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Novel Evasion Mechanisms of the Classical Complement Pathway

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Abstract

Complement is a network of soluble and cell surface-associated proteins which gives rise to a self-amplifying, yet tightly regulated system with fundamental roles in immune surveillance and clearance. Complement becomes activated on the surface of 'non-self' cells by one of three initiating mechanisms known as the classical, lectin, or alternative pathways. Evasion of complement function is a hallmark of invasive pathogens and hematophagous organisms. While many complement inhibition strategies hinge on hijacking activities of endogenous complement regulatory proteins, an increasing number of uniquely evolved evasion molecules have been discovered over the past decade. In this review we focus on several recent investigations which have revealed mechanistically distinct inhibitors of the classical pathway. Because the classical pathway is an important and specific mediator of various autoimmune and inflammatory disorders, in-depth knowledge of novel evasion mechanisms could direct future development of therapeutic anti-inflammatory molecules.

Preface

The human complement system is comprised of a collection of cell surface and circulating plasma proteins that mediate important functions in innate and adaptive immune responses (1). Complement provides protection against microbial infections via activation of a proteolytic cascade that ultimately results in rapid clearance of target cells. Important effector functions of the complement system include: a) labeling microbes for phagocytosis by immune cells; b) recruitment of phagocytes to the site of infection; c) the direct assembly of a pore-forming complex known as the membrane attack complex (MAC) on susceptible membranes; and d) enhancement of adaptive immunity.

Complement evasion molecules have been found in a considerable number of microbial pathogens (2) and hematophagous organisms including mosquitos (3), ticks (4–7), mites (8), and several species of sanguinivorous flies (9–11). Thus, it appears organisms whose

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lifestyles involve contact with blood and related bodily fluids have necessarily evolved mechanisms to evade complement attack. Many organisms are known to co-opt host complement regulatory proteins (12), however, naturally occurring novel inhibitors which directly target complement components are being discovered at an increasing rate. In this review we will focus on a select group of recently discovered classical pathway (CP) specific inhibitors for which detailed mechanistic analysis have been performed (Table 1). These studies reveal a wide breadth of novel molecular strategies now known to specifically target and inactivate the CP.

Complement Activation

Upon encountering foreign or damaged self-cells, complement pattern recognition proteins trigger a series of enzymatic events at the target surface. A central step in the cascade involves the cleavage of complement component C3 into the anaphylatoxin C3a and the opsonic C3b fragment which covalently attaches to the target surface and labels it for phagocytosis. The conversion of C3 is catalyzed by surface-assembled protease complexes termed C3 convertases (C4b2a and C3bBb). When high densities of C3b are deposited on the target surface, C3b molecules associate with the existing C3 convertases to form C5 convertase complexes (C4b3b2a and $C3b_2Bb$) that exhibit substrate preference for cleavage of C5 (13). C5 proteolysis results in formation of C5b, which subsequently binds C6, C7, C8 and multiple copies of C9 to form the lytic MAC. In addition, C5a, the small soluble byproduct of C5 conversion, acts as a potent chemoattractant for phagocytic cells, particularly neutrophils (14).

While C3 and C5 can be cleaved by serine proteases of the coagulation system under certain circumstances (15, 16), prototypical complement activation is triggered by one of three pathways: i) the alternative pathway (AP), ii) the aforementioned CP and iii) the lectin pathway (LP), each of which differ by mode of initiation. The focus of this review remains on novel and direct inhibitory mechanisms of the CP. As such, a detailed description of the molecular events associated with CP activation is necessitated and provided in Fig. 1, while LP and AP activation are only briefly outlined here. The LP is initiated when patterns of sugar moieties on foreign cells are recognized by mannose-binding lectin (MBL) or ficolins, which are themselves non-covalently associated with MBL-associated serine proteases (MASPs). These complexes catalyze C4 and C2 cleavage leading to C4b2a convertase formation (17). On the other hand, the AP C3 convertase, C3bBb, is formed when surface-attached C3b interacts with the protease factors B (fB) and D (fD) (18). In the absence of CP and LP, the AP depends on slow but continuous C3b deposition by soluble $C3(H_2O)Bb$ convertases that occur from interactions of fB and fD with spontaneously hydrolyzed C3 ('tick-over') (19). AP C3 convertases amplify C3 conversion on the target surface as C3b serves the scaffold for assembly of new C3bBb convertases.

Regulation of Complement by the Host

In order to prevent unwanted complement activation on host cells, convertase formation is tightly regulated via soluble and host plasma membrane-bound regulators of complement activation (RCAs). RCAs either act as a cofactor for factor-I-mediated C3b and C4b

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inactivation, or promote dissociation of convertases. An example of such a regulator is the plasmatic C4b-binding protein (C4BP) which destabilizes CP and LP convertases and is a cofactor for C4b degradation (20). Factor H (fH) serves an analogous AP regulatory function as it is a cofactor for factor I-mediated C3b degradation and possesses decay accelerating activity (21). CP activation is controlled at the level of C1 by C1 esterase inhibitor (C1-INH), a serpin that directly inactivates C1 by covalently binding the catalytic site of both C1r and C1s and dissociating the inhibited C1r-C1s-$(C1\text{-}INH)_2$ complex from C1q (22).

