blue), C9 (light blue). The transmembrane β-hairpins forming the wall of the pore are coloured in grey, and includes the detergent belt. **(B)** Cryo-EM reconstruction of the poly-C9 complexes formed in the absence of membranes or detergent (EMDB: 3235; Reference #49). The 22 subunits of the map are segmented, individually coloured and fitted with a pseudo atomic model (PDB:5FMW). **(C)** Cryo-tomography reconstruction of the complete MAC formed on liposomes (EMDB: 3289; Reference #7). The MAC is in teal, the bilayer in grey. All figures are rendered in Chimera [82].

Figure 3 Schematic of the signalling cascades associated with sub-lytic MAC. Pore components are coloured as in Fig.2A.

References:

1. Müller-Eberhard HJ. The membrane attack complex of complement. Annu. Rev. Immunol. 4 (1986) 503-528.
2. Morgan BP. Mechanisms of tissue damage by the membrane attack complex of complement. Complement Inflamm. 6 (1989) 104-111.
3. Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J. 264 (1989) 1-14.
4. Morgan BP. The membrane attack complex as an inflammatory trigger. Immunobiology. 221 (2016) 747-751.
5. Hadders MA, Bubeck D, Roversi P, Hakobyan S, Forneris F, Morgan BP, Pangburn MK, Llorca O, Lea SM, Gros P. Assembly and regulation of the membrane attack complex based on structures of C5b6 and sC5b9. Cell Rep. 1 (2012) 200-207.
6. Serna M, Giles JL, Morgan BP, Bubeck D. Structural basis of complement membrane attack complex formation. Nat. Commun. 7 (2016) 10587.
7. Sharp TH, Koster AJ, Gros P. Heterogeneous MAC Initiator and Pore Structures in a Lipid Bilayer by Phase-Plate Cryo-electron Tomography. Cell Rep. 15 (2016) 1-8.
8. Kinsky SC. Antibody-complement interaction with lipid model membranes. Biochim. Biophys. Acta. 265 (1972) 1-23.
9. Borsos T, Dourmashkin RR, Humphrey JH. Lesions in erythrocyte membranes caused by immune haemolysis. Nature. 202 (1964) 251-252.
10. Hesketh TR, Dourmashkin RR, Payne SN, Humphrey JH, Lachmann PJ. Lesions due to complement in lipid membranes. Nature. 233 (1971) 620-623.
11. Podack ER, Esser AF, Biesecker G, Müller-Eberhard HJ. Membrane attack complex of complement: a structural analysis of its assembly. J Exp Med. 151 (1980) 301-313.
12. Podack ER. Molecular composition of the tubular structure of the membrane attack complex of complement. J Biol Chem. 259 (1984) 8641-8647.
13. Esser AF. Big MAC attack: complement proteins cause leaky patches. Immunol Today. 12 (1991) 316-318.
14. Tschopp J, Podack ER, Müller-Eberhard HJ. Ultrastructure of the membrane attack complex of complement: Detection of the tetramolecular C9-polymerizing complex C5b-8. Proc. Natl. Acad. Sci. USA. 79 (1982) 7474-7478.
15. Tschopp J. Circular polymerization of the membranolytic ninth component of complement. Dependence on metal ions. J Biol Chem. 259 (1984) 10569-10573.
16. Cerenius L, Kawabata S, Lee BL, Nonaka M, Söderhäll K. Proteolytic cascades and their involvement in invertebrate immunity. Trends Biochem Sci. 35 (2010) 575-583.
17. Suzuki MM, Satoh N, Nonaka M. C6-like and C3-like molecules from the Cephalochordate, Amphioxus, suggest a cytolytic complement system in invertebrates J. Mol. Evol. 54 (2002) 671-679.

18. Chondrou MP, Londou AV, Zarkadis IK. Expression and phylogenetic analysis of the ninth complement component (C9) in rainbow trout. Fish Shellfish Immunol. 21 (2006) 572-576.

