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INVITED REVIEW

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Complement component C3 – The "Swiss Army Knife" of innate immunity and host defense

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Summary

As a preformed defense system, complement faces a delicate challenge in providing an immediate, forceful response to pathogens even at first encounter, while sparing host cells in the process. For this purpose, it engages a tightly regulated network of plasma proteins, cell surface receptors, and regulators. Complement component C3 plays a particularly versatile role in this process by keeping the cascade alert, acting as a point of convergence of activation pathways, fueling the amplification of the complement response, exerting direct effector functions, and helping to coordinate downstream immune responses. In recent years, it has become evident that nature engages the power of C3 not only to clear pathogens but also for a variety of homeostatic processes ranging from tissue regeneration and synapse pruning to clearing debris and controlling tumor cell progression. At the same time, its central position in immune surveillance makes C3 a target for microbial immune evasion and, if improperly engaged, a trigger point for various clinical conditions. In our review, we look at the versatile roles and evolutionary journey of C3, discuss new insights into the molecular basis for C3 function, provide examples of disease involvement, and summarize the emerging potential of C3 as a therapeutic target.

KEYWORDS
compstatin, convertase, immune evasion, inflammation, therapeutics

1 | COMPLEMENT C3: A FUNCTIONAL HUB IN INNATE IMMUNITY AND BEYOND

Host defense systems are fundamental pillars of life in almost every organism, and higher species in particular have forged several intricate layers of defense that confer protection from intruders and maintain barrier function. Innate and adaptive immunity, coagulation and contact systems, pattern recognition molecules (PRMs), and antimicrobial peptides all contribute their share to the generation of a forceful defense response. Preformed mediators of defense are a highly important part of this conglomerate; in contrast to adaptive immune responses, which are tailored to a specific pathogen and need time to develop, preformed mediator systems are available instantaneously and allow an immediate reaction to insults such as injury and infection.[1] However, in view of their limited specificity, such first-line-of-defense systems face a challenging task, given that they have to strike a balance between forceful defense against foreign particles and protection of host cells. At the same time, their activity should not stop at the clearance of threatening cells but should also translate into processes that enable adaptive responses and restore homeostasis. In our review, we look at the complement system as an archetype of a preformed mediator of host defense and show how nature industriously and elegantly uses a single molecular scaffold, i.e. complement component C3, to achieve exceptional versatility in coordinating defense responses.

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At the dawn of the 20th century and of modern immunology, studies of the bactericidal activity of blood serum led to the discovery of the complement system, and it soon became evident that this cascade acts as a critical first-line responder of innate immunity.[2] Initially thought to be composed of nine distinct components (i.e. C1-C9), the number of associated molecules grew with our increasing knowledge about the functional and molecular characteristics of complement. Today, we consider some 50 proteins, encompassing both plasma proteins and membrane-bound receptors/regulators, to belong of the complement system, forming a tightly knit and highly cooperative surveillance network.[3,4] Since its initial description as an antibody-"complementing" bactericidal blood component, the perception of complement has dramatically changed and now involves much broader functions in immune surveillance, homeostasis and development, tissue-localized and intracellular activities, and intense cross-talk with many physiological pathways.[3–5] The sensing and elimination of microbial intruders and other potential insults still remains a core function of complement and is largely maintained by the core cascade system (Figure 1A).

1.1 | C3-mediated opsonization as central element in the elimination of threats

Once they encounter a pathogen or other foreign cell, the various PRMs of the complement system detect and bind to distinct surface patterns and induce the cascade via several routes.[3,4,6] Although the classical pathway (CP) is induced by the C1 complex, which primarily binds to antibody complexes and also to other pathogen- or damage-associated molecular patterns (PAMPs, DAMPs), the various PRMs of the lectin pathway (LP) recognize microbial carbohydrates and similar markers (Figure 1A). In all cases, PRM-associated proteases become activated and cleave components C2 and C4 to form a reactive but unstable protease complex on the surface of the targeted cells; this CP/LP C3 convertase binds to and cleaves the abundant plasma protein C3 and induces a transformation that determines the fate of the cell. The convertase-mediated cleavage of C3 releases the small protein C3a and induces a conformational change in the remaining C3b fragment that exposes a highly reactive but short-lived thioester. In close proximity to the initiating surface, the newly formed C3b can covalently bind to hydroxyl or amino groups, thereby opsonizing the cell. Importantly, formation of C3b enables the binding of the protease Factor B (FB), and the resulting pro-convertase (i.e. C3bB) is quickly transformed by Factor D (FD) into an active C3 convertase (i.e. C3bBb) that by itself can cleave more C3 into C3b, thereby creating an amplification loop for C3b deposition (Figure 1C). This mechanism is commonly, yet somewhat misleadingly referred to as the "alternative pathway" (AP) of complement, especially as this route can sometimes contribute up to 80% of the overall response, even after initiation by the CP or LP.[7,8]

Although not pathogen-specific, CP- and LP-mediated initiation still confers some selectivity, as these routes are dependent on the presence of certain microbial surface markers or, as in the case of antibody-mediated CP activation, receive assistance from adaptive immunity. However, complement also employs a simple yet elegant "tick-over" mechanism involving C3 that allows the system to increase baseline activity and probe cells in a less distinctive manner (Figure 1B): Although C3 predominantly circulates in its native form, a small fraction is constantly undergoing spontaneous hydrolysis to result in a distinct conformer termed C3(H_2O). Without changing its composition, C3 thereby modifies its reactivity as a result of structural changes that generate a molecule with a functional spectrum similar to that of C3b. As such, C3(H_2O) can bind FB and form AP C3 convertases that turn C3 into C3b and initiate the AP. Importantly, physical adsorption of C3 on various surfaces, such as microbial cells containing lipopolysaccharides (LPS) and also blood-gas interfaces or activated platelets, can induce a similar conformational activation in C3 (Figure 1B), thereby enhancing and directing the tick-over in the case of less-defined threats.[9] Finally, C3 may be directly cleaved by non-convertase proteases such as coagulation enzymes (e.g. thrombin, plasmin) or tissue kallikreins[10,11]; although these activities are typically low in comparison with the primary substrates, it is possible that this "extrinsic" pathway (Figure 1A) gains relevance under specific disease conditions or in local microenvironments.

Independent of the route of initiation, opsonization with C3b marks the starting point for the generation of a series of potent effectors with the aim of facilitating the clearance of the tagged particles (Figure 1D). In this context, C3b not only amplifies the complement response via convertase formation but also acts as an effector itself that mediated functions in innate and adaptive immunity.[12] Via binding to complement receptor 1 (CR1; CD35) on immune cells, it confers immune adherence that enables the shuttling of opsonized cells to the spleen and liver. Tissue-resident macrophages such as Kupffer cells in the liver also express the complement receptor of the immunoglobulin family (CRIg) that has been shown to bind C3b and induce phagocytosis. As briefly discussed later, binding of C3b to CD46 has an impact on adaptive immunity by influencing T-cell responses. The interaction of C3b with CR1/CD35 and CD46, both of which are members of the regulators of complement activation family (RCA; see below), mediates the degradation of this primary opsonin to iC3b (after removal of the C3f peptide) and/or C3dg plus C3c (Figure 1A). Although C3c is released into circulation, the late-stage opsonins iC3b and C3dg remain bound to the surface and exert potent signaling functions (Figure 1A, D). iC3b, in particular, is a highly versatile effector that not only maintains binding activity for CRIg but also gains affinity for the phagocytic integrin receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), thereby largely enhancing complement-mediated phagocytosis. Intriguingly, there is increasing evidence that the powerful iC3b-CR3 mechanisms are not only employed for microbial clearance but also for immunoediting during tissue development; recent studies have shown that complement is engaged during synaptic pruning with potential implications for the development of schizophrenia.[13,14] Finally, the conversion to iC3b also exposes a binding site for CR2 (CD21); this receptor is expressed on B cells (and follicular dendritic cells) and forms part of the B-cell co-receptor complex. The interaction with CD21 lowers

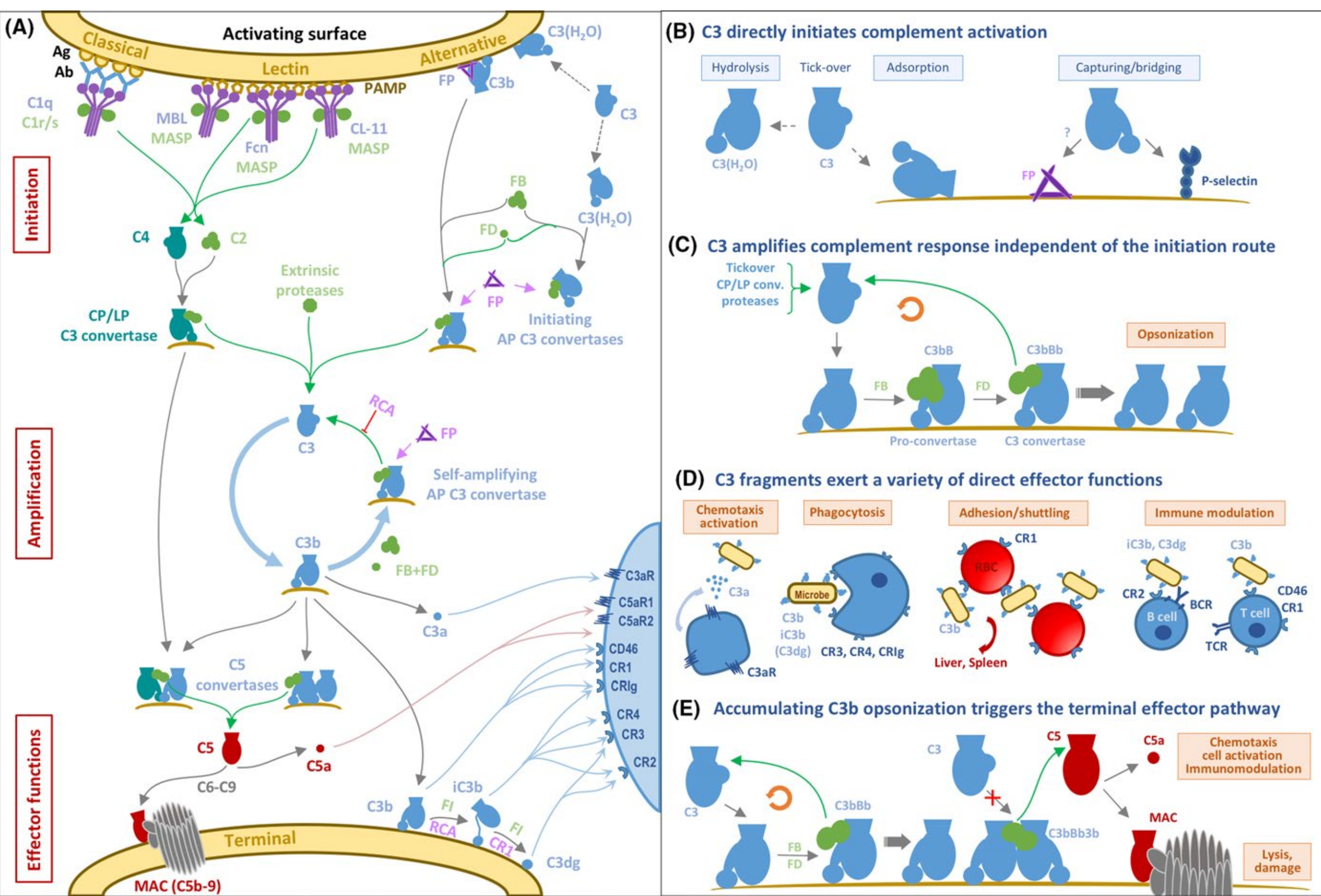

FIGURE 1 The various roles of C3 in complement activation and effector functions. (A) Schematic representation of the complement cascade. Initiation of classical and lectin pathway (CP, LP) via pattern recognition molecules, and of the alternative pathway via tick-over or adsorption, leads to the formation of initial C3 convertases, which activate C3. C3 can also be cleaved directly by extrinsic proteases. Independent of the activation route, the cleavage product C3b can form new convertases after engagement of factor B and D (FB, FD), thereby fueling an amplification loop that leads to rapid opsonization of the target surface with C3b. Increasing C3b densities facilitate the formation of C5 convertases that initiate the terminal pathway with generation of the lytic membrane attack complex (MAC) and the potent anaphylatoxin C5a. At the same time, C3 fragments exert direct effector functions. Although C3a binds to the anaphylatoxin receptor C3aR, C3b and its degradation products iC3b and C3dg interact with a variety of complement receptors (CR) to mediate immune adhesion, phagocytosis, and adaptive immune stimulation. Properdin (factor P, FP) enhances convertase stability, but regulators of complement activation (RCA) promote its decay and enable the degradation of C3b. (B) C3-induced complement initiation via hydrolysis in solution and physical adsorption on surfaces (i.e. "tick-over") or via proposed capturing by bridging molecules such as properdin or P-selectin. (C) Amplification of the initial complement response, driven by the formation of C3bBb complexes via interaction of C3b with FB to form the pro-convertase C3bB and subsequent activation by FD. (D) Examples of major effector functions of C3 fragments, including chemotaxis and cell activation via C3a, phagocytosis of opsonized particles, immune adhesion and shuttling, and adaptive modulation via B- and T-cell stimulation. (E) Induction of the terminal pathway by increasing densities of C3b, leading to the generation of C5a and MAC. Ab, antibody; Ag, antigen; BCR, B-cell receptor; C3aR, C3a receptor; C5aR1, C5a receptor 1 (CD88); C5aR2, C5a receptor 2 (C5L2); CL-11, collectin 11; CRIg, CR of the immunoglobulin family; Fcn, ficolins; FI, factor I; MASP, MBL-associated serine protease; MBL, mannose-binding lectin; PAMP, pathogen-associated molecular patterns; RBC, red blood cell; TCR, T-cell receptor

the activation threshold of B cells and influences the generation of memory cells. Although the binding to CD21 is maintained in the end-stage opsonin C3dg, the interaction with CR4 appears to be lost. Interestingly, recent studies suggest that the interaction profile of C3dg with the related receptors CR3 and CR4 is distinct. C3dg still contains binding sites for CR3 and, at least in the case of densely C3dg-opsonized erythrocytes, the C3dg-CR3 interaction can mediate phagocytosis; the relevance of this mechanism in the context of different (patho)physiological scenarios needs to be further explored. In any case, the deposition and conversion of C3b provides a dynamic platform for tiered effector functions ranging from immune adherence and transport (C3b-CR1) to phagocytosis (C3b/iC3b-CRIg, iC3b/C3dg-CR3, iC3b-CR4) and adaptive immune modulation (C3b-CD46, iC3b/C3d-CD21).