An additional layer of regulation occurs in the case of IgG-mediated CP activation. Due to its low affinity for solution phase monomeric IgG molecules, serum C1 remains inactive. IgG-mediated activation of C1 only occurs through clustering of surface-bound IgG where multivalent binding increases C1q-binding affinity (23). Recently, a study by Diebolder and colleagues led to new insights into the mechanism of IgG-mediated CP activation (24). This investigation showed that activation is affected by hexamerization of IgG on the target surface via Fc-Fc interactions and that hexameric IgG significantly increases C1q-binding and activation (24). While immune complexes represent the canonical target for CP activation, it is important to note that many complement-activating, antibody-independent C1q ligands are known, including specific bacterial surface proteins (25, 26).

General Mechanisms of Complement Evasion

Microorganisms use a wide range of general defense strategies to survive complement attack and this topic has been reviewed thoroughly elsewhere (2, 12, 27, 28). Select examples are presented here for the purposes of illustration. Recruitment of host RCAs to the microbial surface is by far the most common mode of complement evasion among bacteria, viruses, fungi and parasites alike (2). One such example is *Streptococcus pneumoniae,* which binds factor H (fH) via its membrane-bound fH-binding inhibitor of complement (Hic) and hijacks the primary endogenous AP regulator in a functional state (29). Numerous other microbes, including *Neisseria gonorrhoeae* and Group A *Streptococci*, express analogous proteins which adsorb C4BP at the bacterial surface, thereby resulting in down regulation of both the CP and LP (27). *Escherichia coli* and *Helicobacter pylori* have been reported to transfer GPI-anchored CD59 to their membrane, a regulator that prevents C9 polymerization and MAC formation on many host cells (30, 31). In contrast, several viruses surround themselves with membrane-associated RCAs by budding from host membranes (32).

Rather than recruitment of host proteins, certain viruses express host regulator mimics which share sequence homology to the ‘complement control protein’ (CCP) modules that are the most prevalent domains of RCAs (33). Two prominent examples of this type of molecular mimicry are the vaccinia virus complement control protein (VCP) and the smallpox inhibitor of complement enzymes (SPICE) from variola virus. VCP and SPICE both contain four CCP domains and protect virally infected cells from CP and AP activity by serving as factor-I cofactors for C3b/C4b degradation in addition to possessing convertase decay accelerating activities (34–36).

Cobra venom factor (CVF) is the prototypical example of a complement inhibitor that acts by activation and consumption of complement. CVF rapidly depletes C3 and C5 from a

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variety of mammalian sera via the formation of stable CVF-Bb convertases (37). Microbes have also evolved proteins capable of activation and depletion of complement. For example, a secreted form of the ubiquitously-expressed *S. pneumoniae* endopeptidase O (PepO) was shown to activate the CP by binding C1q and inducing depletion of fluid-phase complement (38). A related anti-complement strategy commonly employed by microbes is the proteolytic degradation of complement components by either bacterially-derived or recruited endogenous proteases. For instance, *S. aureus* produces staphylokinase (SAK), a protein that complexes with host plasminogen to convert it into the active serine protease plasmin (39, 40), while *Pseudomonas aeruginosa* degrades these components with specific bacterially-expressed enzymes (41, 42).

Finally, many complement evasion molecules which act by unique mechanisms have now been discovered. The most notable examples come from *S. aureus*, which produces a broad range of evasion proteins that interfere at multiple levels of the complement cascade. These include inhibitors of the C3 or C5 convertases (43–46), molecules that bind C5 to prevent its conversion (47), and an antagonist of the C5a receptor on neutrophils (48). Such complement evasion proteins are among an arsenal of secreted factors used by *S. aureus* to manipulate and subvert both innate and adaptive human immunity (49).

Downregulation of the Classical Pathway via Antibody Targeting

The CP is distinguished from the LP and AP by its ability to be activated by immune complexes (i.e. antibody-antigen). In this regard, there are several evasion molecules that indirectly target the CP via antibody-directed mechanisms. *S. aureus* expresses two Ig-binding proteins; protein A (SpA) and staphylococcal binder of immunoglobulin (Sbi) (50, 51). SpA is a type I membrane protein that binds the Fc regions of IgG with high affinity and thereby blocks C1q-binding sites in these domains (52). Sbi, on the other hand, is a secreted protein that blocks CP activation by binding to Fc domains as well as stimulating the futile consumption of complement by binding directly to C3 (53). Other known IgG-targeting molecules include protein G, which is a cell wall-associated protein of Group C and G *Streptococci* that binds all subclasses of IgG via their Fc regions (54), and the Herpes simplex virus glycoproteins gE and gI (32, 55, 56).