19. Tomlinson S, Stanley KK, Esser AF. Domain structure, functional activity, and polymerization of trout complement protein C9. Dev Comp Immunol. 17 (1993) 67-76.

20. Jensen JA, Festa E, Smith DS, Cayer M. The complement system of the nurse shark: hemolytic and comparative characteristics. Science. 214 (1981) 566-569.

21. Lambris JD, Reid KB, Volanakis JE. The evolution, structure, biology and pathophysiology of complement. Immunol Today. 20 (1999) 207-211.

22. Mondragón-Palomino M, Piñero D, Nicholson-Weller A, Laclette JP. Phylogenetic analysis of the homologous proteins of the terminal complement complex supports the emergence of C6 and C7 followed by C8 and C9. J Mol Evol. 49 (1999) 282-289.

23. Morgan BP, Dankert JR, Esser AF. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis.

J Immunol. 138 (1987) 246-253.

24. Meri S, Morgan BP, Davies A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology. 71 (1990) 1-9.

25. Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem. J. 264 (1989) 1-14.

26. Galat A. The three-fingered protein domain of the human genome. Cell Mol. Life Sci. 65 (2008) 3481-3493.

27. Hughes TR, Piddlesden SJ, Williams JD, Harrison RA, Morgan BP. Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement. Biochem. J. 284 (1992)169-176.

28. Powell MB, Marchbank KJ, Rushmere NK, van den Berg CW, Morgan BP. Molecular cloning, chromosomal localization, expression, and functional characterization of the mouse analogue of human CD59. J Immunol. 158 (1997) 1692-1702.

29. Hinchliffe SJ, Rushmere NK, Hanna SM, Morgan BP. Molecular cloning and functional characterization of the pig analogue of CD59: relevance to xenotransplantation. J Immunol. 160 (1998) 3924-3932.

30. van den Berg CW, Harrison RA, Morgan BP. The sheep analogue of human CD59: purification and characterization of its complement inhibitory activity. Immunology. 78 (1993) 349-57.

31. Dos Remedios NJ, Ramsland PA, Hook JW, Raison RL Identification of a homologue of CD59 in a cyclostome: implications for the evolutionary development of the complement system. 23 (1999) Dev. Comp. Immunol. 23: 1-14.

32. Yeh HY, Klesius PH. Molecular cloning and expression of catfish, Ictalurus punctatus, complement membrane attack complex inhibitor CD59. Vet. Immunol. Immunopathol. 120 (2007) 246-253.

33. Papanastasiou AD, Georgaka E, Zarkadis IK. Molecular cloning of a CD59-like gene in rainbow trout. Mol. Immunol. 44 (2007) 1300-1306.

34. Shi Y, Toet H, Rathinasamy V, Young ND, Gasser RB, Beddoe T, Huang W, Spithill TW. First insight into CD59-like molecules of adult Fasciola hepatica. Exp. Parasitol. 144 (2014) 57-64.

35. Sun C, Wu J, Liu S, Li H, Zhang S. Zebrafish CD59 has both bacterial-binding and inhibiting activities. Dev. Comp. Immunol. 41 (2013) 178-188.

36. Bodian DL, Davis SJ, Morgan BP, Rushmere NK. Mutational analysis of the active site and antibody epitopes of the complement-inhibitory glycoprotein, CD59. J. Exp. Med. 185 (1997) 507-516.

37. Huang Y, Smith CA, Song H, Morgan BP, Abagyan R, Tomlinson S. Insights into the human CD59 complement binding interface toward engineering new therapeutics. J. Biol. Chem. 280 (2005) 34073-34079.

38. Huang Y, Qiao F, Abagyan R, Hazard S, Tomlinson S. Defining the CD59-C9 binding interaction. J. Biol. Chem. 281 (2006) 27398-27404.

39. Biesecker G, Podack ER, Halverson CA, Müller-Eberhard HJ. C5b-9 dimer: isolation from complement lysed cells and ultrastructural identification with complement-dependent membrane lesions. J. Exp. Med. 149 (1979) 448-458.