Although some aspects remain elusive, C3b is also essential for the induction of the terminal pathway of complement effector generation (Figure 1A, E). Increasing the density of C3b during complement activation and amplification leads to a substrate specificity shift in the generated convertases from C3 to its paralog C5. Although the CP/LP C3 convertases are composed of cleavage-generated C4 and

C2 fragments, an association with C3b is necessary for shifting to C5 convertase activity. Once formed, C5 convertases cleave C5 into C5b, thereby releasing the small C5a protein. In contrast to C3b and C4b, C5b does not covalently deposit via a thioester, but rather associates with C6 and C7 to form a complex that can insert into membranes. The binding of C8 and multiple copies of C9 forms the membrane attack complex (MAC) that acts as a lytic pore to damage or kill susceptible cells and organisms.

1.2 | Spreading the danger signal: The role of C3a

Although MAC-induced lysis and opsonin-mediated phagocytosis already represent efficient means of microbial clearance, the translation of local complement activation into cellular signals and the coordination of downstream responses add another critical layer to complement's defensive actions. Alongside the immunomodulatory activities of opsonins, which act in direct contact with target and effector cells (see above), the remote actions of the anaphylatoxins C3a and C5a are highly important for shaping the immune response (Figure 1A). C5a, in particular, is a powerful chemoattractant that signals via two C5a receptors (C5aR1/CD88, C5aR2/C5L2) and exerts activities that range from immune cell activation and priming to smooth muscle contraction and beyond. In addition, C5a-mediated signaling is engaged in a plethora of crosstalk activities with other defense and homeostatic pathways.

C3a has long been considered a less potent cousin of C5a, with a similar activity spectrum but a 10- to 100-fold weaker activity. However, newer studies have suggested that C3a, which signals through the G protein-coupled C3a receptor (C3aR), shows a distinct and context-specific profile that sometimes even counteracts C5a-induced activities.[15] In fact, C3a appears to assist in some unique functions, with our group and others having demonstrated its involvement in such diverse processes as tissue development and regeneration (e.g. in the retina, liver),[16–19] homing of hematopoietic stem cells,[20,21] and migration of neural crest cells,[22,23] among others. Although C3a has also been suggested to signal via other receptors, these activities have remained relatively unexplored (e.g. the binding of C3a to the receptor of advanced glycation end-products[24]) or remain controversial, as in the case of the proposed metabolic effects of C3a and its degradation product C3a-desArg via C5aR2 stimulation.[15,25] Interestingly, C3a has also been reported to act as a preformed mediator of defense outside its role in complement, as it may act as an antimicrobial peptide that binds to and destabilizes bacterial membranes.[26] Although many aspects of its function remain to be fully explored, C3a appears to hold a few more functional cards than initially thought.

1.3 | Tuning C3 activity through interaction with modulators and regulators

The molecular pattern on a cell surface largely defines the binding of PRMs and/or adhesion of C3; complement is therefore intrinsically less likely to be engaged on host cells than on foreign particles. However, given the potentially deleterious actions of an activated complement system on or near host cells, circulating and membrane-bound complement regulators offer an essential layer of protection and confer the necessary selectivity. Although soluble C1 inhibitor and non-proteolytic splice products of MASPs help control the CP and/or LP, membrane-bound CD59 prevents the formation of MAC, and carboxypeptidases tame/modulate anaphylatoxin activity.[27] Given their central role in modulating complement activity, however, the majority of complement regulators directly target C3 and its fragments and complexes.[27,28] These inhibitory proteins typically belong to the regulators of complement activation (RCA) family, which includes the membrane-bound CR1 (CD35), decay acceleration factor (DAF; CD55) and membrane cofactor protein (MCP; CD46), and the circulating regulators C4b-binding protein (C4BP), Factor H (FH), and FH-like protein 1 (FHL-1).[27]

With one exception (see below), these regulators mainly act via binding to C3b.[28,29] This interaction can provide decay acceleration activity (DAA) toward the AP C3 convertase as a result of the steric displacement of the Bb fragment from the C3bBb complex. Binding of some RCAs to C3b also provides a binding site for the protease Factor I (FI), which cleaves C3b into iC3b and/or C3dg (termed cofactor activity [CA]). Among membrane-bound regulators, CD55 only provides DAA and CD46 only CA, whereas CR1 combines both activities and is the only RCA to enable the final degradation to C3dg. Although C4BP has been reported to bind C3b and act as a cofactor, albeit at high molar excess, C4BP is generally considered a CP/LP-selective regulator.[30,31] In contrast, FH and its shorter splice product FHL-1 both show strong selectivity for the AP and provide DAA and CA upon binding to C3b. FH is a particularly interesting regulator because it combines its inhibitory activities with pattern recognition capabilities that allow this plasma protein to recognize self-surface patterns (e.g. glycosaminoglycans or sialic acids that are usually absent from microbial cells), thereby assisting regulation on host cells.[27]

Complement-mediated attack on microbial intruders can therefore be seen as a result of both the presence of foreign signatures and an absence of non-self patterns.[32] This delicate interplay between promoting and preventing signatures can be exploited by modulators to fine-tune complement activity and create a directed and measured response to specific targets.[4] Such modulators typically contain pattern recognition regions and binding sites for complement components, activators, and/or regulators. For example, although properdin is best known for its ability to stabilize the C3bBb convertase, the protein has also been reported to act as an initiator that binds markers such as LPS and recruits soluble C3b (as a starter molecule) to the target surface (Figure 1B); however, the relevance of this bridging function is still debated.[33–35] Although the molecular details remain to be elucidated, there is increasing evidence for a recruitment of C3b by P-selectin on platelets and endothelial cells that could induce or exacerbate complement activation.[36] FH-related proteins (FHRs) contain domains homologous to the C-terminal region of FH, which binds both C3b/iC3b/C3dg and self-surface patterns. As a consequence, they can compete with FH for host surface binding and de-regulate complement activity on certain surfaces such as apoptotic cells.[37,38] Finally, members of the pentraxin family are known as context-specific modulators of

complement activity but seem to mediate their activity via binding to PRMs and regulators rather than by directly interacting with C3 fragments.[39]

As such, C3 and its fragments serve multiple purposes across the entire complement cascade: (i) by acting as an initiator of complement activation through tick-over of C3 and passive adsorption to surfaces (i.e. C3(H_2O)) and potential surface-capturing of C3b by modulators such as properdin or P-selectin; (ii) by driving the amplification loop as substrate (C3) and C3 convertase component (C3b); (iii) by functioning as direct effector proteins to mediate phagocytosis and/or immune stimulation/modulation (C3a, C3b, iC3b, C3dg); and (iv) by enabling the generation of terminal pathway effectors (C5a, MAC) as essential component of the C5 convertases (C3b). In order to comprehend how nature has designed and refined such a potent and versatile defense molecule, it is worth taking a journey from evolutionary to biochemical and deeply molecular aspects of C3.

2 | C3 AS AN EVOLUTIONARILY CONSERVED CORNERSTONE OF IMMUNOLOGY

Delving into the evolutionary history of C3, and of the complement system in general, has provided valuable insight into its multifaceted roles in host immunosurveillance, tissue homeostasis, and adaptive immune regulation.[40,41] Indeed, C3 homologs and C3-mediated activities have been characterized in evolutionarily distant species ranging from the most primitive invertebrates, the Cnidaria, and certain protostomes (e.g. arthropods) to diverse members of the deuterostome lineage of the metazoans, including echinoderms, urochordates, lower vertebrates, and mammals (Figure 2).[41–43] The high degree of phylogenetic conservation and the striking divergence of multiple C3 isoforms in certain vertebrates underscore the indispensable role of C3 in host immune surveillance. Elegant studies employing evolutionarily distant organisms have suggested that C3 preserved its immune sentinel function throughout phylogenesis, being the prime mediator of immune recognition and “non-self” discrimination in a primordial phagocytic system shared by both invertebrates and early vertebrates.[42,43]

Several lines of evidence suggest that a common ancestral complement system comprising a set of primitive C3, FB (i.e. Bf/C2 gene), and MASP proteins was established more than 500 million years ago (Figure 2).[42–44] This primordial immune recognition system, loosely resembling the AP and LP of mammalian complement, is postulated to have responded to immune challenge by sequential proteolytic activation of its main component C3, thereby leading to C3-mediated opsonization of target surfaces, phagocytosis of foreign particles by chemotactically attracted immune cells, and induction of inflammation. This basic system was retained by all deuterostomes and was further diversified through gene duplications and domain rearrangements of the C3, Bf, and MASP genes.[44] These large genomic rearrangements gave rise to the first CP components (C4, C2, and C1r/C1s), while additional domain shuffling and gene duplication events led to the emergence of the terminal lytic pathway during the branching of the gnathostomes/cyclostomes from a common ancestor of jawed vertebrates.[42]

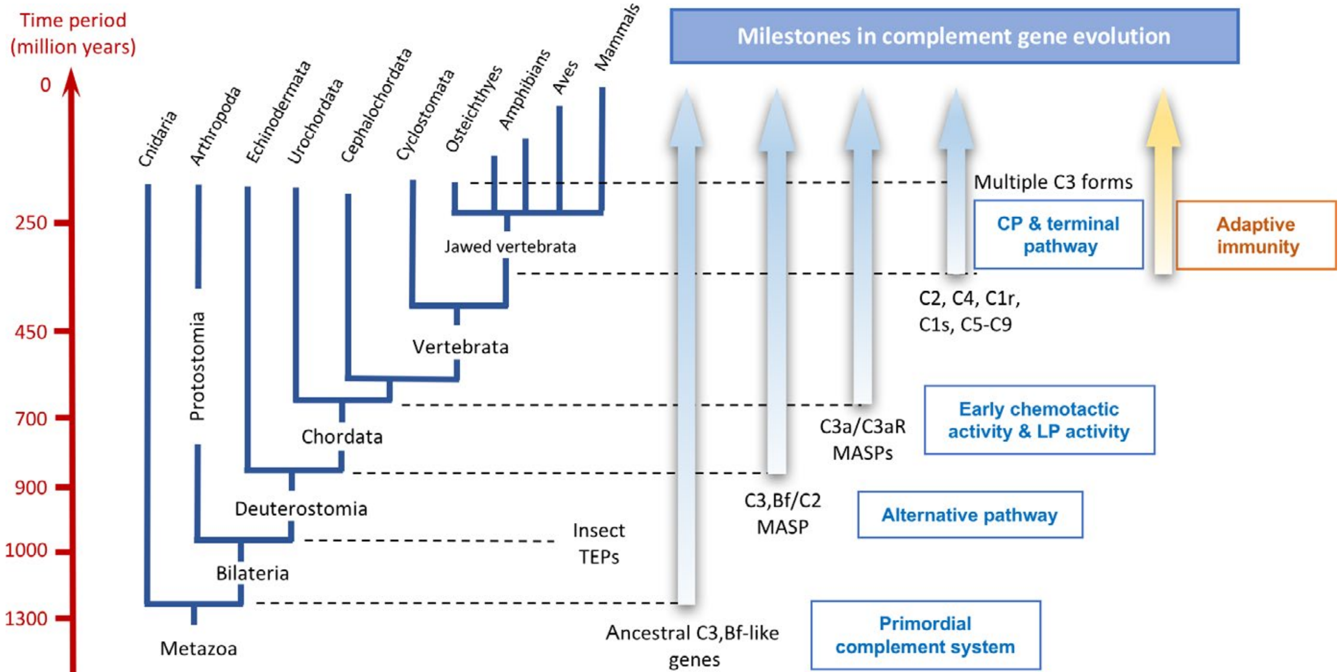

FIGURE 2 Evolution of C3 in the context of innate (complement) and adaptive immunity. The phylogenetic tree shows the early occurrence of ancestral C3 in evolution, the development of thioester-containing proteins (TEP) in insects, and the formation of increasingly mature and versatile alternative and lectin pathway (LP) systems, the striking diversity of multiple C3 isoforms in teleost fish (i.e. osteichthyes), with development of the classical pathway alongside adaptive immunity in vertebrate species

C3 has been highly conserved throughout the evolution of metazoans, with its appearance predating the early divergence of the deuterostomes and protostomes from a common invertebrate ancestor.[41] C3-like molecules identified in the most primitive invertebrates (cnidarian) appear to share the main structural features of their mammalian counterpart, including the thioester moiety, the anaphylatoxin domain, and the cleavage site between the α and β chains.[41] In SeC3, a C3-like gene cloned from the sea coral *Swiftia exserta*, most of the canonical Cys residues of human C3 are conserved, except for those linking the α and β chains. There are structural elements deduced from the cnidarian C3 sequence that suggest the presence of a three-chain structure similar to that of lamprey C3, mammalian C4, and cobra venom factor (CVF).[41] It is noteworthy that a BLAST search for predicted complement domain structures in the genomes of Cnidaria and certain protostomes (i.e. horseshoe crab) retrieved not only C3 but also Bf-related sequences, thus indicating that the core components of the complement system may have already evolved about 900-1000 million years ago.[44]

The echinoderms represent a pivotal link in the evolution of C3, as the very first evidence for the presence of a functional AP was discovered in this phylum of the deuterostome lineage (Figure 2).[45] Indeed, two homologs of the vertebrate C3 and Bf/C2 genes (SpC3 and SpBf, respectively) were cloned in the sea urchin *S. purpuratus*, and it was thought that they might form a primordial AP C3 convertase.[41] SpC3 is synthesized in coelomocytes, a type of innate immune phagocyte that circulates in the coelomic fluid of sea urchins. SpC3 possesses a conserved thioester site with an associated catalytic histidine and displays functional features typical of a C3 opsonin. Consistent with a role for SpC3 in inflammation and opsonophagocytosis, sea urchin coelomocytes have been shown to respond to LPS challenge by upregulating SpC3 expression and increasing their phagocytic activity in an SpC3-dependent manner.[41,46]