Novel Inhibitory Mechanisms of the Classical Pathway

Uniquely evolved AP inhibitors with direct modes of action have been known for over a decade and have been extensively reviewed before (2, 12, 57–61). By contrast, relatively few examples of conceptually similar CP-specific inhibitors have been reported. In many ways this has been surprising given the far upstream position of CP activation within the cascade and its prominent role in recognizing and eliminating many types of pathogens. The myriad of theoretical intervention points at the level of C1 and/or the CP/LP convertase (Fig. 1) further supports the idea that various pathogens, parasites, and opportunists have evolved unique inhibitory molecules that disrupt function of the CP. Several recent studies have borne out these predictions and have revealed a striking level of diversity in CP-specific complement evasion strategies.

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Disruption of the C1 Complex

The productive activation of C1 requires an orchestrated series of intermolecular recognition events coupled to the substrate specificity and catalytic activity of C1s (Fig. 1). While previously activated C1s can indeed cleave C4 and C2 *in vitro*, proteolysis is normally restricted to the context of C1, and there is no known role for C1r or C1s outside of the C1 complex. The importance of complex stability for C1 function is further evidenced by the secondary inhibitory mechanism of C1-INH, which rapidly dissociates two C1r-C1s-(C1-INH)$_2$ complexes per C1 molecule, leaving C1q bound to the activating ligand (22). Recently, two unrelated families of complement evasion proteins have been identified which can bind directly to the collagenous stalk of C1q and disrupt its noncovalent association with the $C1r_2C1s_2$ heterotetramer. By interfering with the C1q/$C1r_2C1s_2$ interaction and inhibiting C1 proteolytic activities, these proteins employ a novel mechanism for specifically targeting and inhibiting the CP.

In 2008, Bonaparte and colleagues reported the first example of this type of CP inhibitor which was discovered in human astroviruses (HAstV), a nonenveloped, icosahedral RNA virus that causes infantile gastroenteritis (62). HAstV virions were shown to suppress CP, but not AP-dependent hemolytic complement activity, and to inhibit formation of the complement activation products C4d, iC3b, and C5b-9 complex under conditions selective for the CP (62). The inhibitory activity for type 1 virions was subsequently isolated to the viral coat protein (HAstV-1 Coat Protein). In a subsequent study, Hair *et al.* demonstrated the dose-dependent inhibition of C1s activation in the context of C1, as well as the displacement of $C1r_2C1s_2$ from the C1 complex by submicromolar concentrations of HAstV-1 Coat Protein (63). Interestingly, HAstV-1 Coat Protein was also shown to inhibit the LP and this inhibitory activity was linked to the ability of the viral protein to bind directly to MBL. HAstV-1 Coat Protein failed to interact with a site-directed MBL mutant, which is known to abolish the interaction of MASP-2 with MBL (64), and thus implicated an analogous protease displacement mechanism for HAstV-1 Coat Protein inhibition of the LP.

The C1q-binding site on the 787 amino acid HAstV-1 Coat Protein was mapped to a 30 amino acid stretch using its limited sequence homology to a known C1q ligand (human neutrophil defensin-1) (65–67). In a very interesting finding, Sharp *et al.* noted that while a 15 amino acid peptide derivative was able to block CP activation it was unable to displace $C1r_2C1s_2$ from the C1 complex, unlike the in-tact HAstV-1 Coat Protein macromolecule (68). These observations strongly suggest that complete displacement of $C1r_2C1s_2$ is not required to inhibit C1, but rather that HAstV-1 Coat Protein likely exerts its inhibitory effect by disrupting the orientation of C1q relative to $C1r_2C1s_2$ within the C1 complex. These data may in part explain the inhibitory mechanism of a different novel CP/LP inhibitor, *Trypanosmoma cruzi* calreticulin (TcCRT), which also binds to the collagenous region of C1q (69, 70). While TcCRT prevented $C1r_2C1s_2$ from binding C1q, it failed to displace $C1r_2C1s_2$ in a preformed C1 complex and only blocked C1s cleavage of C4 in the context of C1 but not the isolated C1s enzyme (70). As with HAstV-1 Coat Protein, TcCRT was recently shown to also block LP activation (71). This observation further supports the concept of a partially overlapping mechanism by these otherwise distinct inhibitors.