40. Podack ER, Tschopp J, Müller-Eberhard HJ. Molecular organization of C9 within the membrane attack complex of complement. Induction of circular C9 polymerization by the C5b-8 assembly. J. Exp. Med. 156 (1982) 268-282.

41. Lovelace LL, Cooper CL, Sodetz JM, Lebioda L. Structure of human C8 protein provides mechanistic insight into membrane pore formation by complement. J. Biol. Chem. 286 (2011) 17585-1792.

42. Aleshin AE, Schraufstatter IU, Stec B, Bankston LA, Liddington RC, DiScipio RG. Structure of complement C6 suggests a mechanism for initiation and unidirectional, sequential assembly of membrane attack complex (MAC). J. Biol. Chem. 287 (2012) 10210-10222.

43. Aleshin AE, DiScipio RG, Stec B, Liddington RC. Crystal structure of C5b-6 suggests structural basis for priming assembly of the membrane attack complex. J. Biol. Chem. 287 (2012) 19642-19652.

44. Schreck SF, Parker C, Plumb ME, Sodetz JM. Human complement protein C8 gamma. Biochim. Biophys. Acta 1482 (2000) 199-208.

45. Brickner A, Sodetz JM. Function of subunits within the eighth component of human complement: selective removal of the gamma chain reveals it has no direct role in cytolysis. Biochemistry. 23 (1984) 832-837.

46. Slade DJ, Lovelace LL, Chruszcz M, Minor W, Lebioda L, Sodetz JM. Crystal structure of the MACPF domain of human complement protein C8 alpha in complex with the C8 gamma subunit. J. Mol. Biol. 379 (2008) 331-342.

47. Slade DJ, Chiswell B, Sodetz JM. Functional studies of the MACPF domain of human complement protein C8alpha reveal sites for simultaneous binding of C8beta, C8gamma, and C9. Biochemistry. 45 (2006) 5290-5296.

48. Stewart JL, Sodetz JM. Analysis of the specific association of the eighth and ninth components of human complement: identification of a direct role for the alpha subunit of C8. Biochemistry. 24 (1985) 4598-4602.

49. Dudkina NV, Spicer BA, Reboul CF, Conroy PJ, Lukoyanova N, Elmlund H, Law RH, Ekkel SM, Kondos SC, Goode RJ, Ramm G, Whisstock JC, Saibil HR, Dunstone MA. Structure of the poly-C9 component of the complement membrane attack complex. Nat. Commun. 7 (2016) 10588.

50. Tschopp J, Engel A, Podack ER. Molecular weight of poly(C9). 12 to 18 C9 molecules form the transmembrane channel of complement. J. Biol. Chem. 259 (1984) 1922-1928.

51. Tweten RK, Hotze EM, Wade KR. The unique molecular choreography of giant pore formation by the cholesterol-dependent cytolysins of gram-positive bacteria. Annu. Rev. Microbiol. 69 (2015) 323–340.

52. Shepard LA, Heuck AP, Hamman BD, Rossjohn J, Parker MW, Ryan KR, Johnson AE, Tweten RK. Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin Clostridium perfringens perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. Biochemistry. 37 (1998) 14563–14574.

53. Shatursky O, Heuck AP, Shepard LA, Rossjohn J, Parker MW, Johnson AE, Tweten RK. The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. Cell. 99 (1999) 293–299.

54. Lukoyanova N, Kondos SC, Farabella I et al. Conformational changes during pore formation by the perforin-related protein pleurotolysin. PLoS Biol. 13 (2015) e1002049

55. Weiland MH, Qian Y, Sodetz JM. Membrane pore formation by human complement: functional importance of the transmembrane β-hairpin (TMH) segments of C8α and C9. Mol Immunol. 57 (2014) 310-316.

56. Esser AF, Kolb WP, Podack ER, Müller-Eberhard HJ. Molecular reorganization of lipid bilayers by complement: a possible mechanism for membranolysis. Proc. Natl. Acad. Sci. USA. 76 (1979) 1410-1414.