Following the divergence of the deuterostomes, C3-like proteins, along with several components of the LP and AP, were identified in urochordate species.[41,47] *Ciona intestinalis* possesses two C3-like genes that encode a thioester, catalytic histidine, α/β-chain processing, and C3 convertase cleavage site; both these C3 molecules are mainly expressed by granular amoebocytes in the hemolymph and are detected in plasma as proteins of approximately 220 kDa.[47] Interestingly, despite a sequence divergence within the C3a region of these C3-like molecules, recombinant forms of *Ciona* C3a-like peptides were found to be able to promote hemocyte chemotaxis, suggesting the presence of early anaphylatoxin signaling in this invertebrate species.[48] Further supporting the existence of a functionally intact C3aR-triggered pathway in invertebrates, a putative C3aR cDNA has been cloned from *Ciona intestinalis* and its expression documented on phagocytic hemocytes.[49] Furthermore, antibody-mediated blockade of the *Ciona* C3aR or pertussis toxin pretreatment of hemocytes could abrogate the directional migration of hemocytes toward a C3a gradient. Collectively, these studies were the first to provide compelling evidence for a conserved G_i protein-coupled C3aR signaling pathway in an invertebrate species.[48,49]

The branching of the cartilaginous fish from their jawless ancestors has defined a major hallmark in the evolution of the immune system.[40,42,43] The first core constituents of an acquired immune response began to appear in chondrichtyes (Figure 2), largely the emergence of MHC-like molecules and ancestral RAG1/2 genes. The concomitant emergence of the first immunoglobulins in cartilaginous fish also contributed to the development of the CP and lytic TP of complement in these species. The rest of the poikilothermic species, from teleosts to reptilians, appear to contain a well-developed complement system resembling that of homeothermic vertebrates (e.g. birds and mammals).[43] In this regard, C3 homologs with highly conserved structural features of the human C3 sequence (i.e. thioester, C3 convertase cleavage, α/β-chain processing, FI cleavage, and N-glycosylation sites) have been described in both amphibian and avian species,[50,51] further underscoring the phylogenetic conservation of key structural elements in C3 that underlie its activation and breakdown cycle.

A prominent landmark in the phylogenetic history of C3 is defined by the divergence of the teleost fish (i.e. osteichtyes) (Figure 2). These bony fish species have been shown to possess a remarkably diversified complement system, with multiple isoforms of C3, C5, and FB in their plasma and distinct immune recognition specificities associated with each of these isoforms.[43,52,53] This diversity in complement also extends to other components, such as C5, C7, and properdin. Initially, the multiple C3 clones that were isolated from teleost cDNA libraries (four in trout, eight in carp) were simply attributed to the tetraploid state of their genome. However, the subsequent identification of multiple C3 isoforms in diploid teleost fish indicated that this striking feature of C3 gene copy variance is not restricted to tetraploid organisms.[54] A fascinating aspect of complement phylogeny revealed in this vertebrate class is that different C3 isoforms are products of distinct genes, rather than splicing variants of a common gene, suggesting that early gene duplication and copy number variation might have led to this remarkable C3 diversity in bony fish. Although most of these C3 isoforms possess the main set of structural features found in mammalian C3, they are strikingly more diverse in terms of structure and function than are their mammalian counterparts. For example, distinct isoforms of C3 present in a single teleost fish are able to bind to diverse complement-activating surfaces (e.g. zymosan, *E. coli*, rabbit and sheep erythrocytes) with varying efficiency.[52] This unique feature suggests that teleost fish have developed a diversified innate immune arsenal at the level of complement, by recruiting distinct C3 isoforms as opsonins and potentially assembling different AP convertase combinations on target surfaces. Such a diversified response should enable teleost fish to expand their innate immune recognition capacity and elicit broader opsonic activity toward a wide spectrum of pathogens and activating surfaces, thereby likely compensating for a poorly developed humoral (Ig-mediated) adaptive immune response.

This concept might have important evolutionary implications for the immune system, especially in view of the decreasing diversity of C3 sequences as we ascend the evolutionary ladder toward higher vertebrates and mammals. Interestingly, teleost C3 genes have been shown to be highly polymorphic, further implying that the selection of different C3 alleles within the population might confer differential immune recognition capacities against various pathogens and non-self surfaces.[43] Furthermore, recent studies in zebrafish have shown

that multiple C3 isoforms within the same organism are differentially expressed after inflammatory challenge and are likely to regulate the magnitude and quality of the host's innate immune response to various inflammatory stimuli. These C3 isoforms have also been postulated to mediate distinct functions during tissue remodeling/regenerative processes in zebrafish.[55] Of particular note, teleost fish are able to generate fully functional C3a following cleavage of C3 by the AP C3 convertase.[56] They also possess a functional C3aR signaling circuit, as demonstrated by the expression of C3aR on trout phagocytic cells and their ability to migrate in response to C3a stimulation.[56]

Comparative phylogenesis has helped to elucidate the structural determinants of C3 (also see below), including its distinct domain architecture, interacting partners, and functional implications. Complement components C3, C4, and C5 belong to the thioester-containing protein (TEP) superfamily that also includes α2-macroglobulin (α2M), the pregnancy zone protein (PZP), CD109 and CPAMD8.[42,57] All of these proteins share homologous structural features, including a thioester domain (TED) with a unique intrachain thioester bond, eight macroglobulin (MG) domains, and a spliced CUB domain holding TED.[58] While the evolutionary origin of the TEP superfamily remains unresolved, comparative phylogenetic analysis of TEP-related sequences from insect species such as *Anopheles gambiae* and *Drosophila melanogaster* have revealed an intriguing relationship between C3, α2M, and insect TEPs.[59,60] Initially thought to represent complement-like orthologs in insects, these insect-encoded TEPs have been postulated to have diverged independently of C3 from a common ancestor before the branching of cnidarians and echinoderms.[42] For reasons that remain to be determined, insects possess only α2M and TEP homologs and have lost C3 from their genomes during evolution. In fact, broader sequence analysis has indicated that insect TEPs are orthologous to human CD109.[42] However, the striking sequence similarity of insect TEPs to thioester-containing complement proteins, their opsonophagocytic activity in insect cell cultures,[60] and their upregulation in immune-related tissues after inflammatory challenge likely indicate an innate immune system with C3-like opsonic activity and a converging evolutionary trait that has been preserved throughout phylogenesis in orthologous proteins.

Appreciating the phylogenetic conservation and functional diversity of complement proteins, it is intriguing to speculate that C3-like proteins were an integral component of a primordial phagocytic system mediating innate immune defense and tissue immunosurveillance in both invertebrates and early vertebrates. This opsonophagocytic circuitry prominently expanded its immune recognition capacity in poikilothermic vertebrates (i.e. teleost fish), by assigning diverse functionalities to multiple C3 isoforms in order to compensate for the absence of a fully developed adaptive lymphocyte response. From an evolutionary standpoint, the emerging roles of C3 and C3-derived effectors (e.g. C3a) in early vertebrate development, as exemplified by elegant studies of tissue morphogenesis and patterning in both amphibian and mammalian embryogenesis, might reflect a primordial and species-transcending function of C3 that has been conserved throughout evolution as a fundamental and survival-promoting trait in all vertebrates.[23,61] Such a fundamental role for complement in vertebrate development would resonate well with its long-standing presence across phylogenesis under approximately 900 million years of evolutionary pressure.

3 | REVEALING THE MOLECULAR SECRETS OF C3: INSIGHT INTO STRUCTURE-FUNCTION ASPECTS

Over almost six decades, a wealth of biochemical data has provided an increasingly refined picture of the functional determinants of this unique immune mediator.[62] C3 is a 185-kDa glycoprotein encoded by the *C3* gene on chromosome 19 (locus p13.3) and secreted as a pre-pro-protein of 1663 amino acids (aa), including a 22-aa signal peptide.[63] Post-translational processing leads to the proteolytic removal of a tetra-arginine linker by a furin-type enzyme and results in a β-chain (75 kDa) and α-chain (110 kDa) that are linked by a single disulfide bridge.[63,64] C3 contains two main glycosylation sites (Asn-917 of the α-chain, Asn-63 of the β-chain; this and subsequent a numbering is based on the mature protein without signal peptide) with attached high-mannose carbohydrate moieties.[63,65] Interestingly, it has been found that C3 can be phosphorylated at various sites by several kinases, in some cases influencing its functional properties[66]; although the (patho)physiological implications remain to be further explored, increased levels of phosphorylated C3 have been detected in patients suffering from systemic lupus erythematosus (SLE).[67]

From a functional perspective, the posttranslational modification of C3 with the most far-reaching consequences is the formation of the thioester bond, achieved by intramolecular transacylation between the thiol group of Cys-988 and the side-chain amide of Gln-991.[68] While this thioester bond is shielded in native C3 (see below), the transformation to C3(H_2O) or C3b exposes and breaks the bond, creating a transient acylimidazol intermediate between Gln-991 and His-1104 that can form covalent ester and amide bonds with hydroxyl groups of carbohydrates or amino groups of proteins, respectively. The ultra-short half-life of this intermediate (approximately 100 μs) restricts covalent deposition of C3b to areas in immediate proximity to the site of activation, with the remainder of the reactive groups being quickly blocked by hydrolysis.

Indeed, the spatiotemporal opsonic reactivity provided by the fascinating transformation of C3 to C3b holds the key to C3's ability to act as an efficient immune surveillance mediator. As an acute phase reactant, C3 needs to be available instantly, act rapidly, and react to various threats but not harm normal bystander cells. Instant availability is achieved by its high plasma concentration of approximately 1.2 mg/mL, making C3 one of the most abundant plasma proteins in circulation. While it is primarily synthesized by hepatocytes in the liver and distributed intravascularly, it is important to note that C3 is produced and secreted by various cells, thereby providing a source of tissue-localized C3.[69] The importance of local C3 production is increasingly recognized[4,69–71]; for example, crossover studies using C3-deficient donor or recipient mice in a kidney transplantation model have revealed that C3 produced by the donor organ rather than the systemic

pool from the recipient is mainly responsible for postischemic renal failure.[72] Further exploration of the C3 distribution will prove important for a better understanding of its defense contributions in peripheral or restricted regions such as the skin, mucosa, central nervous system, and eye. At the same time, new roles have been attributed to intracellular C3, especially in lymphocytes[71]; the existence of a leukocyte complement system has long been postulated,[73] yet we are only now beginning to understand the implications of intracellular complement for T-cell homeostasis/differentiation and other physiological processes.[71,74,75] Certainly, the perception of C3 as an entirely extracellular and intravascular mediator has to be revised, and context/location-specific activities need to be considered.

In the circulation, its high plasma levels and low biological reactivity in the native state allow C3 to act as a sentinel that can be immediately engaged after an encounter with potential insults. In fact, the only confirmed physiological ligands for native C3 are the C3 convertases and extrinsic proteases with C3 cleavage activity; a potential interaction with heme that renders C3 more susceptible to activation has been reported but requires further characterization.[76] It is fascinating that the conversion of C3 to C3b and its degradation products converts one "inert" protein into one of the most multifunctional molecules in our body.[62] Indeed, the complement map database (www.complement.us/cmap) developed by our group,[77] which visualizes known biomolecular interactions between complement proteins and their partners, lists more than 20 endogenous ligands and many exogenous factors, such as microbial immune evasion proteins (see below), for the various forms of C3 (Figure 3). Long before the upsurge in structural biology in recent decades, intricate biochemical analyses based on antibody selectivity and protein fragmentation were employed to meticulously map the interaction sites of the various ligands.[62,78–80] During the past decade, however, the emergence of high-resolution structures for many complement proteins and their complexes, in particular those related to C3, have literally

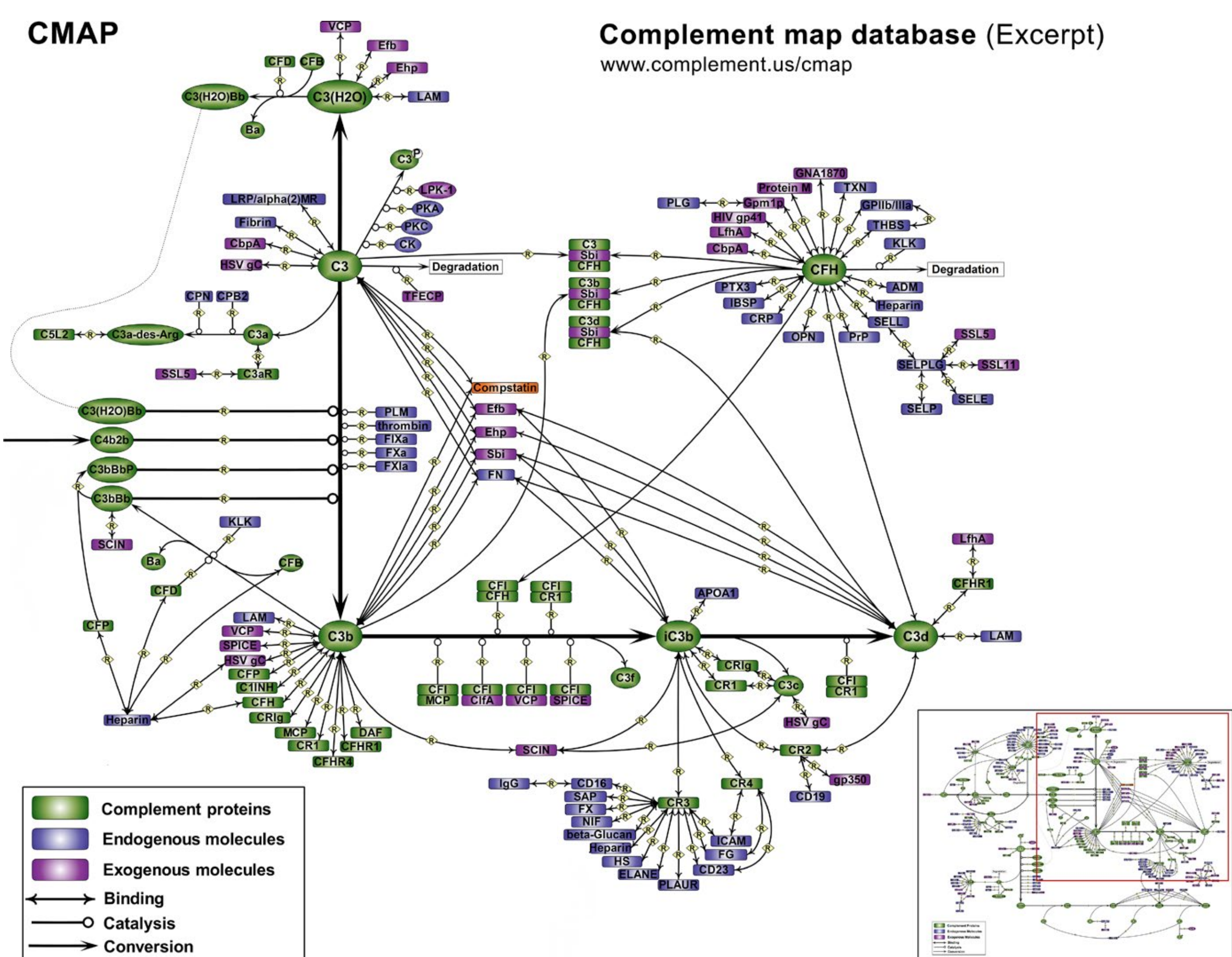

FIGURE 3 Multifaceted connectivity of C3 fragments with endogenous and exogenous ligands. Although C3 engages in very few interactions with endogenous ligands, its activation to C3b and further degradation to iC3b and C3dg generate some of the most versatile ligands of host defense. This excerpt from the main map of the Complement Map database (CMAP; www.complement.us/cmap) shows protein-protein/ligand interactions based on experimental data from literature.[77] Only the area of the main map that involves C3 and its fragments are shown (red box in insert); other elements have been removed