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C1q is a glycoprotein assembled from six copies of three non-identical, interwoven polypeptides (chains A, B, and C) (25). Within the C1 complex, C1r and C1s are arranged as a ring-shaped heterotetramer which is confined by an outer cage-like structure formed by the six collagenous C1q stems (72). Bacteria express a number of cell-surface proteins that are capable of binding to collagenous structures and many of them belong to a group termed microbial surface components recognizing adhesive matrix molecules or MSCRAMMs (73). In 2013, Kang and Ko *et al.* reported that members of collagen-binding MSCRAMMs from a wide range of Gram-positive bacteria, including the *S. aureus* prototype adhesin called CNA, can bind directly to C1q and inhibit CP activation (74). A panel of structure-guided, site-directed CNA mutants which were previously shown to be deficient in collagen binding relative to wild-type CNA, impaired C1q/CNA binding in an identical manner (75). A single point mutation (CNA-Y175K) nearly abolished binding to both collagen and C1q and the relative affinity of this and other CNA mutants closely correlated to their ability to inhibit the CP in hemolytic and ELISA-based complement assays. CNA-like collagen-binding MSCRAMMs from four additional Gram-positive bacteria (*Enterococcus faecalis*, *Enterococcus faecium, Streptococcus equi,* and *Streptococcus mutans*) also bound C1q and inhibited CP activation. Co-immunoprecipitation experiments showed that $C1r_2C1s_2$ was completely displaced from C1q in the presence of 80 μM CNA but not CNA-Y175K, and similarly to HAstV-1 Coat Protein, CNA could interfere with the $C1q/C1r_2C1s_2$ interaction in an ELISA-based competition format. In contrast to HAstV-1 Coat Protein and TcCRT, which have no apparent effect on recognition of complement activating ligands, CNA (but not CNA-Y175K) interfered with C1 recognition of IgM-coated microtiter plates. Interestingly, this effect was specific for C1 as little to no competition was observed when isolated C1q was used. These data suggest that CNA may stabilize a conformation of C1q within the C1 complex that possesses lower affinity for immune complexes. Thus, by recognizing specific collagenous structures, CNA-like MSCRAMMs from Gram-positive bacteria act not only as adhesins, but are able to inhibit the CP by binding directly to C1q and disrupting the stability and ligand recognition properties of the C1 complex.

Inhibition of C1r Proteolytic Activity

C1q recognition is common to the complement inhibitory activities of HAstV-1 Coat Protein, TcCRT, and CNA-like MSCRAMMs, described above. Surprisingly, examples of specific, C1q-independent targeting and inactivation of C1r and C1s have been absent from the literature. Recently, Garcia *et al.* reported that the etiological agent of Lyme disease, *B. burgdorferi*, expresses a lipoprotein termed BBK32 that forms high-affinity, noncovalent complexes with purified C1 ($K_{D,SPR}$ = 3.9 nM) and exhibits half-maximal inhibitory concentrations (IC_{50}) of 34 nM and 110 nM in CP-specific ELISA-based and hemolytic complement assays, respectively (76). When BBK32 was expressed in a normally serum-sensitive *B. burgdorferi* strain (B314), it conferred serum protection in complement killing assays and promoted bacterial attachment to immobilized C1. When isolated components of C1 were evaluated, high-affinity interaction was retained for C1r only ($K_{D,SPR}$ = 15 nM) whereas no detectable interaction was measured for C1q, C1s, or pro-C1s. In agreement with this observation, and the CP-specific function of C1r, BBK32 failed to inhibit the AP or LP at concentrations of BBK32 up to 1μM. The intrinsically disordered N-terminal region of

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BBK32 (residues 21–205) is known to participate in bacterial adherence by binding certain glycosaminoglycans (77) and fibronectin (78) via non-overlapping binding sites. In contrast, the C1/C1r binding activity and CP inhibitory activities were fully retained by the BBK32 C-terminal globular domain (BBK32-C, residues 206–354). A series of biochemical and co-immunoprecipitation experiments revealed that in addition to preventing C1r cleavage of pro-C1s, BBK32 also prevented C1r auto-activation within the context of the C1 complex. Therefore, rather than disrupting the stability of the C1 complex as described for the C1q-binding inhibitors above, BBK32 instead traps C1 as a zymogen by preventing the initial proteolytic activation of C1.

Targeting the CP/LP C3 Proconvertase

Throughout the last several decades, the Gram-positive pathogen *S. aureus* has become a paradigm for understanding host/pathogen interactions and immune evasion. In the early stages of these developments some twenty years ago, McGavin *et al.* identified the so-called Extracellular Adherence Protein (Eap) as a secreted staphylococcal adhesin with the ability to bind several extracellular matrix glycoproteins (79). A large body of literature expanded upon this initial work, and described several surprising outcomes from the study of Eap's effects on various physiological models in mice. In particular, Eap was reported to contain intrinsic anti-inflammatory activities that block leukocyte recruitment to tissues (80–82), to impair various angiogenic responses (83), and to disrupt the overall process of wound healing (83). While Eap was recently described as an inhibitor of neutrophil serine proteases (84), a previously undiscovered link between Eap and the complement system has also been confirmed.