57. Sims PJ, Wiedmer T. Kinetics of polymerization of a fluoresceinated derivative of complement protein C9 by the membrane-bound complex of complement proteins C5b-8. Biochemistry. 23 (1984) 3260-3267.

58. Welby M, Poquet Y, Tocanne JF. The spatial distribution of phospholipids and glycolipids in the membrane of the bacterium Micrococcus luteus varies during the cell cycle. FEBS Lett. 384 (1996) 107-111.

59. Nishibori A, Kusaka J, Hara H, Umeda M, Matsumoto K. Phosphatidylethanolamine domains and localization of phospholipid synthases in Bacillus subtilis membranes. J Bacteriol. 187 (2005) 2163-2174.

60. Berends ET, Dekkers JF, Nijland R, Kuipers A, Soppe JA, van Strijp JA, Rooijakkers SH. Distinct localization of the complement C5b-9 complex on Gram-positive bacteria. Cell Microbiol. 15 (2013) 1955-1968.

61. Ram S, Mackinnon FG, Gulati S, McQuillen DP, Vogel U, Frosch M, Elkins C, Guttormsen HK, Wetzler LM, Oppermann M, Pangburn MK, Rice PA. The contrasting mechanisms of serum resistance of Neisseria gonorrhoeae and group B Neisseria meningitidis. Mol. Immunol. 36 (1999) 915-928.

62. Bhakdi S, Kuller G, Muhly M, Fromm S, Seibert G, Parrisius J. Formation of transmural pores in serum-sensitive Escherichia coli. Infect. Immun. 55 (1987) 206-210.

63. Taylor PW. Complement-mediated killing of gram-negative bacteria: an elusive mechanism. Exp. Clin. Immunogenet. 9 (1992) 48-56.

64. Dankert JR, Esser AF. Bacterial killing by complement: C9-mediated killing in the absence of C5b-8. Biochem J. 244 (1987) 393-399.

65. Berends ETM, Kuipers A, Ravesloot MM, Urbanus RT, Rooijakkers SH. Bacteria under stress by complement and coagulation. FEMS Microbiol Rev. 38 (2014) 1146-1171.

66. Stein JM, Luzio JP. Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. Biochem. J. 274 (1991) 381-386.

67. Head BP, Patel HH, Insel PA. Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. Biochim. Biophys. Acta. 1838 (2014) 532-545.

68. Stratton D, Moore C, Antwi-Baffour S, Lange S, Inal J. Microvesicles released constitutively from prostate cancer cells differ biochemically and functionally to stimulated microvesicles released through sublytic C5b-9. Biochem. Biophys. Res. Commun. 460 (2015) 589-595.

69. Lachmann PJ. Plugs, burns and poisons. Bioessays. 7 (1987) 223-229.

70. Cole, DS, Morgan BP. Beyond lysis: how complement influences cell fate. Clin. Sci. 104 (2003) 455–466.

71. Wang Y, He Q, Qin H, Xu J, Tong J, Gao L, Xu J. The complement C5b-9 complexes induced injury of glomerular mesangial cells in rats with Thy-1 nephritis by increasing nitric oxide synthesis. Life Sci. 79 (2006) 182-192.

72. Soane L, Rus H, Niculescu F, Shin ML. Inhibition of oligodendrocyte apoptosis by sublytic C5b-9 is associated with enhanced synthesis of bcl-2 and mediated by inhibition of caspase-3 activation. J. Immunol. 163 (1999) 6132–6138.

73. Morgan B.P. The membrane attack complex as an inflammatory trigger. Immunobiology. 221 (2016) 747-751.

74. Qiu W, Zhang Y, Liu X, Zhou J, Li Y, Zhou Y, Shan K, Xia M, Che N, Feng X, Zhao D, Wang Y. Sublytic C5b-9 complexes induce proliferative changes of glomerular mesangial cells in rat Thy-1 nephritis through TRAF6-mediated PI3K-dependent Akt1 activation. J. Pathol. 226 (2012) 619–632.

