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CROSS-DISCIPLINARY RESEARCH STIRS NEW CHALLENGES INTO THE STUDY OF THE STRUCTURE, FUNCTION AND SYSTEMS BIOLOGY OF COMPLEMENT

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1. INTRODUCTION

Complement is a pivotal effector arm of the innate immune response that participates in various immunoregulatory circuits via a complex network of protein–protein interactions[1]. The complement cascade is a dynamic network of interactions involving a wide array of soluble glycoproteins, membrane-bound receptors, and fluid-phase or membrane-anchored regulatory proteins[2]. Upon complement activation, a well-orchestrated sequence of protein–protein interactions is initiated that results in proteolytic cleavage of precursor molecules, release of bioactive peptides, and downstream activation of receptors that relay the appropriate signals to the intracellular molecular circuit of complement-targeted cells.

In recent years complement pathobiology has been reiterated with the advent of proteomics and functional genomics, the use of high-throughput analytical approaches, transgenic mouse models, and the exponential growth of research data that implicate several components in processes that go beyond the classical immunologic milieu[3]. Complement components appear to modulate critical developmental processes by intercepting molecular circuits that control the cell cycle, cell migration and proliferation, and the homing of myeloid progenitors into tissues[4].

Furthermore, the need to contain the detrimental proinflammatory effects of complement activation, without eliminating its beneficial properties in host im-

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mune homeostasis, has led researchers to adopt multidisciplinary and high-throughput approaches in a systematic effort to develop rational drug-design platforms and more potent complement-based antiinflammatory therapeutics that might be amenable to clinical protocols. In this respect, emphasis has been placed on the elucidation of key structural elements that govern the dynamics and energetics of protein interactions within the complement system[5]. The integrated use of fine biophysical and in silico approaches in monitoring distinct conformational changes of complement proteins has thus far yielded promising results. This crossdisciplinary approach to complement research highlights the importance of integrating the core structure and dynamics of a biochemical reaction in the context of its pathophysiologic consequences.

Overall, the "systems-wide" impact of complement is supported by evidence that complement-mediated pathways engage in functional "crosstalk" with other biological systems. Complement proteins appear to modulate key developmental and homeostatic processes, both in the course of inflammation and in noninflammatory settings. Here we outline this novel conceptual framework for the study of complement structure and function and integrate it into a wider pathophysiological perspective with examples from health and disease. We present a comprehensive account of how an integrated "systems" approach has contributed to elucidation of the structural–functional aspects of C3–ligand interactions and the rational design of small-size complement inhibitors. We outline the enormous capabilities offered by the integrated study of thermodynamics in protein binding and the bioenergetics of complement protein–protein interactions and consider new conceptual "avenues" that can be explored in elucidating key structural elements of complement function. We also present critical aspects of our studies on viral molecular mimicry and immune evasion and highlight the main mechanistic attributes of the "crosstalk" between complement and various biologic processes.

It is our conviction that complement research will be spearheaded in the next decade by such combinatorial and crossdisciplinary approaches that will address basic biological networks modulated by complement in a global and integrated manner. Furthermore, the mining of biomolecular and textual databases will essentially complement these experimental strategies and enable scientists to form the integrative context for hypothesis-driven scientific discovery.

2. BIOPHYSICAL APPROACHES IN ELUCIDATING COMPLEMENT STRUCTURE AND BINDING ENERGETICS

Cell regulatory networks are the key components of a unified biological system and are defined at the molecular level by the numerous biomolecular interactions that tilt the binding equilibriums and decide the fate of a cellular response or the elicited phenotype upon specific stimulation[6]. Defining the structural elements that underlie the various stages of a binding reaction between interacting

proteins is integral to understanding a cellular response and also for devising means of intercepting, silencing, or enhancing its effect to the benefit of the host. The resolution of the fine structure of proteins by means of x-ray crystallography has assisted scientists to a great extent in defining such structural modules that regulate binding reactions. However, crystallographic data only refer to a static "snapshot" of a given interaction or conformation and fail to consider the complex and dynamic behaviour of the interacting partners in a protein–protein association. In an effort to circumvent this inherent drawback and shed light onto the dynamic nature of complement-mediated interactions, novel biophysical approaches are being adopted that allow the monitoring of the binding dynamics between various complement proteins and receptors[7].