added another dimension to our knowledge of the molecular mechanisms underlying complement activity. The picture emerging from these studies has been one of clever domain rearrangements and profound conformational changes that allow a tailored and coordinated transition from activation and amplification to regulation and effector signaling. In the following sections, we summarize current structural insights into the unique design of C3 and the key processes of complement function.

3.1 | The anatomy of C3: One scaffold that determines many functions

The structure of C3 at 3.3-Å resolution was reported in 2005 and revealed an intricately shaped protein composed of 13 domains (Figure 4A–C).[58] The α- and β-chain each contain six individual domains (Figure 4D); surprisingly, and quite unusually, a thirteenth domain is shared between the two chains. The majority of the β-chain was found to consist of a "key ring"-shaped core formed by five domains with a hitherto-undescribed fold, which were termed MG domains to identify them as a common structural element of the α2M protein family (see above). The five complete MG domains (MG1-5) are followed by a partial MG domain ($MG6^{\beta}$) and a hydrophobic linker domain (LNK) that loops through the core ring. An anaphylatoxin (ANA) domain, which is prone to be released as the C3a fragment, defines the start of the α-chain. This domain is followed by the second part of the MG6 domain (MG6α) to complete the key ring and attach the remainder of the α-chain on top of the MG core. Following another MG domain (MG7), a split CUB domain (CUB^{g}, CUB^{f}) frames and holds the globular thioester-containing domain (TED) and leads to a final MG domain (MG8). A short anchor region finally connects the C-terminal complement domain (CTC; previously termed C345C[29,58]).

The unusual presence of two split domains (MG6, CUB) in C3 indicates that functionally critical elements (i.e. the proC3 processing site, C3a, and CUB-TED) were added by gene insertion during evolution and assumed increasing importance in host defense.[58]

The arrangement of the 13 domains in native C3 largely explains its relatively inert nature when compared to its cleavage fragments. The orientation of the TED is of particular importance, as this domain contains the reactive thioester moiety that mediates covalent binding to targeted surfaces. In native C3, the thioester bond (Cys-988/Gln-991) is intact and tucked away in a hydrophobic pocket at the MG8-TED interface; although shielded from solvent contact, the thioester region still resides close to the protein surface.[58] Upon activation, Gln-991 forms a reactive acylimidazol intermediate with His-1104 that is further stabilized by Glu-1106; approximation of these residues is hindered in native C3, thereby providing another mechanism that prevents ready conversion and explains the slow hydrolysis rate (approximately 0.2%-0.4%/h) in this state.[58,81] Moreover, with the exception of the dimerization site on the MG ring that mediates the binding of C3 to convertases (see below) and the scissile loop of ANA/C3a, all known ligand-binding sites in C3b/iC3b/C3dg are buried or sterically hindered by the arrangement of the α-chain in native C3.

3.2 | Activation in variations: From tick-over to opsonization

Despite sharing the same domain organization with native C3, the hydrolyzed form $C3(H_2O)$ has remained enigmatic, largely because of the absence of a high-resolution structure. Functional studies have long suggested that $C3(H_2O)$ adapts a C3b-like structure, but one that still contains the ANA domain.[81,82] Initial structural evidence was provided

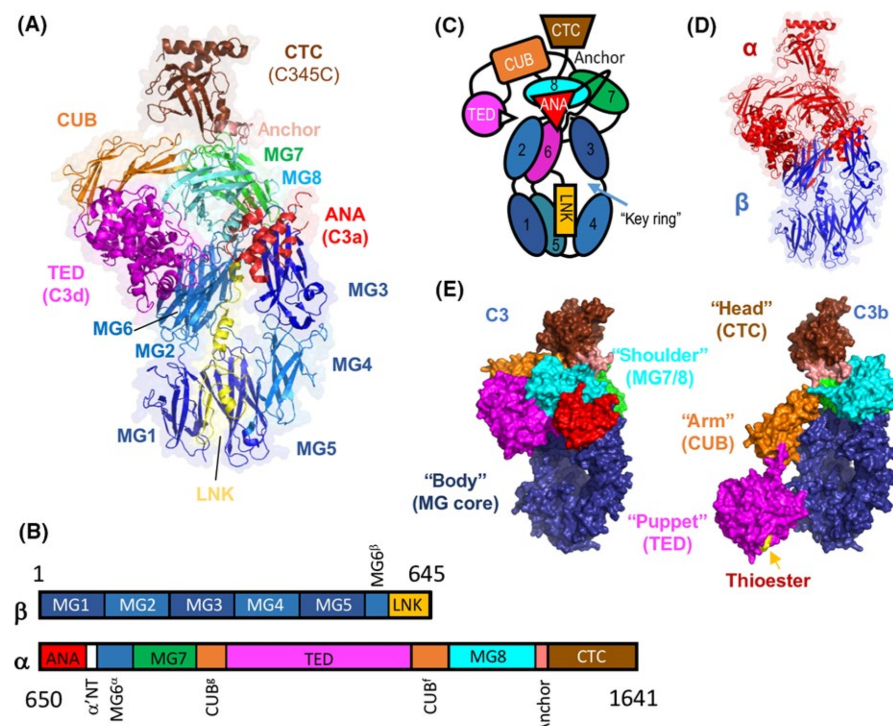

FIGURE 4 Structural anatomy of C3. (A) Crystal structure of native human C3 (PDB 2A73 [58]), with domains colored individually. (B, C) Schematic representation of domain arrangements, shown as linear bars divided into the β-chain and α-chain of C3 (B; amino acid numbering corresponding to mature protein without signal peptide) and as a scheme of their relative orientations in the crystal structure (C). (D) Positioning of the α-chain and β-chain of C3 as shown in a cartoon representation of the crystal structure. (E) Structural transformation of C3 to C3b upon complement activation. The major structural areas based on the analogy of a puppeteer with body, shoulder, head, and arm holding a puppet are shown in surface representations of the crystal structure of C3 and C3b (PDB 2I07 [91]). The location of the exposed thioester bond in C3b is highlighted in yellow

by studies with monoclonal antibodies that revealed comparable neoepitopes after conversion of C3 to either C3(H_2O) or C3b.[78,83] Hydrogen-deuterium exchange mass spectrometry (HDX-MS) identified several areas with changes in solvent accessibility upon hydrolysis of C3, particularly in the α-chain and its TED domain.[84] Detailed mechanistic insights were offered by electron microscopy (EM) studies suggesting that native C3 represents a “kinetically trapped” conformational state, in which the bulky ANA domain acts as a “door stop” to prevent the formation of the reactive conformer.[85] In an unfavorable high-energy (and therefore rare) event, potentially enabled by unfolding of MG6, ANA may slip through the key ring of the MG core, leading to an irreversible conformational change with an extended TED-CUB and exposed thioester. Such displacement of the ANA domain in C3(H_2O), with rearrangement of CUB and TED alongside MG7 and MG8, has been supported by quantitative cross-linking mass spectrometry (QCLMS).[86] Although EM, QCLMS, and independent small-angle X-ray scattering studies indicate an arrangement of the TED-CUB-MG core interface that is slightly different in C3(H_2O) and C3b,[85–87] the relevance and functional implications of the difference are not entirely clear. Binding studies have indicated distinct yet sometimes conflicting differences in the interaction profiles of the two proteins toward FB and FH,[88,89] but additional insight from structural and/or functional assays will be necessary to reveal the full picture of this important complement initiator.

In contrast to its functional cousin, C3b is the best-characterized C3 fragment, with several high-resolution crystal structures describing this key opsonin in isolation or in complex with various ligands.[29,90–95] With the notable exception of one study, which has meanwhile been retracted,[96,97] all C3b-containing structures agree on the domain arrangement and have provided a rationale to explain the difference between the essentially inert C3 and the highly reactive C3b. The 4-Å crystal structure of C3b in isolation, published in 2006,[91] showed that the β-chain of C3b remains largely unchanged when compared to C3, thereby identifying the key ring built by domains MG1-6 as a stable core element. In contrast, the α-chain in the activated form has undergone profound conformational changes, with the CUB in an extended position, the TED located at the other side of the MG core with the thioester approximately 95 Å from its original location, and the MG7-MG8 domains rearranged. The analogy of a “puppeteer” composed of a body (MG1-6, LNK), shoulder (MG7-8), neck (anchor), head (CTC), and arm (CUB), who holds a puppet (TED) was previously coined; in C3, the puppet is held up to the shoulder, whereas it is dropped in C3b, leading to an extended arm and a twisted shoulder (Figure 4E).[91] This dropping of TED is considered critical for bringing the short-lived acylimidazole intermediate of the reactive thioester into close proximity with the target surface; a single electropositive patch around the thioester moiety may help orient the reactive C3b on negatively charged surfaces such as bacterial cells.[91]

Of equal importance is that the fact that the transformation of C3 to C3b reveals several previously cryptic binding sites that define its versatile involvement in activation, regulation, and signaling. For example, the repositioned CUB is involved in binding both the convertase precursor FB and most regulators of the RCA family, including CR1/CD35, CD46, CD55, and FH.[29,90,92] In the case of CRIg, the binding site for this receptor is located in the key ring area (MG3-6, LNK) that is present in both C3 and C3b; here, the specificity for C3b is determined by rearrangements of the MG3 and LNK domains upon activation.[94]

3.3 | Fueling the powerhouse of complement: The inner workings of the AP C3 convertase

In many physiological and pathological situations, the C3 convertase of the AP is arguably the most powerful component of complement activation; the assembly, spatiotemporal activity, and regulation of this complex often determines the fate of a probed cell. In the absence of true specificity, it is expected that distinct safety mechanisms have evolved to prevent uncontrolled activity of the convertase. Surprisingly, and in contrast to most other steps in the cascade systems of host defense, the two enzymes involved in AP C3 convertase formation, i.e. FB and FD, circulate as mature enzymes rather than in zymogen form. Moreover, the same protein scaffold acts as both substrate (i.e. C3) and part of the enzyme complex (i.e. C3b). The fascinating story how this unique constellation is translated into an efficient yet controlled mechanism of complement activation and amplification was only fully reconstructed in recent years with the help of several structural studies by our group and others (Figure 5).

All the individual pieces of this puzzle had been contributed earlier via the crystal structures of C3 and C3b,[58,91] FB and its Bb fragment,[98,99] and FD.[100] However, the primary obstacle to putting the pieces together and achieving a structural image of the AP C3 convertase was the fact that the C3bBb complex irreversibly dissociates within a few minutes, thereby preventing successful crystallization. An attempt to solve the structure of the pro-convertase as an initial step involved the use of CVF, a snake-derived C3b/C3c that forms more stable convertases (see below), and a gain-of-function double mutant of FB (D254G/N260D[101]) to increase the stability of the complex.[102] The resulting crystal structure at 2.2-Å resolution revealed that CVF and presumably, by analogy, C3b mainly interact with FB at its head and shoulder region via domains MG2, MG6, MG7, CUB, and CTC. Most of the contacts are provided by the Ba segment of FB, whereas the interface with the Bb segment is restricted to the CTC domain of CVF. In agreement with the long-standing notion that convertase formation is magnesium-dependent, the distorted metal-ion-dependent adhesion site (MIDAS) of FB was thought to adopt a high-affinity state in the CVF-bound form that chelated a Mg^{2+} ion together with the C-terminus of CVF. In contrast, and quite surprisingly, the rest of the FB structure, and in particular the catalytic region, shows little to no change when compared to free FB, thereby leaving critical questions concerning convertase formation unresolved.