75. Fosbrink M, Niculescu F, Rus V, Shin ML, Rus H. C5b-9-induced endothelial cell proliferation and migration are dependent on Akt inactivation of forkhead transcription factor FOXO1. J. Biol. Chem. 281 (2006) 19009–19018.

76. Gao L, Qiu W, Wang Y, Xu W, Xu J, Tong J. Sublytic complement C5b-9 complexes induce thrombospondin-1 production in rat glomerular mesangial cells via PI3-k/Akt: association with activation of latent transforming growth factor-beta1. Clin. Exp. Immunol. 144 (2006) 326–334.

77. Towner LD, Wheat RA, Hughes TR, Morgan BP. Complement membrane attack and tumourigenesis: A systems biology approach. J. Biol. Chem. 291 (2016) 14927-14938.

78. Niculescu F, Rus H, Shin ML. Receptor-independent activation of guanine nucleotide-binding regulatory proteins by terminal complement complexes. J. Biol. Chem. 269 (1994) 4417-4423.

79. Niculescu F, Rus H. Mechanisms of signal transduction activated by sublytic assembly of terminal complement complexes on nucleated cells. Immunol. Res. 24 (2001) 191-199.

80. Villar VA, Cuevas S, Zheng X, Jose PA. Localization and signaling of GPCRs in lipid rafts. Methods Cell Biol. 132 (2016) 3-23.

81. Jane-Wit D, Surovtseva YV, Qin L, Li G, Liu R, Clark P, Manes TD, Wang C, Kashgarian M, Kirkiles-Smith NC, Tellides G, Pober JS. Complement membrane attack complexes activate noncanonical NF-κB by forming an Akt+ NIK+ signalosome on Rab5+ endosomes. Proc Natl Acad Sci USA. 112 (2015) 9686–9691.

82. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera--a visualization system for exploratory research and analysis. J. Comput. Chem. 25 (2004) 1605-1612.

Figure 1.