Taking advantage of a recombinant protein library that represents secreted *S. aureus* proteins, Woehl, Stapels, and coworkers used a biochemical screening approach to identify Eap as a dose-dependent inhibitor of the CP (85). Interestingly, Eap also inhibited activity of the LP to a similar extent, as judged by C3b deposition in a pathway-specific ELISA. The fact that Eap was able to potently block C3b deposition via the CP and LP, but had no effect on the activation of C4 to C4b suggested that Eap acted on either the fully-assembled C3 convertase shared by the CP and LP (i.e. C4b2a), or an isolated component of this proteolytic complex. While Eap binds directly to C4, C4b, and C4c with nanomolar affinity, its interaction with C4b ($K_{D,Alpha}$=185 nM) appears to be paramount from a functional perspective. In this context, Eap binding to C4b results in dose-dependent inhibition of C4b binding to the pro-protease, C2. It remains to be determined whether Eap's influence on C4b binding to C2 arises through steric or allosteric events, since the published data do not address this issue directly. Despite this limitation, it seems clear that the ultimate consequence of Eap interaction with C4b is inhibited formation of the CP/LP C3 proconvertase, which in turn hampers downstream formation of the active CP/LP C3 convertase. On balance, this mechanism presents numerous parallels to that of the *S. aureus* AP inhibitor, Efb-C, which instead blocks generation of the AP C3 proconvertase, C3bB (86).

Most natively occurring regulators of the complement system consist of tandem repeats of the CCP4 domain (33, 87), though as numerous studies with factor H have shown, not all of

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these repeats are required for binding to their complement targets or for manifestation of complement regulatory activities (88, 89). Eap shares a similarly modular architecture, as it consists of sequential ~110 residue repeating domains that are connected by short polypeptide linkers (90, 91). Although a gene encoding Eap is found in 98% of all *S. aureus* strains (92), it occurs in isoforms that vary between 4 and 6 domains (80, 84). While these isoforms appear largely equivalent in terms of their activity in functional assays, mechanistic investigations of Eap's effects on the complement system have been carried out exclusively with the four domain isoform expressed by *S. aureus* strain Mu50 due to its tractable biophysical properties (85, 91). Deletion analyses of this Eap variant have established that a truncation consisting of domains 3 and 4 (i.e. Eap34) has similar C4b binding affinity ($K_{D,Alpha}$=525 nM), interferes with C2 binding, and retains complement inhibitory properties comparable to full-length Eap ($IC_{50,LP}$ =227 nM). These results demonstrate that Eap is modular at both the structural and functional level, which is an attribute that appears to be common amongst complement regulators regardless of their origin.

A perplexing feature of many *S. aureus* immune evasion molecules is that obvious structural and/or functional homologs do not appear to exist in other organisms. However, a secreted protein from Group B *Streptococcus* was recently discovered which shares a remarkable level of functional and mechanistic similarity to Eap. Pietrocola *et al.* identified the gene COH1_1804 in a library of putative surface-retained antigens from *S. agalactiae* strain COH1 which lacked any further cell-surface retention motifs (93). A recombinant form of this ~15 kDa protein blocked C3b deposition by both the CP and LP in a dose-dependent manner, leading the authors to rename it CIP for Complement Interfering Protein. Further study demonstrated that CIP bound to C4 and C4b, with the latter complex exhibiting low-nanomolar affinity ($K_{D,SPR}$=95nM). Similarly to *S. aureus* Eap, CIP binding to C4b interfered with formation of the C4b2 proconvertase complex, though it had no effect on formation of the AP C3 proconvertase, C3bB. Remarkably, while CIP and Eap both bind C4b and interfere with formation of the C4b2 proconvertase, it does not appear that these proteins represent true homologs of one another. Not only does CIP share very limited amino acid identity with Eap (15%) (93), structure prediction suggests that CIP adopts a thioredoxin-class fold that is significantly different from the tandemly repeating structural domains characteristic of Eap (91, 94). Thus, it seems more likely that CIP and Eap are a product of distinct evolutionary lineages that have selected for potent inhibitors of the CP and LP. Indeed, strategies that block the furthest upstream event shared by the CP and LP (i.e. formation of the C4b2 proconvertase) certainly meet this criterion.

Undefined Mechanisms of Classical Pathway Inhibition

In addition to the better characterized CP inhibitory mechanisms described above, a novel CP-specific inhibitory molecule has been discovered in the blood-feeding sand fly *Lutzomyia longipalpis* (10). Ferreira and colleagues identified LJM19 (renamed to salivary anti-complement from *Lu. longipalpis* or SALO) as the molecule responsible for the complement inhibitory activity in sand fly salivary gland homogenates (SGH). Recombinant SALO was a potent inhibitor in CP hemolytic assays ($IC_{50} \approx 100$ nM), while two paralogous proteins LJS169 and LJS192 were devoid of inhibitory activity. Moreover, antibodies raised against recombinant SALO were able to reverse CP inhibition by SGH. SALO inhibition

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was CP-specific as concentrations up to 2 μM exhibited no effect in AP or LP assays. However, SALO did not directly block the activity of isolated C1s in C4 cleavage assays and did not interfere with C1q binding to immobilized IgG. Hence, while the specificity for CP inhibition remains clear, the complement target and mechanism of CP inactivation is currently not well defined for this novel inhibitor.