An important breakthrough in this endeavor came with the use of the bacterial complement inhibitor SCIN (see below), which had previously been shown to trap the AP C3 convertase in a kinetically stable yet inactive state.[103,104] Indeed, in vitro convertase formation in the presence of SCIN resulted in stable complexes that could be crystallized at 3.9-Å resolution.[93] The crystal structure showed a dimeric

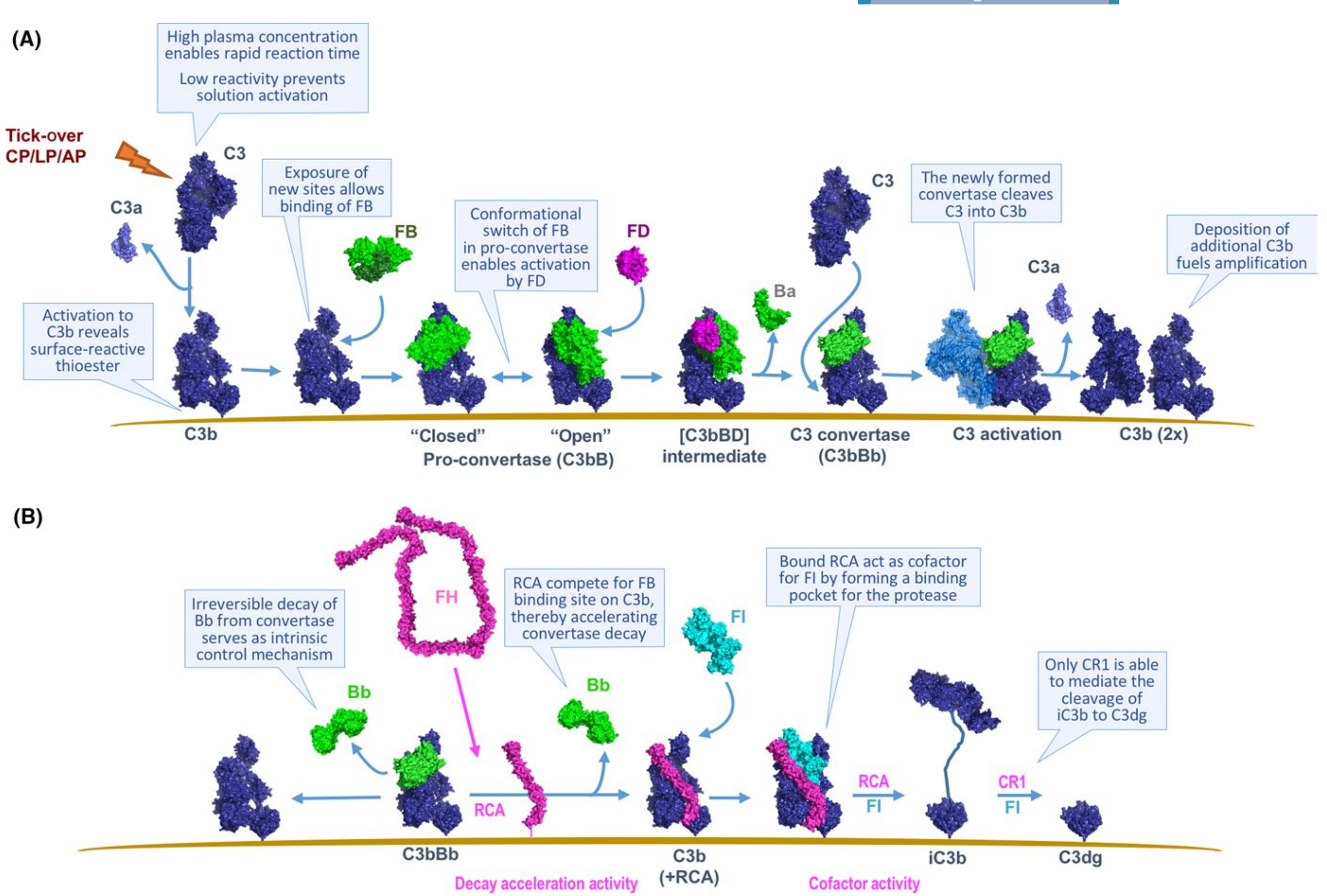

FIGURE 5 Molecular mechanisms driving C3 convertase formation, activity, and regulation. (A) Convertase formation and C3 activation. Upon activation of C3 and deposition of C3b, factor B (FB) binds to the newly exposed sites on C3b and undergoes a conformational transition between a closed "loading" and an open "activation" state. Factor D (FD) binds to the open form of the pro-convertase (C3bB) and cleaves Ba to leave the final AP C3 convertase C3bBb. The C3 substrate binds to C3bBb via a C3:C3b dimerization site and, upon presumed movement of the C3b-bound Bb, becomes activated to C3b, which can undergo new convertase formation. (B) Regulation of convertase activity. Although the C3bBb complex dissociates within a few minutes, with the Bb segment not being able to re-associate, regulators of complement activation (RCA) accelerate convertase decay by competing with the FB/Bb binding site on C3b. Bound RCA proteins also form a joint binding site for factor I (FI) that enables the cleavage of C3b to iC3b and, in case of CR1/CD35, to C3dg. To generate this figure, the structures of C3 (PDB 2A73), C3b (2I07), C3a (4HW5), C3d (1C3D), $C3b_2Bb_2SCIN_2$ (2WIN; only C3bBb part shown), C3bB (2XWJ), C3bBD* (2XWB), C3b-FH[1-4] (2WII), FB (2OK5), Bb (1RRK), FD (1DSU), and FI (2XRC) were used in PyMOL. For visualization purposes, the closed form of C3bB was generated by using FB from CVF-FB (3HS0), Ba was extracted from the FB structure, full-length FH was composed of five copies of FH[1-4], and a hypothetical iC3b model was prepared using the structures of C3d and C3c (2A74). The model of C3 bound to C3bBb was prepared as described in,[93] and the model of C3b-FH[1-4]-bound FI was created according to [107]

and symmetric complex formed by two C3b, Bb, and SCIN molecules each (i.e. $C3b_2Bb_2SCIN_2$). When extracted from this structure, the two C3bBb complexes were highly comparable and showed that, like the CVF-FB structure, Bb was mainly bound to the CTC domain of C3b, suggesting that the high-affinity MIDAS state is maintained. As a result of this interaction, Bb appears to "dangle" from the top of C3b in the final AP C3 convertase. The dimeric nature of the SCIN-stabilized convertase complex also revealed critical information about potential substrate binding and processing. Intriguingly, the data showed that the two copies of C3b were dimerizing at a region that is retained in both C3 and C3b, pointing to a potential contact site between the C3 substrate and convertase-forming C3b. Indeed, replacement of one C3b copy in the dimeric complex with native C3 resulted in a model in which the protease domain in Bb is directed toward the ANA domain of C3. Although the catalytic site and scissile loop are still 30 Å apart, it was speculated that the presumed conformational flexibility of the CTC domain would allow Bb to swing closer to the C3 cleavage site in the uninhibited substrate-convertase complex and allow activation of C3.[93]

With a refined model of the assembled convertase in hand, the question concerning the formation of the complex was reinvestigated. Under improved conditions, including Ni^{2+} instead of Mg^{2+} ions and again involving the FB gain-of-function mutant, it was possible to obtain a structure of the C3bB pro-convertase at 4-Å resolution.[90] Surprisingly, the domain arrangement of C3b-bound FB differed significantly from that observed in free FB and in CVF-FB.[90,99,102] Although the contact interface of the Ba segment and of Bb with the CTC domain of C3b remained largely the same, the serine protease (SP) domain of FB underwent a large rotation (84°), thereby forming

additional contacts with the MG2 and CUB domains of C3b. It was speculated that the arrangement observed in CVF-FB resembles a closed "loading state" of FB, which dynamically transitions to an open "activation state" that would allow the binding of FD.[90,102,105] Indeed, co-crystallization of a functionally inactive mutant of FD with the pro-convertase resulted in a C3bBD* complex, confirming that FD specifically binds to the open form of C3b-bound FB.[90] This interaction brings the catalytic site of FD into close proximity with the scissile loop of FB to allow its cleavage and the release of Ba. Importantly, FD interacts with C3bB via a hitherto unidentified exosite distant from the catalytic site. Moreover, FD possesses a self-inhibitory loop that prevents ready access to the catalytic center in circulation but is rearranged in the bound form to induce an active conformation only upon binding.

Taken together, these structural and additional binding studies provide fascinating insights into an intricate molecular mechanism tailored for instant, yet controlled, tagging of targeted particles (Figure 5).[90,93] Both C3 and FB are present in rather high plasma concentrations (1.2 and 0.2 mg/mL, respectively) to allow instant availability but do not react with each other in circulation. Similarly, the lower concentration of FD (0.002 mg/mL), its self-inhibitory loop, and the missing binding site in the closed form of unbound FB prevent a solution reaction between these two partners. The initial generation of an active form of C3, i.e. C3b or C3(H_2O), by any means on a target surface provides a binding platform for FB as the result of the exposure of new binding areas, primarily on the CUB domain. A fast initial interaction of the Ba segment of FB with this region and Mg^{2+}-specific binding of the MIDAS to the CTC domain of C3b enable a quick but specific "loading" of the protease component to form the pro-convertase C3bB. Once bound, dynamic switching of the SP domain to an open conformation of FB allows the binding of FD; only then is the self-inhibitory loop of FD allosterically removed to give access to the catalytic center. Binding and cleavage of the scissile loop in FB release Ba and produce the final C3bBb convertase. Although Ba initially restricts C3's access to the dimerization site in the pro-convertase C3b, its dissociation now allows the binding of the C3 substrate to the convertase. The presumed flexibility of the anchor and CTC interface likely enable the Bb part to swing closer to the scissile loop to cleave the ANA domain; the feasibility of such a movement has been supported by the arrangement of CVF in complex of C5.[106] Release of C3a removes the "door stop" that kinetically traps C3 and initiates the profound conformational change to C3b that quickly brings the newly formed, short-lived thioester intermediate into contact with the nearby surface and exposes another FB binding site to fuel amplification (Figure 5A).

This tiered interaction of three components, each necessitating a conformational transition, already presents an intricate means of intrinsic control with respect to convertase formation.[90] The low stability of the assembled convertase, with a half-life of approximately 3 minutes at 37°C, adds another layer of control. Binding studies have shown that the Ba segment is essential for pro-convertase formation because it provides rapid, Mg^{2+}-independent loading and brings the MIDAS into contact with the C-terminus of C3b. After the release of Ba, the Bb-C3b continuously dissociates, but Bb cannot rebind in the absence of the Ba segment; even in the presence of Mg^{2+}, no significant binding of Bb to C3b can be observed (Figure 5B).[93] Thereby, convertase activity relies on a constant supply of FB and the generation of new C3bBb complexes to maintain or even amplify the opsonic response. Moreover, this mechanism provides a basis for the ability of complement regulators to extrinsically control complement activation on host surfaces and confer selectivity between self and non-self (Figure 5B).

3.4 | A tale of two activities: A common binding mode defines regulatory functions

The N-terminal four domains of the soluble AP regulator FH exert both decay acceleration and CA. Crystallization of the C3b-FH[1-4] complex at 2.7-Å resolution therefore provided an important starting point for studying the molecular basis of these two major regulatory mechanisms.[92] The structure revealed that FH[1-4] occupies a large, continuous contact area that covers an entire flank of C3b; despite this extended binding site, the interaction of FH[1-4] with C3b results in only a rather weak affinity of approximately 10 µM. The N-terminal portion of FH[1-4] binds at the top of C3b (the MG7 domain and α′-NT region), partially occupying the area where FB and Bb bind. Indeed, superposition of C3b-FH[1-4] with the C3bBb complex from the SCIN-stabilized convertase structure reveals a profound steric clash between FH and Bb that can be exacerbated by the N-linked glycan in Bb and electrostatic repulsion between the two proteins. It is therefore reasonable to propose that the DAA of FH is achieved by a competition for the Bb binding site on C3b, leading to a displacement of Bb and accelerated decay of the AP C3 convertase (Figure 5B). Cofactor activity, on the other hand, can be explained by the observation that C3b-bound FH[1-4] forms a new binding platform for FI that is mainly formed by CCP domains 1-3 of FH and the CUB domain of C3b.[92] Subsequent docking studies have confirmed and extended this model and have shown that the binding of FI to this joint C3b-FH site releases allosteric inhibitory restraints in FI and brings the catalytic center of FI close to the scissile loop in the CUB domain of C3b to enable the first cleavage (between Arg-1281 and Ser-1282) to $iC3b_1$.[107]

Recent studies have further refined our molecular understanding of RCA-mediated regulatory functions by comparing the co-crystal structures of C3b with CR1/CD35 (domains 15-17), MCP/CD46, DAF/CD55, and the viral RCA mimic SPICE (see below).[29] The comparative analysis of these structures demonstrated that all these regulators invoke the same general binding mode as FH[1-4]. However, marked differences in the exact contact interface and the resulting binding strength of different domain interactions help explain their distinct regulatory profiles. For example, although the dual-activity regulator FH[1-4] engages all four N-terminal domains for C3b binding, the CA-selective MCP almost exclusively binds via its CCP3-4 domain at the lower half of C3b that is involved in forming the FI-binding site, and the DAA-conferring DAF predominantly occupies the areas of C3b in proximity to the FB site. Despite these differences, the common binding mode suggests that all regulators have evolved from a common origin and that MCP and DAF have enhanced their respective functions at the cost of dual activity.[29] In addition to improving our understanding of the molecular mechanisms of complement regulation, these

C3b-RCA structures have already become and will continue to be essential for deciphering the functional consequences of the disease-related mutations and polymorphisms of RCA proteins.[4,29,92,108]

3.5 | Access and restriction: Explaining the receptor specificity of C3-derived opsonins

One of the key features of complement-mediated opsonization is the unique ability to engage various immune receptors in a sequential, context-specific manner. In this regard, complement regulators not only impair the activation and amplification loop but also process the opsonins to forms that exhibit distinct signaling profiles. Interestingly, C3b contains all the receptor contact areas as preformed sites but cannot engage the entire spectrum of signaling tasks. The gain and loss of ligand binding during the breakdown from C3b to iC3b and C3dg is elegantly mediated by a series of remarkable transitions (Figure 6).