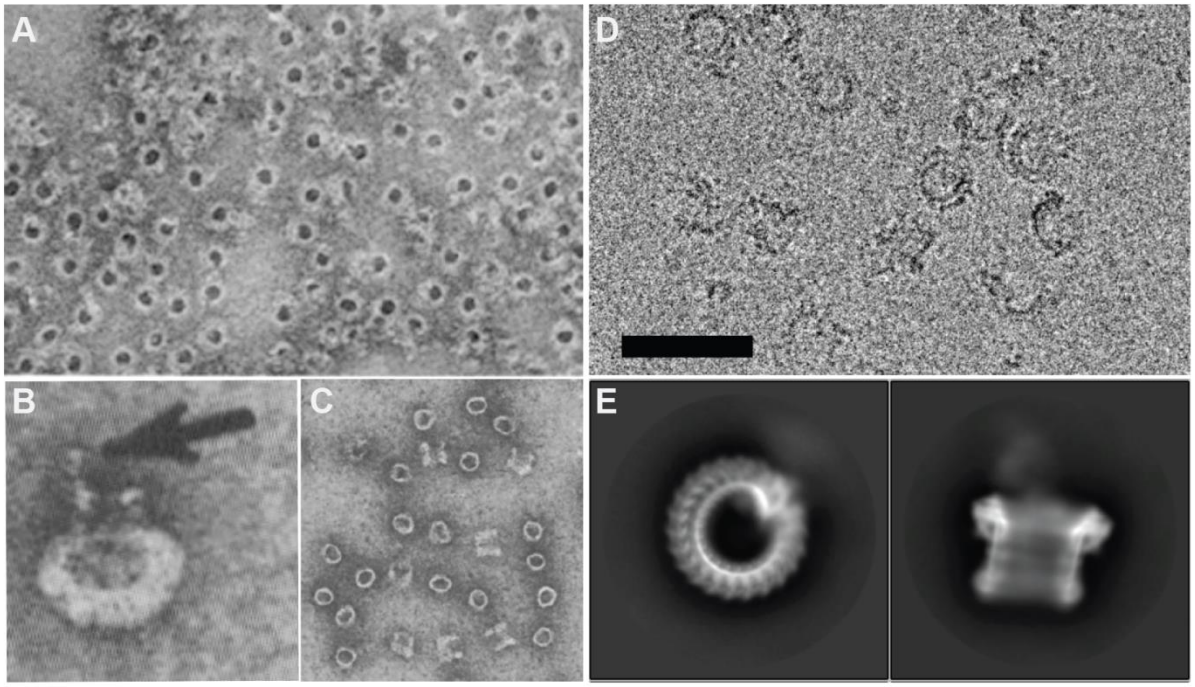


Figure 2.