Such approaches also take into account a wide array of interactions that contribute to the formation of binding interfaces, including hydrophobic interactions among non-polar side chains, hydrogen bonding interactions, electrostatic interactions, and van der Waals interactions. Furthermore, these approaches also consider the electrostatic nature and shape constraints of the interacting partners within a complex, two parameters that dictate to a great extent the mechanism by which the optimum and more stable configuration is selected for recognition and binding[7].

In this respect, recent studies have yielded important information regarding the dynamics that govern complex interactions between various complement components, using a crossdisciplinary platform that integrates biochemical, physicochemical, and computational methods. Defining the binding interface and interacting structural elements of C3d and its receptor CR2 has been a major challenge in this direction. The application of electrostatic potential calculations has essentially complemented the available crystallographic[8–10] and site-directed mutagenesis data[11] and has indicated that the dynamics of the C3d–CR2 interaction is strongly dependent on the force of electrostatic fields applied between the two interacting molecules. Indeed, the analysis of the electrostatic potential of each protein in free form and in complex with each other has revealed that this interaction follows a two-step association model comprising distinct stages of recognition and binding[5]. The design of theoretical site-specific mutations within the C3d moiety further supports this two-step association model[5]. It is anticipated that such integrative approaches combining available crystallographic data, biochemical approaches, and biophysical calculations will shed more light on the complex C3d–CR2 association and provide a comprehensive platform for the development of effective complement therapeutics.

3. THERMODYNAMICS OF COMPLEMENT PROTEIN BINDING

Distinct thermodynamic changes occur during a binding reaction, and the monitoring of such changes allows for a dynamic study of protein–protein interactions. Isothermal Titration Calorimetry (ITC) is a method that allows the longi-

tudinal study of the thermodynamic changes that occur during protein complex formation[12]. It is essentially used for calculating the heat that is released in a biochemical reaction as a function of time and yields information on the stoichiometry, enthalpy, association constant, and free energy of binding. A distinct feature of ITC is that it can discriminate between entopy and enthalpy changes, thereby providing information on distinct chemical and structural (conformational) changes[13] that contribute to protein binding. ITC has recently been applied for the study of energetics of the interaction of C3 with its inhibitor, compstatin[14]. Thermodynamic measurements have indicated that the binding of compstatin to C3 is 1:1 and occurs through hydrophobic interactions with possible conformational changes in C3 or compstatin. Some protonation changes, occurring at the binding interface, have also been observed by ITC analysis[14]. analysis will be extended to the energetics of various protein–protein interactions, with a goal to obtain the energetic parameters of complement activation and regulation pathways.

4. PROBING CONFORMATIONAL CHANGES OF COMPLEMENT PROTEINS WITH HYDROGEN/DEUTERIUM EXCHANGE AND MASS SPECTROMETRY

Hydrogen/deuterium exchange has traditionally been used to understand the formation of protein core or stable intermediate or transient states in pathways of protein folding, because it provides a noninvasive method for identifying protected (or de-protected) exchanging amides. The same principles can be applied to studies of protein–protein association, where the loss in solvent-accessible surface area upon association can be correlated with amide protection from exchange for the amides that lose their contact with solvent. Recent advances in the use of mass spectrometry allow for rapid collection of data of free and complexed proteins[15–17]. Comparison of mass spectra of free and complexed proteins provides the sites of interaction without the need of previously available structural data. Hydrogen/deuterium exchange coupled to mass spectrometry has recently been used to probe the conformational changes of the C3 molecule in its transition from a native to a hydrolyzed state[18], and it is becoming clear that such a methodology could provide valuable insight into the structural determinants that govern the interaction of C3 with various ligands and receptors (e.g., C3d–CR2).

5. COMBINATORIAL AND IN SILICO PROTEIN DESIGN: IN SEARCH FOR MORE POTENT C3 INHIBITORS

Deregulated activation of complement on the surface of host cells and consumption of complement proteins in the fluid phase have been associated with detrimental proinflammatory effects leading to local tissue damage, perturbed ho-

meostasis and remote organ failure in several pathological states[19]. Over the years considerable effort has been devoted to the discovery of selective complement inhibitors that can intercept the complement cascade at distinct steps, thus neutralizing its deleterious effects in the progression of disease pathology[19]. Several complement inhibitors are currently under development, including small-size organic compounds, synthetic peptides, and also large monoclonal antibodies[20]. Compstatin, a potent small-size complement inhibitor that acts at the level of C3 by blocking all three pathways of complement activation, was discovered by screening a phage-displayed random peptide library for C3-binding peptides[21]. This molecule stands out as a promising complement inhibitor that might be amenable to therapeutic applications in the clinic due to its small size, cost-effective and large-scale synthesis, and its ability to shut down all three pathways of complement activation by blocking the proteolytic cleavage of native C3 by the C3 convertases.