As discussed above, the activation of C3 to C3b leads to an extension of the CUB-TED interface that contains binding areas for members of the RCA family. Alongside their regulatory activities, some of the membrane-bound RCAs also act as adhesion and/or signaling receptors. For example, CR1 on erythrocytes is important for shuttling C3b-opsonized particles to the spleen and liver and is known to facilitate antibody-mediated phagocytosis via Fc-receptors.[6,109] The interaction of CD46 with C3b, on the other hand, can induce or modulate intracellular signaling events on various lymphocytes, including the induction of a productive Th1 phenotype in T cells.[110,111] Finally, the binding of CRIg to the key ring of C3b enables phagocytic clearance of opsonized particles by Kupffer cells and other tissue-resident phagocytes[94,112]; of note, CRIg may also exert AP-specific regulatory functions by interfering with the binding of C3 to C3bBb and was recently reported to act as a direct PRM for Gram-positive bacteria.[112,113]

The CA of certain RCAs initiates the degradation of C3b to iC3b by FI through the release of the C3f peptide from the CUB domain. There is currently no crystal structure of iC3b available, but EM studies have provided important but somewhat conflicting insights into the structural consequences of this transformation. Although some models predict a disordered CUB with a distant and flexible orientation between the C3d and C3c parts of the molecule,[85] other EM studies suggested a more compact conformation resembling the tucked-in TED in native C3.[114] Recently performed HDX-MS experiments by our group comparing C3b and iC3b have found full solvent exposure across the entire CUB domain, suggesting complete unfolding and a loss of the interaction between TED and the MG core and supporting the initial EM results indicating a distant, flexible configuration (Ricklin and Lambris, unpublished observations). In this model, the removal of C3f and subsequent unfolding remove the RCA and FB binding sites on the previously ordered CUB domain and explain the loss of convertase formation and most RCA-related regulator/receptor functions in iC3b; however, the binding to CRIg remains intact.[112]

Conversely, an intact CUB sterically restricts the access of CR2/CD21 to its binding site on the TED in C3b; unfolding of CUB eliminated this blockage and provided an explanation for the selectivity of CR2-mediated adaptive immune signaling for iC3b and C3dg. A similar situation likely applies to the integrin receptor CR3 (CD11b/CD18): Recent studies have revealed that at least one major binding area of the receptor is located on the TED.[115,116] Although distinct from the CR2 site, this CR3 contact region is also sterically hindered by CUB in C3b and only becomes accessible in iC3b. In contrast to CR2, however, additional binding sites on the C3c segment may be involved in the interaction with iC3b.[115] Interestingly, the related CR4 (CD11c/CD18) does not seem to bind the same site on TED but rather interacts primarily with the C3c part of iC3b, most likely at the MG3/

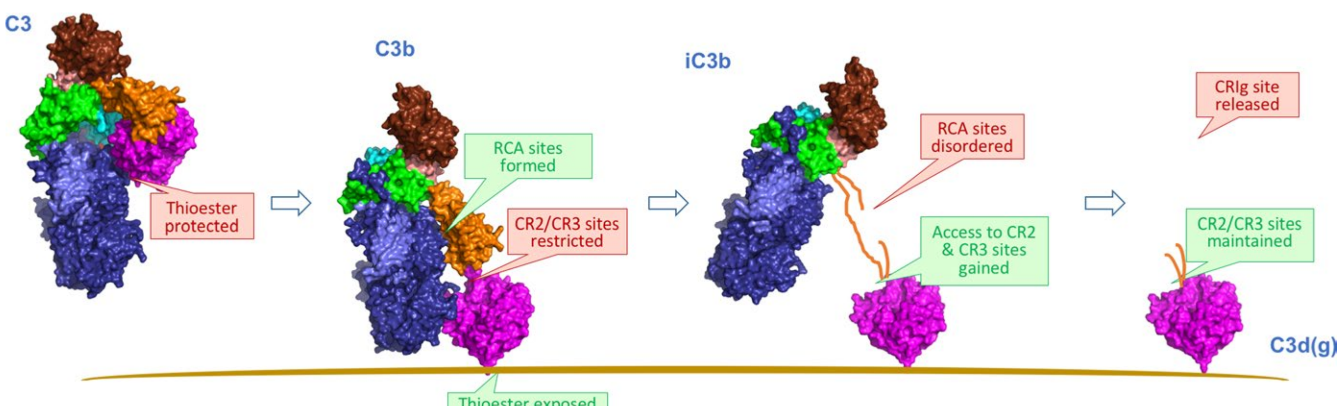

FIGURE 6 Structural transformations guide the differential ligand binding of C3-derived opsonins. During the activation of C3 to C3b and the subsequent degradation of C3b to iC3b and C3dg, the ligand binding and signaling profile of the opsonins change to allow for a differential immune response. The extension of the TED-CUB interface in C3b enables binding of regulators and receptors of the RCA family (i.e. CR1/CD35, CD55, CD46, FH, FHL-1) and, in the presence of a cofactor, factor I (FI). The FI-mediated cleavage in CUB to form iC3b presumably leads to a degradation of the domain, thereby removing the RCA-binding site but providing access to sites for complement receptors CR2 and CR3 that were sterically restricted in C3b. These sites are also present in C3dg, although the release of the C3c segment eliminates the MG key ring that harbors a binding site for CRIg in C3b and iC3b. The structures of C3 (PDB 2A73), C3b (2I07) and C3d (1C3D) were used for this figure; the hypothetical structure of iC3b was generated using the structures of C3c (2A74) and C3d connected by a freeform line. The color scheme defined in Figure 4 was used to color individual domains

MG4 interface.[115,117] As this area is preserved between C3b and iC3b, intracellular transformations would not account for the iC3b selectivity of CR4; the authors therefore speculated that the more flexible orientation of iC3b on the surface determines opsonin selectivity.[117] Additional studies may be needed to fully explore the distinct binding modes of CR3 and CR4 toward complement opsonins.

The final transformation of iC3b to C3dg can only be mediated by CR1/CD35, but the molecular determinants of this enhanced CA are still being investigated. Although it has been shown that C3dg (40 kDa) can be further processed to C3d (35 kDa) after cleavage of the C3g peptide by various proteases in vitro, this transformation is currently not considered to be physiologically relevant under most circumstances.[118] With the removal of the C3c segment, including its MG core, C3dg loses the ability to bind to CRIg. The interaction with CR2, however, and the associated B-cell stimulation function, are fully preserved in C3dg. In fact, the comparatively small size, high stability, and maintained B-cell activity make multimeric C3d a candidate for the development of novel vaccine adjuvants.[119,120] Although CR3 has long been considered to have strict opsonin specificity for iC3b, the above-mentioned molecular studies have confirmed the existence of a high-affinity binding site in the TED domain.[115] Direct binding studies confirmed that the recombinant α_MI domain of CR3 interacts with iC3b, C3dg, and C3d but not with C3b.[116] In vitro, we were further able to show that densely C3dg-opsonized erythrocytes from patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) are recognized and phagocytosed in a CR3-dependent manner, suggesting a physiological role for the C3dg-CR3 interaction.[116] Whether this route is dependent on C3dg density and how it compares kinetically with other uptake mechanisms in vivo remain to be further investigated.

4 | A FORCE THAT NEEDS TO BE CONTROLLED: C3 AS A MICROBIAL IMMUNE EVASION TARGET

The central role of C3 and the AP C3 convertase in the clearance of microbial intruders renders the C3 axis a major target in the immune evasion strategies employed by many human pathogens.[28,121–123] Indeed, evolutionary pressure has led to the emergence of intricate molecular mechanisms to impair C3-mediated opsonization and effector functions at various stages. Among these mechanisms, the recruitment of soluble RCA appears to be a particularly common approach employed by bacteria (e.g. *Neisseria* and *Streptococcus* species), fungi (e.g. *Candida* and *Aspergillus* species), and certain parasites (e.g. *Plasmodium falciparum*). Although microbes typically lack self-surface patterns, these pathogens express specialized proteins or surface structures that adsorb circulating regulators such as C4BP or FH onto their surfaces (Figure 7A).[121,124] For example,

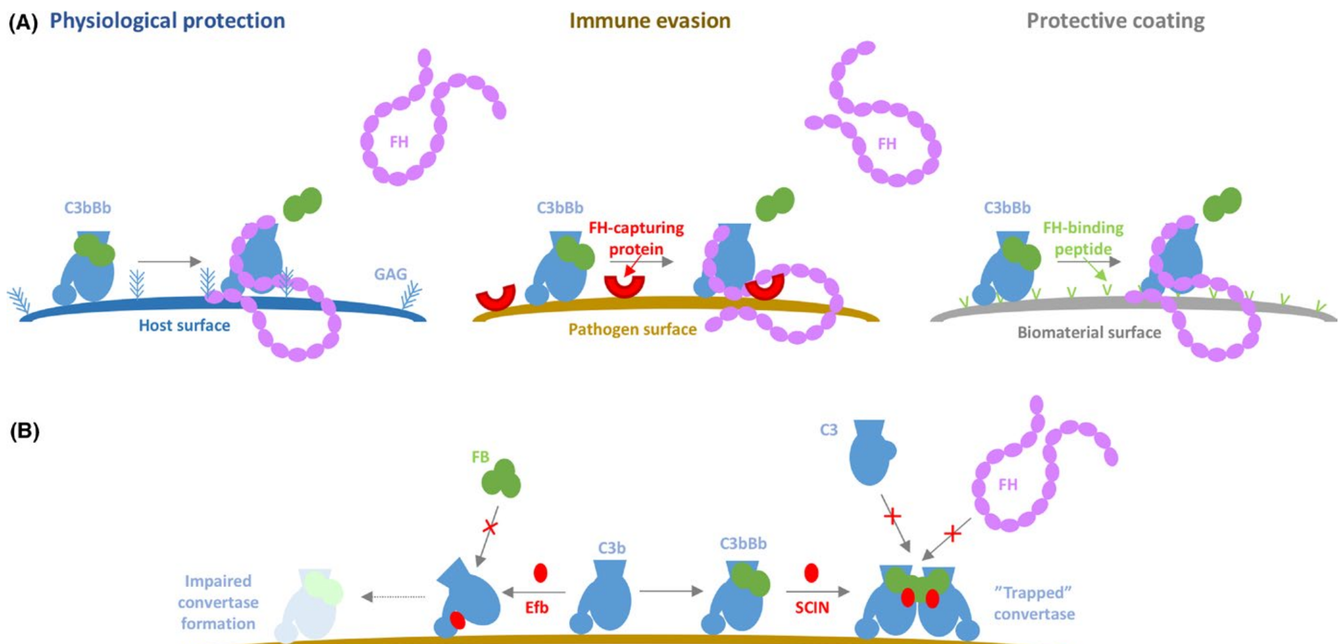

FIGURE 7 Examples of immune evasion strategies employed by human pathogens. (A) Capturing of host complement regulators to provide protection from complement attack. Factor H (FH) is able to recognize polyanionic host surface markers such as glycosaminoglycans (GAG) to direct complement regulation to self-cells (left). Although microbial surfaces typically lack such structures, many pathogens express specialized proteins that capture FH from circulation to protect their surfaces. This successful microbial evasion strategy can also be exploited for therapeutic purposes, for example by coating biomaterials with FH-binding peptides. (B) Convertase inhibition by staphylococcal evasion proteins. Bacterial proteins of the Efb and SCIN families both impair complement activity but use distinct mechanisms. Efb binds to the TED and induces a conformational change in C3b that largely reduces binding of FB and generation of AP C3 convertases (i.e., C3bBb). SCIN, on the other hand, stabilizes the assembled AP C3 convertase in an inactive (dimeric) form that prevents binding of the C3 substrate and renders the convertase unresponsive to decay acceleration

N. meningitidis expresses an FH-binding lipoprotein (fHbp) that mimics the polyanionic carbohydrates on host surfaces and potently and species-specifically binds human FH.[125] Indeed, subvariants of fHbp are part of the meningococcal vaccines Bexsero (GSK) and Trumenba (Pfizer).[126] Recently, the pneumococcal protein PspC was shown to bind FH in a manner that induces a more active conformational state of the regulator than might be otherwise achieved on GAG-covered host surfaces.[127] Malaria parasites appear to engage complement in various ways during their life cycle,[128] and sexual stages of *P. falciparum* have been reported to recruit FH and FHL-1 via the surface protein Gp50.[129] In all these and many other cases, pathogens exploit the physiological host's complement regulators to control convertase activity and degrade C3b. Of note, this successful strategy of non-self protection may be translated into therapeutic approaches (Figure 7A); for example, our group has developed a FH-binding peptide that can be coated on biomaterial and cell surfaces, recruits FH and thereby protects the coated material from complement attack via the AP.[130,131]

Orthopox viruses have found a distinct approach for harvesting the regulatory power of RCA: rather than recruiting host regulators, they express RCA mimics. Despite being much smaller than FH, some of these mimics combine strong DAA and/or CA activities with surface-binding capacity in just four domains.[132] Examples include the smallpox inhibitor of complement enzymes (SPICE) and the vaccinia virus complement control protein (VCP); even though these proteins only differ in 11 aa, their complement-inhibitory activity can vary by several orders of magnitude.[133] In a recent structural analysis, we have shown that SPICE indeed exhibits the same general binding mode for C3b as does human RCA, most closely resembling the binding of CD46 and FH.[29] Viral RCA mimics are not unique to poxviruses, though, and are also expressed by certain herpes viruses (e.g. KAPOSICA from the Kaposi's sarcoma-associated herpesvirus).[132] In addition, some herpes viruses also utilize non-RCA-based complement inhibitors.[62,132] For example, glycoprotein C from herpes simplex type 1 (gC-1) has been shown to bind to C3c-containing forms of C3 and impair the binding of properdin and C5, thereby blocking AP-mediated hemolysis.[134] Of note, gC-1 is among the very few ligands with activity toward native C3, even though its main inhibitory activity appears to be exerted at the level of C3b.