Perspectives

All immune evasion molecules discussed here (Table 1) are capable of specifically targeting and inhibiting the CP. However, distinctions can be made amongst the C1q-binding inhibitors like PepO, CNA-like MSCRAMMs, and HAstV-1 Coat Protein, which may potentially exploit the well-recognized complement-independent functions of C1q (95, 96). For instance, *S. pneumoniae* (97) and *Bacillus anthracis* (98) have both been shown to facilitate C1q-dependent adherence and host cell invasion. It is therefore interesting to speculate on the potential role of C1q-binding CP inhibitors on non-complement related functions of C1q, especially in cases where displacement of $C1r_2C1s_2$ from C1q occurs. In the same light, consideration should be given to the modular and multi-functional nature of many of the CP-specific inhibitors presented here. The relevance of other host protein binding activities of inhibitors like BBK32 (e.g. fibronectin-binding) has yet to be evaluated, however, a functional synergism may in fact exist. Indeed a synergistic function involving a component of the coagulation system and complement has already been shown for the *S. aureus* AP-inhibitor Efb which bridges fibrinogen and C3b and promotes bacterial survival through a sophisticated immune shielding mechanism (99).

In addition to blocking the CP, several inhibitors presented here have also been shown to prevent activation of complement by the LP. In the case of the C4b-binding proteins Eap and CIP this dual-inhibitory property can be attributed to the intersection of these two pathways at the level of the C3 proconvertase, C4b2. In contrast, HAstV-1 Coat Protein and TcCRT bind the collagenous stalk of C1q and a similar collagen-like structure is present in the LP pattern recognition molecules MBL/ficolins (100). As this site also harbors the cognate protease binding sites (i.e. MASP-1/-2), HAstV-1 Coat Protein and TcCRT are able to effectively inhibit both pathways. Although CNA-like MSCRAMMs also disrupt $C1r_2C1s_2$ by binding the C1q collagen stem, it is currently unknown if these proteins are capable of binding LP pattern recognition molecules. TcCRT, which blocks ficolin-initiated but not MBL-initiated LP activation, suggests that specificity for individual pattern recognition molecules can exist, akin to what has been previously observed for the associated host proteases (101, 102). Interestingly, to date there are few known inhibitors of the LP that do not also block CP activation. The discovery of molecules such as BBK32 and SALO which act exclusively on the CP, the existence of LP-specific synthetic inhibitors (103), and the more ancient evolutionary relationship of the LP to the CP (104) make it particularly likely that future studies will serve to uncover novel inhibitors specific for the LP that originate from natural sources.

The conserved sequence and structural relationships of CNA-like MSCRAMMs led to the discovery of a broad new class of CP-specific inhibitors found in many Gram-positive bacteria. Surprisingly, this type of structure-function convergence appears to be the

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exception rather than the rule for complement evasion molecules. As has been noted for the *S. aureus* AP complement inhibitors (e.g. SCINs, Efb, and Sbi), molecules such as BBK32, Eap, and SALO have no obvious sequence correspondence to genes outside of their respective genera. However, it appears several structurally divergent complement evasion molecules have evolved to share common complement inhibitory mechanisms. This concept is illustrated by the apparent lack of sequence/structure relationships between the CP/LP procovertase-targeting inhibitors (Eap/CIP) and is further supported by the otherwise unrelated C1 disrupting proteins (CNA-like MSCRAMMs/HAstV-1 Coat Protein/TcCRT). Although the inhibitory mechanisms of complement evasion are clearly constrained by the structure and function of their cognate complement targets (i.e. C1, convertases, etc.), the structure of functionally related complement inhibitors is seemingly much less restricted. This observation strongly suggests that future efforts aimed at discovering novel complement evasion molecules will require empirically-driven approaches rather than sequence informatics or other candidate-based methodologies.

Despite its protective role, the dysregulation of complement is a hallmark of many autoimmune diseases and inflammatory conditions including ischemia/reperfusion injury, atypical hemolytic uremic syndrome, age-related macular degeneration, rheumatoid arthritis, antibody-mediated transplant rejection, and cancer (105, 106). Considerable need exists for the pharmacological treatment of complement-related diseases, and the development of novel complement-directed therapeutics has gained significant momentum over the past decade (106–108). The involvement of excessive CP activation in human disease has been recently cast into the spotlight due to its causal link to schizophrenia (109) and Alzheimer's disease (110), not to mention other devastating diseases where the contribution of the CP to pathology has been longer appreciated (111, 112). At a minimum, the naturally occurring inhibitors discussed here represent promising conceptual and/or mechanistic templates for the development of evolutionarily-optimized, CP-specific inhibitors. While issues related to immunogenicity likely prevents their direct use for therapeutic intervention, the true utility of these naturally occurring inhibitors may not be fully realized until drug-like compounds which mimic their properties can be engineered.