The complement inhibitory activity of compstatin has been ascertained in various in vitro, in vivo, ex vivo, and in vivo/ex vivo interface models[22-30].

In a systematic effort to characterize the structural basis of the inhibitory activity of compstatin and design more potent analogs, a wide array of combinatorial, biophysical and in silico approaches have been used[31,40].

Determination of the solution structure of compstatin by NMR-based strategies[31] paved the way for the rational design of more potent analogs through successive rounds of sequence and structure optimization. Instrumental to the success of these optimization approaches has been the integrated use of biophysical methods and computational modeling[33-40].

In conjunction with the high-throughput screening approaches, compstatin was also subjected to in silico combinatorial design, using a novel two-step computational optimization methodology. Interestingly, this round of theoretical design yielded a sixfold more active analog than the parent peptide with sequence Ac–I[CVYQDWGAHRC]T–NH_2[41-44]. In addition to these rounds of experimental and combinatorial peptide design, a recent rational design effort was undertaken to generate analogs of compstatin with higher inhibitory activities, incorporating in its structure non-natural and D-aminoacids[45]. This approach was largely based on the hypothesis that the aromatic rings of y and w may contribute to the function of compstatin. This approach has led to identification of a more potent compstatin analog that exhibits 99-fold greater inhibitory activity and contains a non-natural aminoacid in its sequence[45]. The peptides derived from such computational and rational design approaches are now in the process of being tested experimentally, and a new generation of compstatin analogs (approx. 270-fold more active than the parent peptide with incorporation of non-natural aminoacids in the sequence) are being produced in heterologous expression systems (Katragadda et al, unpublished observations).

In conclusion, the integrated use of rational experimental and computational (in silico) peptide design approaches has provided a unique and cross-disciplinary platform for the discovery of more effective complement therapeu-

tics targeting the C3 activation step in the complement cascade. Such integrated approaches should be integral to any drug design effort that involves peptide screening, synthesis, and structure manipulation.

6. DEFINING THE STRUCTURAL DETERMINANTS OF VIRAL IMMUNE EVASION: THE C3B/SPICE/VCP INTERACTION

Considerable effort has been placed in the field of antiviral vaccine design toward elucidating the mechanism by which certain herpes and orthopox viruses escape the host immune response, through structural and functional mimicry of complement regulatory proteins[46]. SPICE and VCP are two secreted viral homologs of complement regulatory proteins that bear CCP modules and mediate immune evasion in the host by interacting with C3b and preventing complement-mediated neutralization of virus[47,48]. Strikingly, despite the fact that it is 1000-fold more potent than VCP in deactivating human C3b, SPICE differs from VCP in only 11 aminoacid residues[48,49]. The generation of VCP–SPICE chimeras consisting of VCP and SPICE CCP modules has recently led to identification of the critical aminoacids that render SPICE a more potent inhibitor of complement[49].

Furthermore, electrostatic potential calculations using these chimeric proteins in interaction with human C3b have revealed an essential role of electrostatic forces in driving the VCP/C3b interaction. Electrostatic modeling has suggested a two-step association model for VCP/C3b that involves electrostatically driven recognition and enhanced binding. These studies revealed that a predominantly negative C3b and a predominantly positive VCP variant favor their electrostatically driven recognition and enhance their association. An increase in the positive charge of VCP variants occurs by mutations of acidic amino acids, which reduce the negative character of the electrostatic potential at the vicinity of SCR-2 and SCR-3 and enhance the positive character of the electrostatic potential at SCR-1[49]. Electrostatic modeling of the VCP/C3b interaction, in conjunction with site-directed mutagenesis studies testing the ability of different VCP/SPICE variants to inhibit complement activation, have provided an integrated framework for better understanding the structural basis and dynamics of the VCP/SPICE–C3b interaction and the molecular mechanism by which viral RCA homologs mediate immune evasion.