Potentially, the most versatile arsenal of complement-modulating strategies, many of which target C3 activation, has thus far been reported for the major human pathogen *Staphylococcus aureus*.[121,123] Although mechanisms that engage or enhance RCA binding have also been described for *S. aureus*,[135–137] two unique protein families act through direct interactions with C3b. Members of both the extracellular fibrinogen-binding protein (Efb) and staphylococcal complement inhibitor (SCIN) family have been shown to impair complement activation by interfering with the AP C3 convertase (Figure 7B). Intriguingly, especially considering the structural similarity between the two families, they employ highly distinct mechanisms to achieve this goal.[28,138] The C-terminal domain of Efb (i.e. Efb-C) strongly binds to TED-containing forms of C3, including native C3, C3(H_2O), C3b, iC3b, and C3dg.[139,140] Despite being the most likely target for a C3-directed complement inhibitor, C3b was found to be the weakest binder in an initial interaction profiling. This result was attributed to the fact that, according to available crystal structures, the Efb binding site is not readily accessible in C3b.[140] It was subsequently revealed that C3b-bound Efb-C acts like a wedge to stabilize a conformation of C3b in which the TED is dislocated from the position that it assumes in all the available crystal structures.[135] Propagation of this distortion along the CUB domain into the shoulder region of C3b allosterically impairs the binding of FB to C3b, thereby largely reducing the formation of the AP C3 convertase (Figure 7B).[135] In addition, Efb-C has been reported to interfere with B-cell activation, based on its ability to block the interaction between C3dg and CR2/CD21, which is part of the B-cell co-receptor complex.[141] SCIN proteins, on the other hand, have been shown to directly and unrestrictedly interact with the shoulder region of C3b.[93,103] Binding to this functional hotspot on C3b distinctively affects the interaction with FB and FH. Although SCIN partially impairs the binding of intact FB to C3b, the main inhibitory effect is achieved via its ability to potently intercalate with C3bBb in the assembled convertase. As a consequence, SCIN stabilizes the AP C3 convertase and traps it in an inactive state that prevents further cleavage of C3 (Figure 7B).[93,103] As mentioned above, this stabilizing effect was successfully utilized to enable the crystallization of the otherwise unstable C3 convertase.[93] The resulting SCIN-stabilized convertase is essentially resistant to decay acceleration by FH.[103] Furthermore, SCIN-induced dimerization masks functional sites in C3b, including those involved in signaling via CR1 and CRIg.[142]

Although most organisms employ C3 for defensive purposes, or try to evade its antimicrobial activity, at least one family of predatory animals has learned to shape the potent effector function of C3 into a tool for stunning its prey. CVF, found in the snake venom of certain cobra species, shares profound sequence homology with C3b/C3c; although the three-chain protein is devoid of a TED, it still contains a CUB domain.[143] In plasma, CVF is able to assemble AP C3 convertases with human FB and FD that are kinetically much more stable than C3bBb (half-life of 7 hours compared to approximately 3 minutes) and virtually resistant to RCA-mediated regulation. As a consequence, CVF leads to a depletion of C3 stores with rapid release of anaphylatoxins, which together increase the vascular permeability and facilitate distribution of the venom in the prey. As mentioned above, CVF has been used to study the formation of the pro-convertase; in addition, it has been utilized in animal models to transiently deplete C3, and a humanized form has been considered for therapeutic purposes.[143] A C3-like protein is also found in the venom of spiders belonging to the *Loxosceles* species, which includes the brown and the Chilean recluse spiders (*L. reclusa* and *L. laeta*, respectively). Whether these proteins constitute part of the spiders' complement system or rather contribute to the venom activity, by analogy to CVF, remains to be elucidated. Interestingly, however, the major component of *Loxosceles* spider venom is sphingomyelinases, which have previously been shown to increase complement activation.[144]

5 | TOO MUCH, TOO LITTLE: DISTURBED C3 BALANCE AND ITS CLINICAL CONSEQUENCES

In view of the fundamental role of C3 in numerous biological processes that are vital for the proper functioning of our bodies, it is predictable that the disruption of C3 function as a result of gene mutation, leading to protein deficiency or abnormal activity, would result in pathological conditions.[4,108,145] Indeed, complete deficiency of C3 has been associated with increased susceptibility to bacterial infections in early childhood. Meningitis and respiratory tract infections are the most prominent and include otitis, pneumonia, tonsillitis, and sinusitis, mainly caused by *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, *S. aureus*, and *N. meningitidis*. In addition to infections, renal and lupus-like diseases have been reported in 25% and 15% of C3-deficient patients, respectively.[146,147] To date, 40 cases of complete deficiency of C3 have been described worldwide, of which 21 have had the clinical diagnosis validated by molecular analysis.[146–149] Notably, the onset of symptoms starts at about 2 years of age, and by the time of the case reports, 29% of the patients have had at least one sibling die at a very young age (less than 2 years old), indicating a critical role for C3 in childhood, when the adaptive immune system and antibody responses are not fully developed.[150] Interestingly, a recent report describing 41 French patients who were diagnosed with distinct complement deficiencies during adulthood did not include any C3-deficient patients.[151] Together, these numbers raise the question as to whether C3 deficiency is relatively undiagnosed in infants who die at a very young age, especially in locations where the likelihood of encountering a microbial challenge is substantial. Unfortunately, only a single report provides information on the health condition of a patient several years after the diagnosis, stating that the clinical consequences of the C3 deficiency diminished as the patient aged, with milder and occasional infections in the period from 18 to 34 years of age.[152] Awareness of the health status of C3-deficient individuals after reaching adulthood would be of great value to us in understanding the relative contribution of C3 to immunity during aging.

In line with the concept that C3dg acts as adjuvant in the activation of B cells and antibody production,[119] differential ability in mounting antibody responses toward specific antigens after vaccination of C3-deficient patients has previously been reported.[153] Furthermore, extremely low levels of total IgG4 have been determined in some of the patients.[153–156] In addition, recent reports have described impaired T- and B-cell responses ex vivo when lymphocytes from C3-deficient patients are used.[149,154,157] Because information about these patients is scant after the initial report describing the deficiency, it is unclear how these observations relate to in vivo situations. Decreased plasma levels of C3 are also found in patients with deficiencies or functional problems in the complement regulators FI or FH and in individuals presenting with auto-antibodies against the C3 convertase of the AP, also known as C3 nephritic factor (C3-Nef). In these circumstances, complement is constantly activated, and C3 is rapidly consumed from the circulation, resulting in a secondary deficiency of C3. Although FI or FH deficiency is generally associated with increased susceptibility to infections, the presence of C3-Nef or functional defects in FH are mainly correlated with kidney diseases such as C3 glomerulopathy (C3G) and atypical hemolytic uremic syndrome (aHUS), reflecting the pivotal role of FH in the regulation of complement activation.[146,158–160] Evidence acquired from a healthy individual presenting with an abnormally low concentration of C3 in circulation, as a result of having one functional and one null C3 allele, indicates that C3 concentrations of 0.18 mg/mL (approximately 18% of normal C3 levels) are sufficient to maintain a proper activation of complement response and avoid complement-related diseases.[148]

Now that the development of complement therapeutics is in vogue, with numerous compounds in or close to the clinical phase for various diseases,[145,161,162] it is worth considering the biological impact of the therapeutic inhibition of C3. Such an approach commonly raises concerns over infections in the treated individuals. Although this is a legitimate consideration, and patients undergoing C3 inhibition therapy will likely need to be immunized against meningitis and potentially other bacterial diseases, there is no evidence pointing to a vital role for C3 in adults who have a fully developed adaptive immunity. In fact, as mentioned above, aging appears to be inversely correlated with infections in a C3-deficient patient,[152] and our unpublished data showed an absence of infection in adult non-human primates (n=10) in which C3 was completely inhibited for a month by means of the C3 inhibitor Cp40 (AMY-101; see below).[163] Clearly, only clinical experience will elucidate the real implications of C3 inhibition; however, it is noteworthy that pharmacologic inhibition of C3 is clearly different from C3 deficiency, as C3 is constantly produced by the liver and other tissues even during C3 inhibition, and activity may be quickly regained once the therapy ends. In many conditions, the degree of C3 inhibition required to alleviate disease symptoms is unknown. Once clinical experience is obtained, an ideal therapeutic scenario might be accomplished in which C3 inhibitors can be titrated in accordance with disease requirements, allowing minimal amounts of free C3 to remain available to react in the event of a microbial challenge.

In addition to C3 deficiency, variants of the C3 protein produced by gene mutations that affect its structure and/or binding properties have been correlated with pathologic conditions. A C3 polymorphism resulting from a single amino acid substitution (p.Arg^{80}Gly), first described four decades ago, defines two C3 variants known as S (slow) and F (fast), based on their differential mobility on high-voltage agarose gel electrophoresis. The allelic frequency of C3F markedly varies among races, being prevalent in Caucasian populations. Although the molecular consequences of this polymorphism are still being explored, the C3F variant appears to be associated with an unfavorable outcome in several diseases, including IgA nephropathy, partial lipodystrophy, and systemic vasculitis.[164] Meanwhile, numerous distinct C3 variants have been correlated with certain pathologic conditions, particularly with age-related macular degeneration (AMD), aHUS, and C3G.[165–173] Complement dysregulation is a common feature in these diseases, producing a situation featuring chronic inflammation and tissue damage.[4] Notably, the exact involvement of complement can differ between diseases and even between patients. In this context, functional evaluation of protein variants within the AP has shown that

a specific repertoire of variants (or “complotype”) likely determines the complement activation profile and disease progression in each individual.[174,175] As such, inheritance of more active variants of C3 or FB or less active variants of the regulators FH, FI, or MCP favors the activation of the AP and inflammation, while the opposite tips the balance toward a less inflammatory phenotype.

Genetic variants of complement genes, including C3, strongly contribute to the risk of AMD, a progressive degenerative disease of the retina and a major cause of blindness in the elderly.[176] Although several aspects of the pathological mechanism of the disease remain unclear, extracellular deposits located in the retina (termed drusen) have been shown to contain C3 and other complement activation products, indicating the presence of local, complement-mediated inflammation.[177] C3 variants also dictate the risk of kidney diseases such as aHUS and C3G. In aHUS, characterized by microangiopathic hemolytic anemia, thrombocytopenia, and thrombosis, dysregulated complement activation leads to local inflammation in the renal endothelium, with consequent tissue injury.[178] In contrast, in C3G, a group of renal pathologies characterized by detection of C3 in the glomeruli and minimal or no deposition of immunoglobulins, complement activation is poorly regulated in the fluid phase; the result is systemic consumption of C3 with an excessive generation of activation fragments that deposit in kidney tissue (which is poor in complement regulators), culminating in inflammatory lesions in the glomeruli and renal fibrosis.[179] In such diseases, the outcome is determined by how the mutation affects the regulation of C3b or the stability of the AP C3 convertase. The majority of C3 mutations affect the interactions with the regulators FH, MCP, CR1, and also FB. In these patients, the dissociation of the C3 convertase is inefficient, and C3b has a reduced rate of conversion to iC3b, leading to increased complement deposition and tissue damage.[167,180–182]

In addition to the diseases described above, C3 is an acute phase protein and can be involved in virtually any inflammatory condition. Increased levels of C3 and/or C3 activation fragments in the circulation are observed in numerous conditions, including neurological and cardiovascular diseases, obesity, asthma, cancer, periodontitis, and transplant rejection.[4,145,183–189] Excessive complement activation contributes to the disease pathogenesis by fueling the inflammatory process and inducing tissue damage, highlighting the profound importance of the tight regulation of complement activation in the maintenance of homeostasis.[4,108,145]

6 | THERAPEUTIC CONTROL OF C3 ACTIVATION

Given the growing recognition of complement’s involvement in various disease processes and the technological advances made in recent decades, the complement system has moved into the spotlight of pharmaceutical and biotechnological companies. Because of its early and widespread involvement in danger sensing and inflammatory processes, complement is now considered an attractive therapeutic target for a broad spectrum of immune, thrombo-inflammatory, and age-related diseases, as well as in transplantation medicine and beyond.[4,108,162] With two compounds in the clinic, several candidates in clinical trials, and numerous promising concepts in preclinical development, complement-targeted drug discovery has become a thriving field, and we refer to specialized reviews for an overview of past and recent advances.[161, 145,162,190–191]

The central role of C3 in complement activation, amplification, and effector generation naturally renders this protein an interesting target for therapeutic intervention. So far, however, the complement-inhibiting drugs available in the clinic, i.e. the anti-C5 antibody eculizumab (Soliris, Alexion) and various preparation of C1-INH (e.g. Cinryze, Shire) target peripheral steps such as initiation or effector generation. Fortunately, several candidate drugs acting at the level of C3 activation are currently in development; they either act on C3 itself or on the C3 convertase (Figure 8).[190,192–194]

With its high plasma concentration (approximately 5 μM) and metabolic turnover, native C3 has traditionally been considered a challenging target for therapeutic intervention. Moreover, and in contrast to “druggable” targets in the classical sense such as serine proteases, kinases, or GPCRs, the functional activity of C3 primarily relies on conformational changes and protein-protein interactions that are not easy to impair. However, largely thanks to the unprecedented biochemical and structural insights into C3 and its various functions discussed above and the tireless work of several academic and industrial groups in advancing the initial concepts, we are closer to having the first C3-targeted drugs reach clinical accessibility than ever before.[190,192,194] In addition to offering an urgently needed new avenue for treating complement-related diseases that do not adequately respond to existing options, the availability of potent molecules that inhibit distinct steps of C3 activation should further advance our knowledge of the role of C3 in health and disease.

