THERAPEUTIC MODULATION OF THE COMPLEMENT SYSTEM: CLINICAL INDICATIONS AND EMERGING DRUG LEADS

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THERAPEUTIC MODULATION OF THE COMPLEMENT SYSTEM: CLINICAL INDICATIONS AND EMERGING DRUG LEADS

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The complement system is a multi-tasking gatekeeper of innate immunity that intricately interacts with other key defense systems, such as the endothelial barrier, contact activation and coagulation systems, in maintaining tissue immunosurveillance and homeostasis. Its rapid and forceful activation in the bloodstream not only ensures the effective containment of microbial infections through potent cytolytic mechanisms, but also alerts the adaptive immune compartment to ensure the mounting of a proper humoral immune response against foreign antigens. However, there is a lurking 'dark side' that can lead complement astray, fueling a self-perpetuating vicious cycle of inflammation, exuberant immune activation and irreversible tissue injury that collectively exacerbate both acute and chronic pathologies. Indeed, complement dysregulation or excessive activation have been widely recognized as key pathogenic drivers in a wide spectrum of inflammatory or immune-mediated diseases. Targeted modulation of the complement system at various points of the cascade has revealed promising therapeutic targets for ameliorating disease scores in a number of conditions ranging from ocular, neurodegenerative and thromboinflammatory disorders, to cancer, periodontal diseases, chronic hemolytic anemias, ischemia-reperfusion organ injury, antibody-mediated transplant rejection and hemodialysis-triggered inflammation.

Elegant pre-clinical studies employing a diversified toolbox of highly specific complement inhibitors in rodent or primate models of disease have opened new avenues of therapeutic exploration by providing proof of concept for the therapeutic efficacy of complement modulation. At the same time, the clinical experience gained during this last decade with the sole complement-specific drug currently in the clinic, eculizumab, has rekindled the interest of biopharmaceutical companies in developing new and potent complement therapeutics for complement-driven diseases.

In this respect, the complement field is witnessing a new surge of clinical trials that are evaluating the safety, PK/PD profile and clinical efficacy of promising drug candidates in a number of clinical conditions driven by complement imbalance or over-activation.

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EDITORIAL
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Editorial: Therapeutic Modulation of the Complement System: Clinical Indications and Emerging Drug Leads

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Keywords: complement system, clinical trials, therapeutics, diseases, drug candidates

Editorial on the Research Topic

Therapeutic Modulation of the Complement System: Clinical Indications and Emerging Drug Leads

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Over the last two decades, our perception of the complement system as an innate immune sentinel that is solely responsible for pathogen elimination has been fundamentally transformed to that of a multi-tasking immune system that intricately coordinates both innate and adaptive immune responses (1). This is achieved through extensive crosstalk of complement effectors with multiple pattern recognition and proinflammatory signaling systems both in the intravascular space and in intracellular compartments. Complement is considered a fundamental humoral branch of innate immunity that rapidly responds to danger signals coordinating with other key defense systems, such as the endothelial barrier, contact activation, and coagulation systems (2). Through these reciprocal interactions, complement contributes to the maintenance of host immunosurveillance and tissue homeostasis. Its rapid and forceful activation in the bloodstream not only ensures the effective containment of microbial infections through opsonophagocytic mechanisms, but also alerts the adaptive immune compartment to ensure the mounting of a proper humoral response against foreign antigens. However, there is a lurking "dark side" that can lead complement astray, fueling a self-perpetuating vicious cycle of inflammation that results in persistent immune activation and irreversible tissue injury in both acute and chronic pathologies (2). Indeed, complement dysregulation or excessive activation have been recognized as key pathogenic drivers in a wide spectrum of inflammatory, immune-mediated, and age-related neurodegenerative diseases (3).

More than a decade after the clinical approval of the first complement-specific drug, the C5-targeting monoclonal antibody eculizumab (Soliris, Alexion), the complement drug space is ripe with new opportunities for therapeutic intervention at multiple steps of the cascade (4). The clinical success of complement-based therapy has been further consolidated through the recent approval of eculizumab in two indications of the neurological spectrum: generalized myasthenia gravis (gMG) and neuromyelitis optica spectrum disorder (NMOSD) (3). Furthermore, several drug candidates acting upstream of C5, or on downstream effectors, have advanced to Phase III trials in renal, hemolytic and ocular indications, promising broader or more tailored clinical benefit

than anti-C5 (3). Taken together, these game-changing developments have laid the groundwork for advancing a new generation of complement therapeutics to the clinical stage, in a spectrum of indications ranging from ocular, neurodegenerative, and thromboinflammatory disorders, to cancer, periodontal diseases, chronic hemolytic anemias, ischemia-reperfusion organ injury, antibody-mediated transplant rejection, and hemodialysis-triggered inflammation (5–7).