The important contribution of electrostatic forces to the formation of protein complexes is also highlighted in a recent study discussing the crystal structure of human C3[66]. The findings presented in this study suggest that C3 takes up in solution a tertiary conformation that presents a “dipole” surface. Such a conformation strongly supports the electrostatic nature of C3 interactions, providing invaluable insight into the biophysical parameters (such as electrostatics) that drive the interaction of C3 with its multiple physiological ligands and receptors.

It is our strong conviction that the reliable prediction and monitoring of the dynamic behavior of interacting proteins will essentially rely on an integrative platform combining both experimental and theoretical/biophysical approaches such as a survey of electrostatic forces.

7. A "SYSTEMS BIOLOGY" PERSPECTIVE OF INNATE IMMUNITY: NEWLY IDENTIFIED "CROSSTALKS" BETWEEN COMPLEMENT AND DIVERGENT BIOLOGICAL NETWORKS

Biomolecular (structural and sequence) databases have been populated with an enormous amount of data generated by means of high-throughput screening and genome-wide profiling techniques. These databases essentially contain the core information on how complex biological networks are regulated at the transcriptome and proteome levels. The challenge facing contemporary bioscience is finding the means of managing these databases in such a way as to extract gene/protein associations that can model or predict the molecular circuits by which individual cells and organisms elicit their responses to various stimuli[6]. Systems biology is the field that integrates such approaches and helps create a comprehensive context for interpreting and predicting gene and pathway associations and also generates new knowledge in a *systematic*, hypothesis-driven way[50]. Integral to the success of such a systems-wide approach is the use of new text-mining algorithms that are being developed in an effort to enable scientists to efficiently extract biological information from scientific literature databases[51]. Text mining platforms enable researchers to manage complex ontogenies and cluster biologic entities in a meaningful manner that can shed light on novel systems associations[52].

Accumulating evidence suggests that inflammatory circuits interact with divergent pathways in modulating basic biological responses that do not necessarily pertain to inflammation and the immune response per se[3]. In this respect, complement components have been linked to regulatory networks that not only modulate innate immunity but also affect developmental, metabolic, and homeostatic responses[53] .

An integrated survey of the scientifc literature using a high-throughput bioinformatics approach called "systems literature analysis" has revealed novel associations of complement with a wide array of biological processes that extend well beyond the innate and adaptive immune response[53]. Distinct associations of complement with such noninflammatory processes have also been verified experimentally. Indeed, recent studies using complement gene knockout models and highly selective complement receptor antagonists have demonstrated the involvement of complement in developmental processes, such as *limb and liver regeneration*[54,55]*, stem cell engraftment/mobilzation, and trafficking of hematopietic precursors to the bone marrow*[56]. The main attributes