6.1 | Taming the convertase

As the powerhouse of complement activation and amplification, the C3 convertases are an obvious promising choice to alleviate the detrimental effects of complement activation; this assertion is underscored by the fact that the majority of complement regulators, as well as many evasion proteins produced by human pathogens, interfere with the assembly, activity, or stability of the convertases (see above).[28] Importantly, these endogenous and exogenous convertase inhibitors already provide potent templates and starting points for the development of therapeutic convertase inhibitors. In the case of CP/LP C3 convertases, the clinically available C1-INH preparation (indicated for the treatment of hereditary angioedema) partially acts at this level by blocking serine proteases (i.e. C1r/C1s, MASPs) that are involved in cleaving C4 and C2, yet it also inhibits non-complement enzymes. Antibodies and molecules that specifically inhibit either C1s or members of the MASP family are therefore being developed.[161,190] The indications for such compounds are mainly focused on diseases with an established strong involvement of a distinct initiation pathway, as is the case for the CP in autoimmune hemolytic anemia for instance. For indications with a more complex initiation profile or predominant

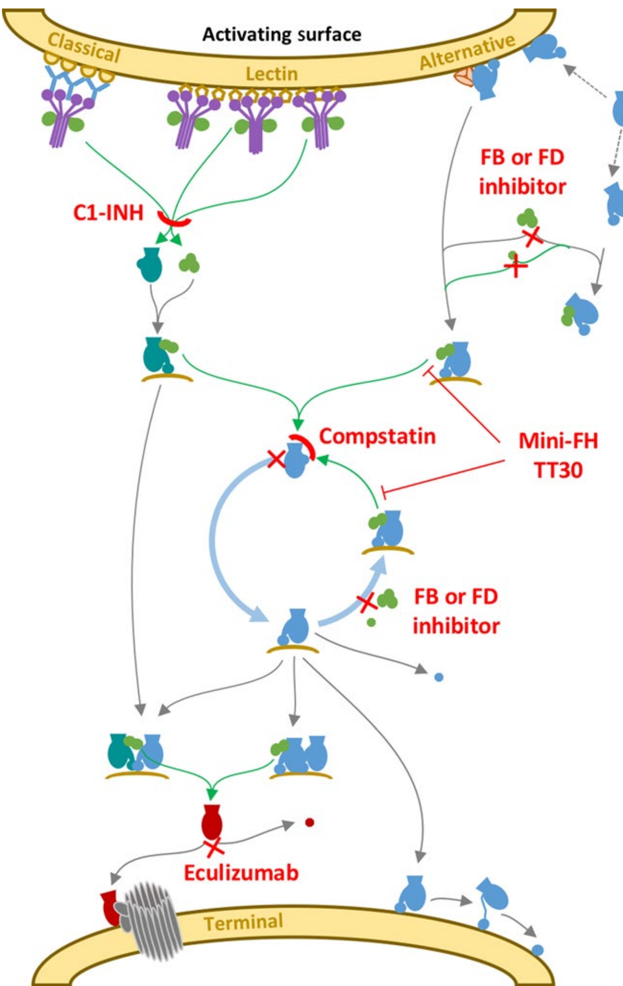

FIGURE 8 Therapeutic intervention strategies at the level of C3. Inhibition of complement activation at the level of C3 is primarily achieved through three major routes. Although FB and FD inhibitors (small molecules or antibodies) prevent the formation of the AP C3 convertase, engineered versions of complement regulators such as mini-FH or TT30 block convertases by accelerating their decay and enabling the proteolytic degradation of C3b by factor I; both approaches are specific for the AP. C3 inhibitors of the compstatin family (e.g. Cp40) bind to C3 and protect the substrate from being activated by any of the C3 convertases. Complement-targeted drugs available in the clinic, i.e. the anti-C5 mAb eculizumab and C1-inhibitor preparations, which all act at peripheral steps of the cascade, are also shown in the scheme

exacerbation via the amplification loop, targeting the AP C3 convertase might prove more rewarding.

As in the case of the above-mentioned approach for the CP/LP, inhibiting the serine proteases that drive the formation of the active convertase is a suitable approach.[190,192,194] Currently, the main focus appears to be on FD, which is the partner with the lowest abundance and which represents a bottleneck in convertase formation. For example, Genentech has developed an antibody fragment (lampalizumab) that binds to the exosite of FD and prevents its binding to the pro-convertase[195]; in phase II clinical trials, intravitreal injection of lampalizumab has shown positive effects in a subset of patients suffering from a dry form of AMD (geographic atrophy), and Genentech has recently initiated phase III trials. Although lampalizumab appears to best suited for local application in the eye, at least two companies are working on small-molecule FD inhibitors that may be administered orally for systemic applications such as in PNH. Achillion recently started phase I trials with their lead compound ACH-4471, and a FD inhibitor from Novartis appears to be in preclinical development. Of note, Novartis is also working on small-molecule inhibitors of FB. It will be interesting to follow the clinical development of these low molecular weight inhibitors and learn more about their pharmacokinetic and pharmacodynamic behavior under clinical conditions. Other approaches to influence convertase assembly and stability are the use of antibodies against C3b, typically binding at the shoulder region and preventing binding of FB, or against properdin.[161,190,192]

Even though the utilization of the natural convertase-regulating power of RCA proteins has long been considered, the direct use of physiological inhibitors such as FH or soluble CR1 has proved to be challenging, partly because of their comparatively large size (150 and 240 kDa, respectively). More recently, engineered smaller versions of human RCAs have entered the spotlight and shown some interesting benefits concerning tissue/cell targeting and efficacy. TT30, a fusion protein combining CCP domains 1-5 of FH with CCP1-4 of CR2, has played a pioneering role in this approach.[196] Although the FH part provides full AP regulatory activity, the CR2 segment mediates binding to iC3b and C3dg, thereby directing the regulator toward cells that are under complement attack. TT30 and related proteins have shown promising effects in various disease models, including PNH and ischemia-reperfusion injury[197,198]; Alexion has evaluated TT30 in a phase I trial but has not announced further development plans.

Another approach to reducing the size of FH while conferring enhanced targeting properties has been taken by our group and others.[199–201] Analysis of the crystal structures of C3b-FH[1-4] and C3d-FH[19-20] have shown that the two functionally important termini are situated in close proximity and can be bridged to produce a mini-FH protein with 6 instead of 20 domains.[199] Interestingly, the recombinant construct not only maintains binding affinity for C3b and GAG but also shows higher activity in AP inhibition models than does FH. This mini-FH achieves stronger binding to the late-stage opsonins iC3b/C3dg, likely because of the better accessibility of the C3d binding site in FH[19-20] after the removal of the middle segment and because the connectivity between the domain areas plays an important role.[202] As a consequence, mini-FH analogs are ideally targeted toward host surfaces under complement distress. Despite missing the surface-recognition part of FH, the splice variant FHL-1 may also be considered as a therapeutic option (e.g. for local applications) because of its small size and presumably improved tissue penetrance.[203] More recently, CRIg and the LP regulator MAP-1 have been explored as means of directing regulators to sites of ongoing complement activity.[204,205] The modular organization of most complement regulators has certainly opened fascinating possibilities for inhibitor design.

6.2 | Protecting the substrate: Direct inhibition of C3 by compstatin analogs

Convertase inhibitors can exhibit powerful activity but are typically pathway-specific and prevent C3 activation via either the CP, LP, or AP. In some situations, however, total blockade of C3-mediated opsonization and effector generation is desired. Such an effect can be achieved by peptides of the compstatin family, which bind to a functional hotspot on the C3 substrate and prevent its activation by any of the C3 convertases.[206,207] Originally discovered by our group in 1996 during phage-display peptide library screening,[208] the compstatin family has since grown to include several distinct analogs with unique properties, two of which are currently in clinical development.[190,206]

The phage display-derived compstatin was identified as a cyclic peptide of 13 amino acids that binds to human C3 (and C3c-containing fragments) and potently inhibits both AP and CP/LP-induced C3b deposition.[208] Importantly, compstatin showed a narrow species specificity toward C3 from humans and non-human primates.[209] From a molecular perspective, the cyclic nature of the peptide was found to be essential, and several key residues were identified.[207] Based on this information, and in the absence of structural data, the first rounds of optimization using natural amino acids resulted in an analog (4W9A) that was acetylated at the N-terminus and included two amino acid substitutions (V4W and H9A), thereby gaining a 13-fold increase in affinity.[207,210] The use of non-natural amino acids, and in particular use of an indol N-methylated variant of Trp in position 4, improved the activity gain to more than 260-fold over the original compstatin.[207,211,212] Early on, some of these compstatin analogs showed promising efficacy data, for example in ex vivo models of kidney transplantation or biomaterial-induced complement activation.[213–215] The lead analog at that point (2006) was licensed to Potentia Pharmaceuticals; this analog performed successfully in phase I trials in AMD but did not reach the desired endpoints after administration of much lower doses in subsequent phase II trials. Meanwhile, the technology was transferred to Apellis Pharmaceuticals; although the unmodified compound (APL-1) is being considered for use in COPD, a PEGylated derivative (APL-2) has been clinically developed for local administration in AMD and for systemic application in PNH.[161,190]

The lack of a crystal structure made it challenging to obtain a rational optimization of this promising lead compound. In addition, its molecular mechanism of action has long been unclear, as biochemical analyses have localized the compstatin binding area to a part of the β-chain that was considered distant from both the C3a cleavage site and the FB binding region.[216] These gaps were filled in 2007 with the publication of a crystal structure of C3c in complex with the compstatin analog 4W9A.[217] The structure revealed that compstatin binds to a shallow groove at the interface between MG4 and MG5 on the outside of the key ring of C3. An early hypothesis that this binding might interfere with the interaction of the C3 substrate and the convertase was subsequently confirmed by the structure of the SCIN-stabilized C3bBb complex (see above).[93,217] Superposition of these structures indeed showed that the compstatin binding site is located on the C3/C3b dimerization interface, which is presumed to be essential to allowing the binding and activation of C3. Although the binding of a second compstatin molecule to C3b of the convertase complex can enhance this effect in the case of the AP, the potent inhibition of CP/LP-mediated complement activation suggests that blockage of the substrate is sufficient to achieve the inhibitory effect.

Binding site analysis and the use of tools from peptide and medicinal chemistry, including backbone N-methylation, have enabled the generation of next-generation analogs with a marked improvement in C3 binding affinity and inhibitory activity.[163,206,211,218] The lead analog, Cp40, was the first analog with subnanomolar binding affinity for C3 (K_D=0.5 nM), marking an approximately 5000-fold improvement over the initial compstatin.[163] Structural analysis showed that N-methylation of Gly induces a bound-like conformation of Cp40 in solution, which may improve target binding. Moreover, the addition of a D-Tyr at the N-terminus extends the binding site and forms additional contacts with C3.[163] Despite the strong activity gain, Cp40 maintains its species specificity for human/primate C3. The tight target binding and the high plasma concentration of C3 also contribute to the unique pharmacokinetic properties of Cp40, which remains in the circulation much longer than do typical peptide drugs.[163,206] Studies in NHP have demonstrated that target-saturating drug concentrations can be maintained with repetitive subcutaneous injections of as little as 1 mg/kg Cp40 and administration intervals of 12-24 hours, or even less frequent intervals [(163, 206) and Ricklin & Lambris, unpublished observations].

Next-generation compstatin analogs, and in particular Cp40, have been used in a variety of disease models and have shown promising preclinical efficacy in PNH,[219] hemodialysis-induced complement activation,[220] C3G,[221] sepsis-associated organ damage,[222,223] xenotransplantation,[224–226] hemorrhagic shock,[227] malarial anemia,[228] and periodontal disease,[185,229,230] among others. The last model is especially intriguing because periodontal disease is primarily caused by a microbial pathogen (i.e. *Porphyromonas gingivalis*), making complement inhibitors a rather counterintuitive choice. However, it has been shown that *P. gingivalis* actively induces an inflammatory milieu to gain a competitive advantage, and in the process causing dysbiosis, inflammation, and bone loss; the anti-inflammatory effect of complement inhibition by Cp40 re-shifts the balance and markedly improves both immunological and clinical parameters.[229,230] This examples nicely illustrates that, as discussed above, complement inhibition needs to be considered in the specific context involved; in some indications, taming the deleterious effects of complement-mediated inflammation may be more important than controlling infection. Cp40 was licensed by Amyndas Pharmaceuticals and is currently being developed for the treatment of PNH, ABO-incompatible kidney transplantation, C3G, and periodontal disease.

6.3 | Moving ahead toward C3 inhibitors in the clinic

Although the development of C3-level complement inhibitors with clinical potential took substantially longer than expected, several highly promising candidates are now waiting in the wings. The overcoming of technical hurdles played an important role in

this advancement. As demonstrated by Cp40, feasible treatment schemes for maintaining target-saturating levels may even be achieved for abundant targets such as C3. Although pharmacokinetic parameters may be even further improved by chemical modifications, for example by employing PEGylation or albumin-binding moieties,[219,231] the cost-benefit ratio needs to be carefully considered in each case. Another game-changer in the development of therapeutic C3-targeted drugs is the regained trust in complement inhibition in general, including the blockage of C3.[4,190,192] The clinical experience has been largely positive and, in the case of eculizumab, the potential risk of infection by meningococci can be addressed by vaccination. For C3-targeted inhibitors, such anti-infective strategies may need to be expanded to other encapsulated bacteria such as pneumococci. Importantly, the risk of C3 inhibition needs to be considered in the right context, as local, systemic, acute, and chronic treatment present highly distinct situations. The clinical trials that are being conducted at this point and in the near future will be critical for advancing our current mainly hypothetical questions about the benefits and risks of blocking C3 activation in humans to evidence-based discussions.

7 | CONCLUSION

Along with the new perception of complement as a versatile modulator in health and disease, our knowledge about its central component C3 has taken profound strides forward in recent years. Although genetic studies and animal models have revealed ever new roles for complement in physiological processes that reach far beyond the clearance of intruding pathogens, the use of refined biochemical and structural tools has provided an unprecedented insight into the fascinating mechanisms that make C3 the most multifunctional and transformable immune mediator of our already elaborate defense machinery. This impressive body of experimental data helps us grasp the molecular basis for complement's metamorphosis from an omnipresent yet non-reactive sentinel to a powerful effector and mediator. In true analogy to a Swiss Army knife, the exposure of new sites and tools in a context-specific manner enables C3 and its fragments to induce and/or amplify the complement response, mark particles for elimination, aid their transport and clearance, and contribute to subsequent cellular and adaptive immune reactions. However, this central position also renders C3 vulnerable to involvement in disease processes triggered by genetic alterations or inappropriate activation on host surfaces, as well as to microbial immune evasion. Fortunately, several promising concepts have emerged in recent years to tame the power of C3 in a therapeutic context, and the prospect of soon gaining clinical experience with drugs targeting at the level of C3 is encouraging and exciting. Such clinical insight, together with continuous progress in genetic and molecular studies, will provide an important driving force for further improving our understanding of C3 in host defense, innate immunity, and beyond. It would not be surprising if this amazing protein still had more unexpected functions in its repertoire.

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CONFLICT OF INTEREST STATEMENT

D. R. and J. D. L. are co-inventors of patents and/or patent applications describing complement inhibitors and their clinical use. J. D. L. is the founder of Amyndas Pharmaceuticals.

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