In addition to this expanding clinical landscape, the growing commitment of the biopharmaceutical industry in the complement drug space is readily reflected in the recently announced multi-million acquisitions of complement-dedicated startups by global healthcare companies (8). These widely publicized and lucrative corporate decisions have not only bolstered confidence in the clinical potential of complement intervention but also raised awareness about regulatory issues pertinent to drug market competition, the prevalence of monopolizing practices in the complement drug space and the relationship between true patient benefit, optimal drug or target selection, and incurred patient costs.

This Research Topic attracted leading academic and clinical experts in complement pathobiology and clinical translation, providing a forum to critically discuss the latest developments in complement drug discovery, from a disease-oriented perspective. While the list of selected clinical indications is certainly not exhaustive, it does illustrate the diversity of therapeutic approaches currently adopted in the field. Our topic includes examples of transformative clinical research that may soon change the treatment landscape in several complement-mediated diseases, while challenges faced along the drug discovery path are also discussed. Overall, emphasis is placed on the potential of the drug development pipeline to deliver to the clinic new complement-targeted therapies tailored to specific diseases.

Hemolytic conditions fueled by complement dysregulation have long remained in the crosshairs of the biopharma industry. Complement dysregulation is recognized as the main pathogenic driver in paroxysmal nocturnal hemoglobinuria (PNH) and as a major exacerbator of autoimmune hemolytic anemias (i.e., cold agglutinin disease, CAD) (3). In fact, PNH has served as a model for benchmarking new complement therapeutics in the clinical setting. While anti-C5 therapy has transformed the clinical course of PNH abrogating intravascular hemolysis and lowering thrombotic risk, there is still an unmet clinical need with regard to residual anemia that is mainly attributed to extravascular C3-mediated hemolysis. In this topic, Risitano et al. provide an overview of the clinical programs targeting complement upstream of C5 and propose that proximal complement inhibition (at the level of C3 or AP convertase) may drastically improve the hematological response in PNH patients who respond insufficiently to anti-C5 agents. The clinical success of anti-C5 therapy in atypical hemolytic uremic syndrome (aHUS) has fueled discussions on the pathogenic involvement of complement dysregulation in a broad spectrum of thrombotic microangiopathies (TMAs). Gavriilaki et al. provide an overview of the clinical landscape and pathophysiological "conundrum" of complement-mediated TMAs and critically address the feasibility of complement inhibition in a spectrum of TMAs with a pathogenic involvement of complement.

Excessive complement activation has been linked to multiple pathological sequelae associated with traumatic or infectious insults leading to multiple organ dysfunction (4). In this topic, Karasu et al. discuss the broad spectrum of pathophysiological consequences of deregulated complement activation in polytrauma, hemorrhagic shock, and sepsis. They also review complement therapeutics which have shown promise as treatment options for these severely debilitating conditions.

Owing to its distinct anatomy and physiology, the kidney glomerulus is endowed with increased susceptibility to complement-mediated damage (9). Complement dysregulation underpins a wide range of glomerular pathologies and several complement therapeutics are currently in clinical development for renal indications. Zipfel et al. present an overview of the pathophysiological traits of several renal diseases linked to complement dysregulation, including aHUS, anti-neutrophil cytoplasmic antibody mediated vasculitis (ANCAV), C3 glomerulopathy, and IgA nephropathy. They also discuss ongoing clinical trials evaluating the efficacy of complement inhibitors in each of these renal indications. Remaining in the renal space but focusing more on the clinical promise of complement therapeutics in kidney transplantation and organ accommodation across HLA and ABO barriers, Tatapudi and Montgomery discuss the pivotal pathogenic role of complement effectors in acute antibody-mediated rejection (AMR) during renal transplantation. They provide a comprehensive evaluation of various anti-complement agents in ongoing clinical trials of kidney AMR, weigh in on the potential of complement modulation in highly sensitized renal graft recipients and discuss the prospects and challenges of complement-based intervention in xenotransplantation. From a different perspective, van Zanden et al. focus on the pathophysiology of early tissue damage in the deceased organ donor, attempting to bridge a knowledge gap about the role of complement effectors in this process. The authors discuss preclinical evidence indicating that complement inhibition in the donor might be a promising therapeutic strategy to improve the quality of various donor organs for transplantation.