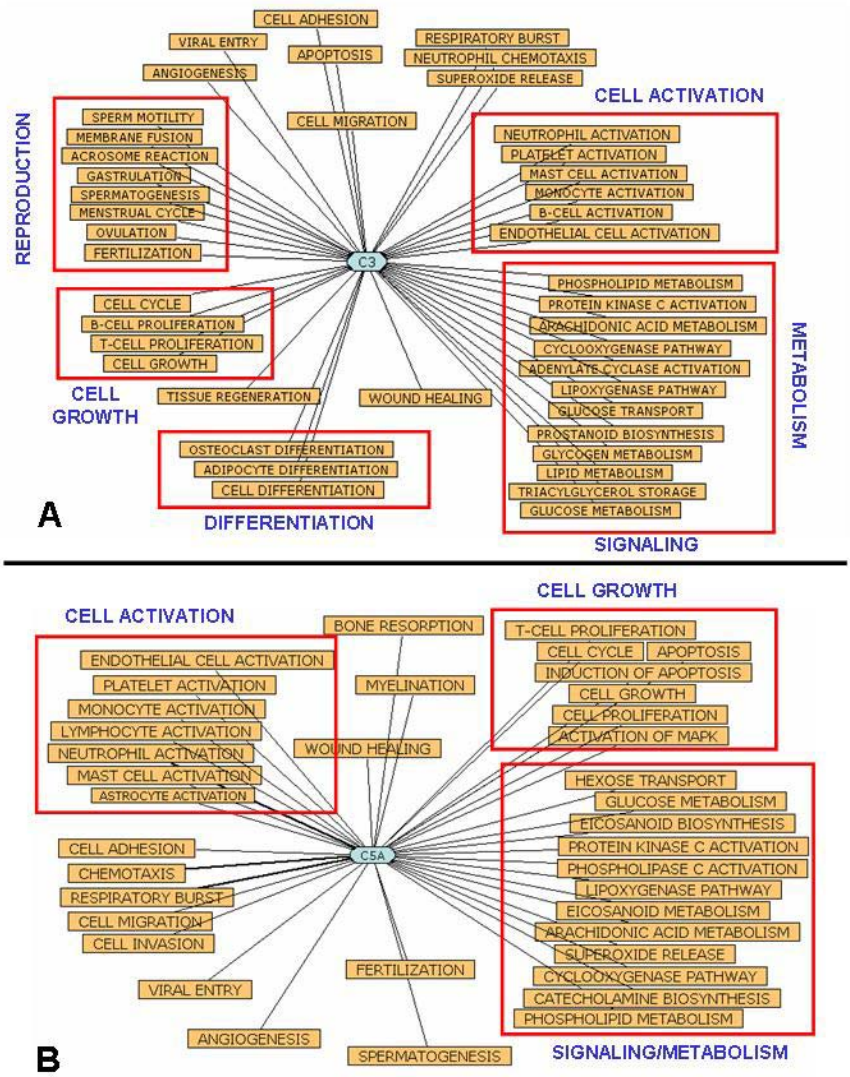

Figure 1. An illustration of a systems-wide overview of complement, as a complex network of protein–protein interactions that regulate the activation state of the cascade and also extend links to divergent biological processes; the systems associations of representative complement components have been retrieved through mining of the entire MEDLINE database.

and mechanistic aspects of these newly identified crosstalks are discussed below. These processes have been selected as examples illustrating the multifaceted nature of the system, and the crossdisciplinary approaches that should be adopted in trying to elucidate its functions in diverse pathophysiological settings.

Limb and lens regeneration in urodele amphibians represent the most challenging models for addressing key developmental questions that pertain to cell dedifferentiation, morphogenesis, and pattern formation. The role of complement components in this complex network of interactions that regulate cell fate decisions, tissue remodeling, and regeneration in lower vertebrates is discussed in greater detail in Chapter 5 by Tsonis et al.

7.1. Complement Intercepts Cytokine-Driven Regenerative Networks in the Liver

Acute toxic and viral injury or surgical liver resection triggers a robust proliferative response in the liver that culminates in full restoration of hepatic structure and function within days after the insult[57]. Essential priming signals that drive the cell cycle re-entry of quiescent liver cells are provided by hormones, cytokines, and hepatic growth factor-mediated signaling pathways. Recent studies have underscored a previously elusive role of innate immunity in the regulation of the regenerative response of the liver. With the use of complement-deficient mouse strains it was demonstrated that complement components C3 and C5 and their downstream anaphylatoxin-mediated pathways provide essential signals that lead to activation of latent hepatic transcription factors and subsequent release of cytokines that mediate the early priming phase of liver regeneration[58,55]. Similarly, in acute hepatotoxicity models it was shown that complement is required as a hepatic survival factor that contributes to the restoration of the liver parenchyma by promoting cell-cycle re-entry and proliferation of hepatocytes[59,58]. These studies, collectively, provided evidence for crosstalk between complement receptor-mediated pathways and cytokine-driven signaling networks in modulating the early regenerative response in the liver. The global impact of inflammation on the regenerative response of the liver and the main mechanistic aspects of the involvement of complement in the early stages of hepatocyte regeneration are discussed in Chapter 2 by DeAngelis et al.

Further delineating the mechanisms by which complement proteins and receptors interact with other signaling networks in the regenerating liver will provide insight into the molecular pathways that drive the early growth response of the liver and “prime” quiescent hepatocytes to re-enter the cell cycle.