The appearance of low molecular weight complement inhibitors, such as Compstatin (113, 114), has challenged the notion that the large protein-protein interfaces upon which the complement cascade is predicated cannot be targeted by much smaller drug-like compounds. To this point, small peptide mimics of *S. aureus* SCIN-derived AP inhibitors have been reported (115), and a peptidic derivative of HAstV-1 Coat Protein (PIC1) has shown efficacy as complement inhibitor in *in vivo* (68, 116). The ability of SCIN-derived peptides and PIC1 to preserve the inhibitory activities present in full-length proteins is consistent with the idea that immune evasion molecules, which often appear to target relatively small functional "hot-spots" on their host targets, hold promise as templates for drug design. However, unlike the more recently discovered CP-specific inhibitors reviewed here, *S. aureus* AP evasion proteins and Compstatin have benefited from an abundance of detailed structural studies which have primed them for therapeutic development (43, 86, 115, 117–122). Obtaining a detailed understanding of the structural basis for CP-specific inhibitors will be a critical step forward in tapping their potential for treatment of complement-related diseases. In this regard, the availability of published high-resolution crystal structures for nearly all CP

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complement components (72, 123–128) including a detailed structural model of the C1 complex (72) stands to significantly bolster these efforts in the years ahead.

Conclusions

Organisms whose life-cycle involves direct contact with blood, lymph, and related bodily fluids must develop protective mechanisms to evade complement. Here we have reviewed a set of recent investigations that have identified direct inhibitors of the CP and revealed a fascinating level of diversity in modes of CP-specific inhibition (Fig. 1). Each of these proteins interferes with the activity of the initiating protease complex of the CP, C1, or acts at the level of the CP/LP convertase. As research continues to grow in this area it seems likely that additional CP-specific evasion mechanisms will be discovered. Indeed, preliminary disclosures of naturally-occurring leech-derived peptide inhibitors of C1s activity (patent publication numbers: CA2318358 A1 and WO2001098365 A2) and the development of a potent anti-C1s monoclonal antibody (TNT003) (129, 130), suggest that C1s can be successfully targeted by diverse molecules. Finally, the discovery of molecules like SALO and TcCRT has highlighted an emerging field of evasion molecules derived from parasites and opportunists (e.g. blood feeders). While great effort has been already expended on discovering evasion molecules from bacterial pathogens, the study of hematophagous organisms represents a seemingly understudied yet important frontier in complement research. Increased attention in the areas of vector borne disease makes it extremely likely that novel complement regulators will be discovered in the near future from either these vectors or the pathogens they transmit.

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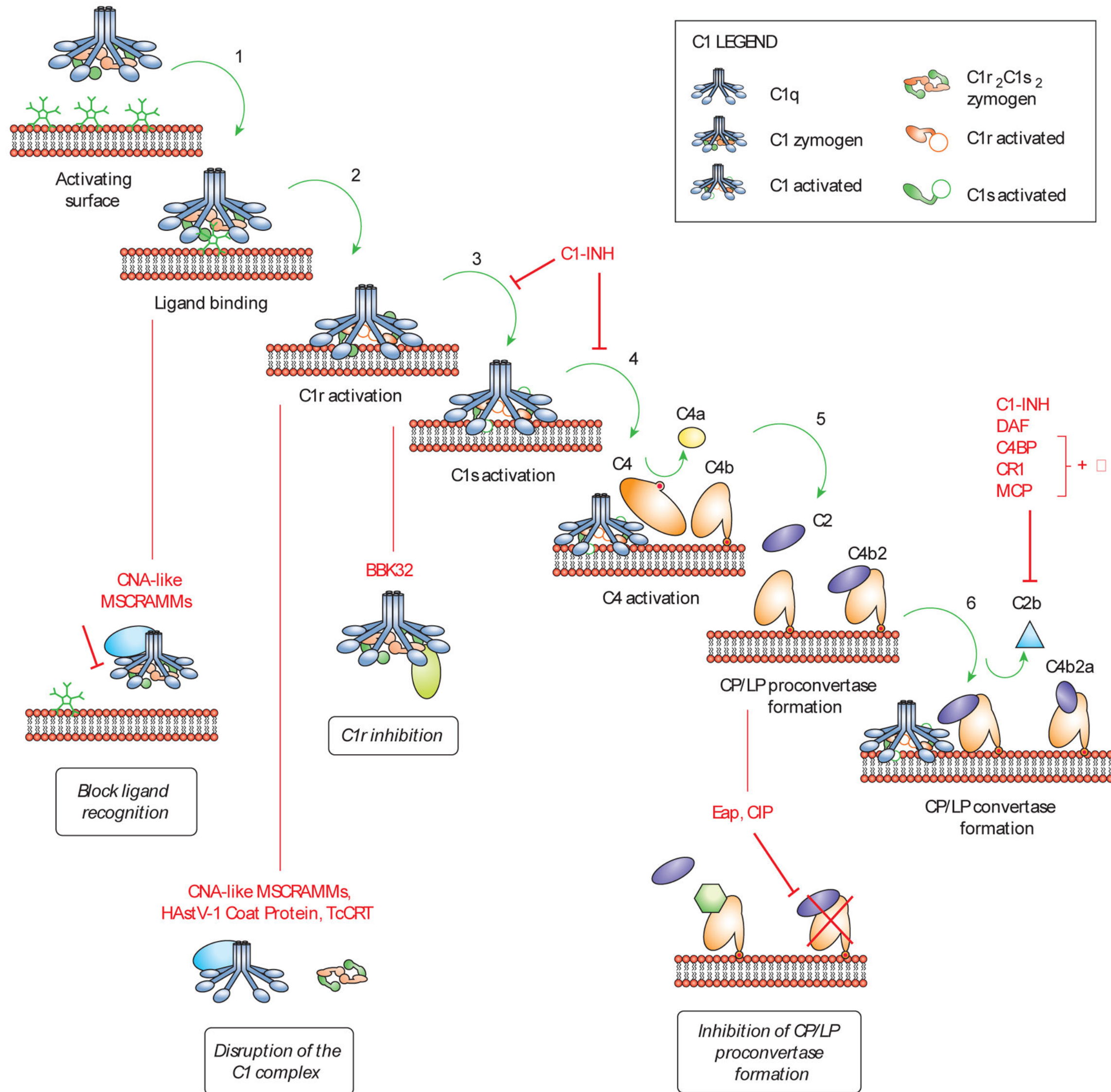