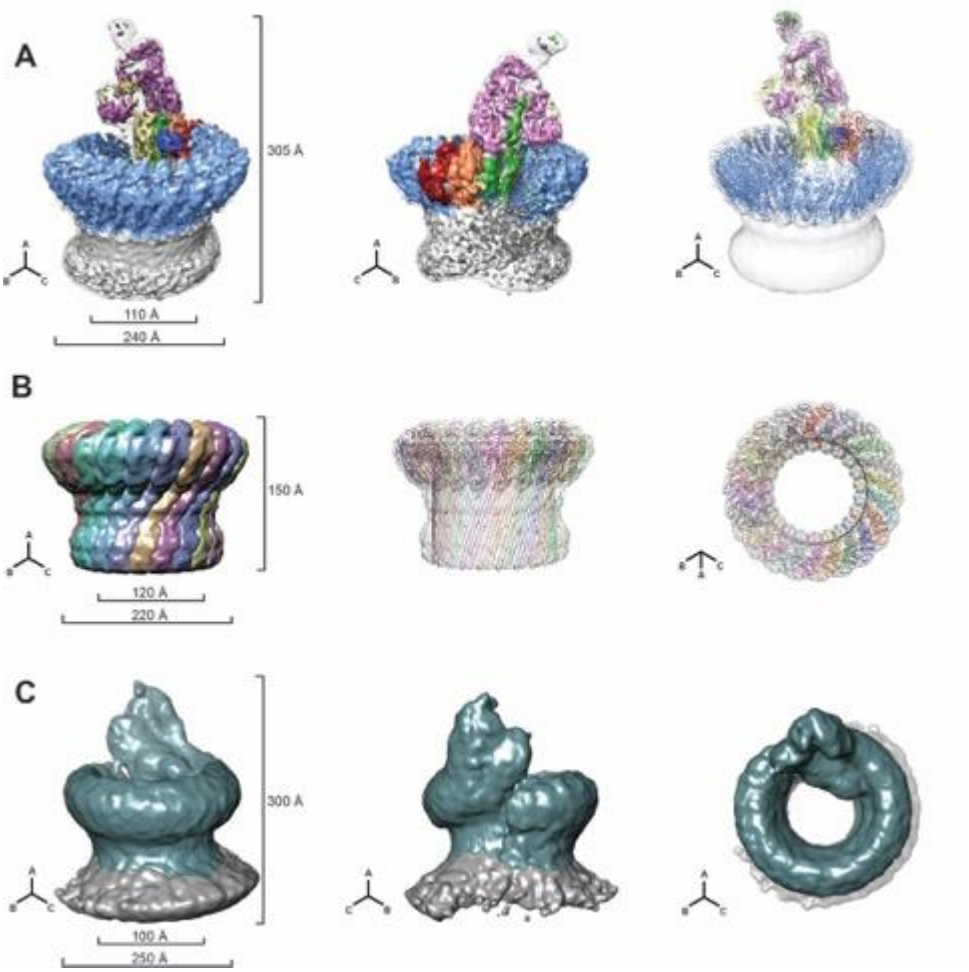
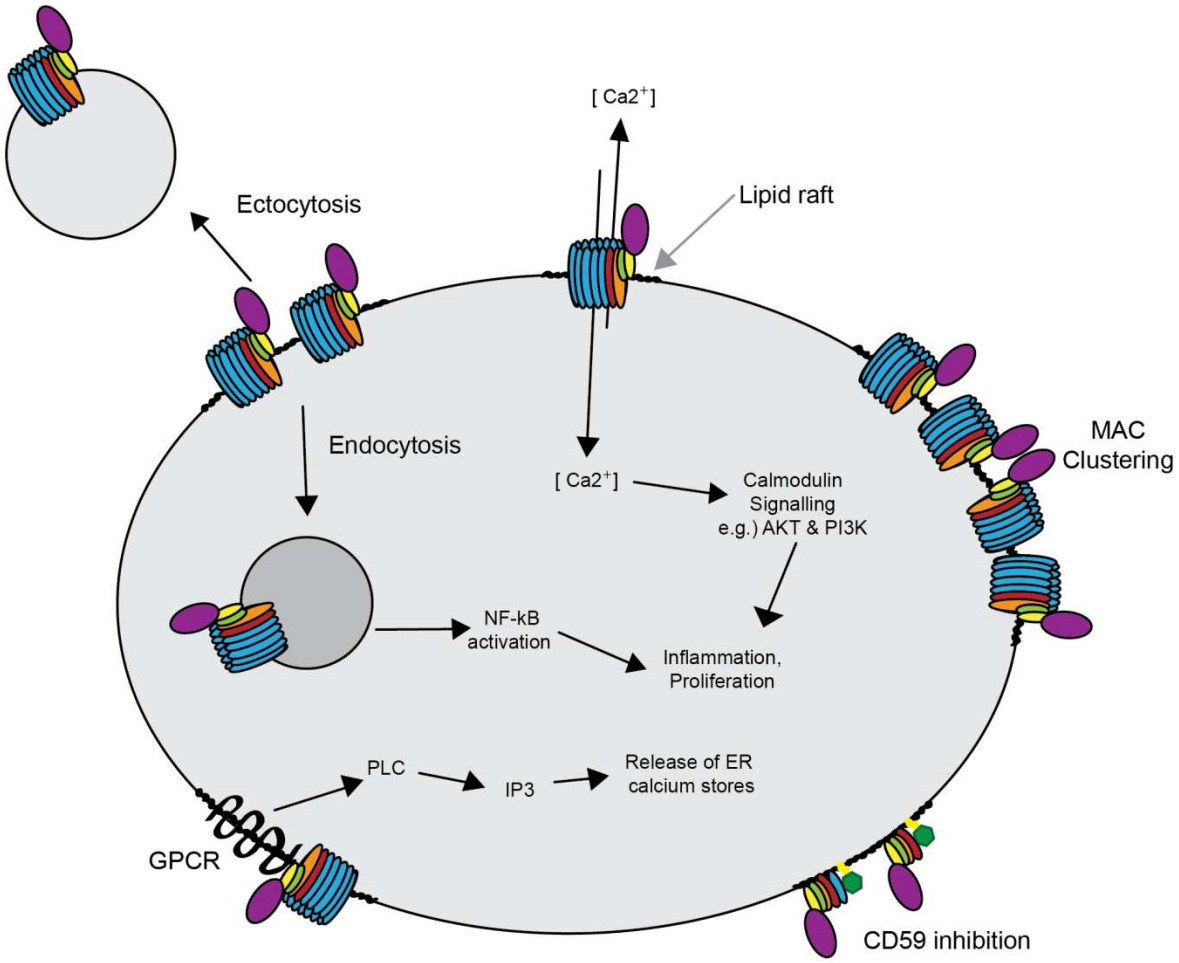


Figure 3.


[Ca2+]
Ectocytosis
Lipid raft
Endocytosis
[Ca2+]
Calmodulin
Signalling
e.g.) AKT & PI3K
MAC
Clustering
NF-kB
activation
Inflammation,
Proliferation
PLC
IP3
Release of ER
calcium stores
GPCR
CD59 inhibition