7.2. A Complement–Chemokine "Crosstalk" Regulates Hematopoietic Stem Cell Engraftment

Complement regulatory molecules and receptors have been implicated in protecting blood cells from autologous complement-mediated lysis and promoting their inflammatory recruitment during the course of infection[60]. However, the distribution/expression of complement components in pluripotent hematopoietic precursors (HSCs) and early-committed myeloid/lymphoid progenitors at different stages of human hematopoiesis still remains ill-defined. Furthermore, the potential interaction of complement with chemokine and growth factor-dependent signaling networks that affect stem cell differentiation and regulate various homing activities of these cells in settings that elicit "danger" signals for the innate immune system (such as myeloablative injury, chemotherapy) has remained under intense scrutiny.

Recently, however, it was demonstrated that human $CD34^+$ hematopoietic progenitor cells express the C3a anaphylatoxin receptor, and that C3a receptor signaling synergistically promotes *a*-chemokine stromal-derived factor-1 (SDF-1/CXCL12)-dependent responses of HSCs that are associated with trans-endothelial migration, MMP, secretion and chemotaxis[56]. These findings were further supported by the enhanced hematopoietic recovery observed in irradiated mice that had been pretreated with C3a-primed Sca-1^+ hematopoietic progenitors[56].

Further studies have shown that C3a and its receptor, C3aR, promote retention of hematopoietic progenitor/stem cells in the bone marrow during stem cell mobilization in mice[61]. Taken together these studies support a crosstalk between complement and the SDF-1/CXCR4 signaling axis that appears to regulate the homing of hematopoietic stem cells into the bone marrow and, in synergy with chemokines, modulates the anchorage of progenitor cells in the bone marrow microenvironment, preventing their uncontrollable release to the peripheral circulation. Recent evidence that provides further insight into the role of complement in hematopoietic stem cell engraftment and supports its potential clinical application in stem cell transplantation/mobilization will be discussed in more detail in Chapter 3 by Reca et al.

7.3. Complement Modulates Coagulation Processes

The inflammatory and coagulation cascades converge at several molecular targets, and both are considered amongst the earlier homeostatic responses to infection[62]. Perturbations in the interactions between these two systems have been associated with morbidity and mortality in several infectious diseases that progress to disseminated thrombosis and multi-organ failure[63,62]. Earlier studies have indicated a role for complement in procoagulant pathways by showing that interception of C5aR results in distinct changes of pro/anti-fibrinolytic protein cascades[63,64], and also in the induction of Tissue Factor in endothelial cells and

monocytes[65]. Neutrophils together with complement are considered among the earliest "innate sensors" for incoming inflammatory signals[64]. They are rapidly chemoattracted by C5a and migrate through vascular endothelium barriers into tissues that have been exposed to inflammatory insults.

To date, the potential interaction of complement and neutrophils in triggering the procoagulant response has remained elusive. Surprisingly, it was recently shown that antiphospholipid antibody-induced complement activation and downstream signalling via C5a receptors in neutrophils leads to the induction of tissue factor (TF), a key initiating component of the blood coagulation cascade (Ritis K. et al. [submitted]). Inhibition studies using the C3 inhibitor compstatin revealed that APS-autoantibodies trigger complement activation, which in turn leads to generation of C5a and induction of a TF-dependent coagulant activity in C5aR-bearing neutrophils. These findings provide further support to the concept that innate immunity and coagulation share common targets and identify a novel "crosstalk" between the complement and coagulation cascades that might be exploited therapeutically for the treatment of complement-associated thrombogenic diseases.

8. FUTURE PERSPECTIVES

The complement system constitutes an attractive paradigm of how a complex network of protein–protein interactions can mediate divergent biologic activities, ranging from host defense and immune surveillance to modulation of adaptive immunity, stem cell trafficking, organ regeneration, and triggering of coagulation cascades. In the age of the "omics" revolution, new analytical tools and high-throughput profiling platforms are made available to complement researchers for elucidating key structure–function relationships and defining the distinct and dynamic conformational changes that underlie the wide array of complement protein interactions. Indeed, the recent resolution of the three-dimensional structure of the central component of the system, C3, and its proteolytic fragment, C3c, provides new insights into the conformational dynamics of complement activation and lays the groundwork for future structural and guided-mutagenesis studies that will unravel the complex regulation of the system[66]. Furthermore, the integrated consideration of available 3D structures, genome-wide profiling data, and the application of novel biophysical approaches to monitor the dynamics and energetics of complement protein interactions are anticipated to open up new avenues of opportunity for rational design of more effective complement therapeutics.

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