Figure 1. Classical pathway activation and novel mechanisms of complement evasion molecules

C1 is the multicomponent initiating complex of the CP, and is formed in a Ca^{2+}-dependent manner by a heterotetramer of two modular serine proteases ($C1r_2C1s_2$) in complex with the bouquet-like CP pattern recognition molecule, C1q. C1r and C1s exist natively as zymogens, and thus, C1 circulates in blood as a large (~790 kDa) inactive complex. The CP is activated through a series of six conceptually distinct steps (green arrows). **(1)** Zymogen C1 binds directly to an activating surface via the globular heads of C1q. C1q-binding activating ligand (i.e. IgM or hexameric IgG immune complexes, or non-antibody ligand) is represented here as a green pentameric structure and omitted for clarity in subsequent steps. **(2)** Ligand binding induces conformational changes in C1q leading to an open angle of the collagenous region and subsequent repositioning and autocatalysis of the C1r zymogen dimer. **(3)** C1r cleaves C1s forming fully activated C1. **(4)** Activated C1s binds C4, enzymatically liberates C4a, and C4b covalently attaches to the activating surface via its now exposed thioester group (denoted with a red sphere). **(5)** Surface-attached C4b serves as a platform for the

formation of the CP/LP proconvertase by binding to C2. **(6)** The final step of CP activation involving C1 occurs when C4b2 is converted to the active CP/LP convertase, C4b2a, by C1s cleavage of C2 and release of C2b. The activity of CP/LP convertases is tightly controlled by the endogenous complement regulators DAF, C4BP, CR1, MCP, and fI. Steps 3, 4, and 6 are regulated *in vivo* by C1-INH, a serpin that covalently inactivates both C1r and C1s and displaces an inhibited C1r-C1s-$(C1\text{-}INH)_2$ complex from C1q. To date, four types of mechanistically distinct, naturally occurring, novel inhibitors of the CP have been reported (red lines). The C1q-binding CNA-like MSCRAMMs from Gram-positive bacteria (dark blue oval) stabilize a form of C1 which has low affinity for immune complexes and thus prevents the initiating recognition event of the CP. Meanwhile, by targeting the collagenous region of C1q and displacing and/or disrupting the $C1r_2C1s_2$ heterotetramer, CNA-like MSCRAMMs, HAstV-1 Coat Protein (human astroviruses), and TcCRT (*T. cruzi*) (collectively represented by a dark blue oval) disable the initiating protease of the CP. On the other hand, *B. burgdorferi* BBK32 (green oval) traps zymogen C1 by binding C1r and preventing its autocatalytic and C1s cleaving activities. Finally, the C4b-binding proteins Eap (*S. aureus*) and CIP (*S. agalactiae*) (together represented by a green hexagon) interfere with the formation of the CP/LP proconvertase and therefore prevent generation of the fully-active CP/LP convertase, C4b2a.

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Table 1

Novel Inhibitors of the Classical Complement Pathway

CP Evasion Molecule	Organism(s)	CP Complement Target	Inhibitory Mechanism	Ref.
BBK32	*Borrelia burgdorferi*	C1r	Inhibition of C1r proteolytic activity	76
CIP	*Streptococcus agalactiae* (group B *Streptococcus*)	C4b	Inhibition of CP/LP proconvertase formation	93
CNA-like MSCRAMMs	*Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis, Enterococcus faecium, Streptococcus equi*, and other Gram-positives	C1q	Displacement of $C1r_2C1s_2$ tetramer from C1q and inhibition of C1q/IgM recognition	74
Eap	*Staphylococcus aureus*	C4b	Inhibition of CP/LP proconvertase formation	85
HAstV-1 Coat Protein	Human astroviruses, serotype 1	C1q	Displacement of $C1r_2C1s_2$ tetramer from C1q	62–64
TcCRT	*Trypanosoma cruzi*	C1q, C1r, C1s	Competition of $C1r_2C1s_2$ tetramer with C1q and disruption of C1s activity within the C1 complex	69–71