The discovery of complement gene polymorphisms that significantly elevate the risk of developing age-related macular degeneration (AMD) has sparked a fertile investigation into potential therapeutic avenues for treating ocular inflammation in AMD (3). Here, Park et al. review the evidence linking AP dysregulation with retinal inflammation and photoreceptor loss in AMD, and critically discuss ongoing clinical programs focused on therapeutic targeting of C3, FD, properdin, C5, and MAC in patients with geographic atrophy, a dry form of advanced AMD for which there is currently no approved therapy.

The role of complement in the development of chronic neuroinflammatory disorders has been documented in elegant preclinical models (10). Moreover, the recent approval of anti-C5 therapy as a treatment option for NMOSD and gMG has rekindled the interest of big biopharma in developing complement therapeutics in the neurological space. In this topic, Carpanini et al. discuss the challenges and opportunities

of targeted complement modulation in acute or chronic CNS disorders and explore ways to improve drug access into the brain and tailor anti-complement therapies for CNS diseases. Focusing on complement intervention in acute ischemic stroke, Clarke et al. review the impact of complement inhibition on cerebral tissue injury and repair following ischemic damage. Importantly, they argue that successful clinical translation of complement therapies in stroke patients will depend on selection of the right therapeutic window, with early intervention during the ischemia-reperfusion phase favored over delayed intervention during cerebral tissue repair.

Complement acts as a "double-edged sword" in cancer immunity, potentiating antibody-mediated tumor cytolysis, but also promoting tumor-associated inflammation and immunosuppression in the tumor microenvironment (5, 11). The protumorigenic activities of complement have attracted considerable attention in recent years and complement therapeutics have recently entered clinical development as modalities for boosting the anti-tumor efficacy of immune checkpoint inhibitors. In this regard, Pio et al. provide a comprehensive overview of the central role of complement in the cancer-immunity cycle and discuss the emerging prospects of introducing complement modulation in combination immunotherapies for various cancer types. Besides cancer, complement activation has long been implicated in the pathogenesis of autoimmune diseases but clinical translation in this area has remained a conundrum. In this Topic, Thurman and Yapa discuss the evidence linking complement deregulation to autoimmune pathologies and evaluate the feasibility of complement inhibition in the clinical setting. Periodontal disease is fueled by a dysbiotic oral microbiota that thrives on oral inflammation and leads to the destruction of tooth supporting tissues. Hajishengallis et al. summarize the body of evidence supporting the pathogenic involvement of complement in oral inflammation, discuss the therapeutic efficacy of C3-targeted intervention in primate models of natural or induced periodontal disease and provide a robust rationale for ongoing clinical trials of C3 inhibitors in human periodontitis.

With a growing interest in clinical complement intervention and an expanding list of ongoing clinical trials, the need for robust patient stratification ahead of clinical trials is accentuated and the use of sensitive diagnostic tools and bioassays for monitoring complement activation in patients receiving anti-complement therapy has become a priority. Mohebnasab et al. address this priority by providing an overview of the appropriate technology platforms for complement biomonitoring and assess the challenges for reliable complement diagnostics in a number of clinical disorders.

Hardly ever before has the complement "space" witnessed such a vibrant and transformative set of defining developments in all fronts, as in recent years. Indeed, molecular and structural insights have revealed new functions and mechanisms of this innate immune system, while more refined animal disease models, high-throughput analytical tools, and high-dimensional technologies are now illuminating new pathogenic pathways in a tissue-specific manner and at unprecedented single-cell resolution. More importantly, human clinical studies have now begun to unveil significant causative associations between distinct complement pathways and processes underlying disease progression or therapy response. Encouraging results from ongoing clinical trials with complement inhibitors are paving the way for new and more efficacious therapies in both rare and more common clinical indications.

It is our conviction that this timely collection of review articles faithfully illustrates both the breadth and scope of research innovation, elegant therapeutic drug design and clinical translational effort invested in the complement drug space.

AUTHOR CONTRIBUTIONS

DM drafted the manuscript. ER and JL reviewed the manuscript and provided critical revisions. All authors contributed to the conception and design of this work, read the final version, and approved the submitted manuscript.

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Conflict of Interest: JL is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for therapeutic purposes. JL is the inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. JL is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan].

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REVIEW
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Complement Therapeutics in the Multi-Organ Donor: Do or Don't?

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Over the last decade, striking progress has been made in the field of organ transplantation, such as better surgical expertise and preservation techniques. Therefore, organ transplantation is nowadays considered a successful treatment in end-stage diseases of various organs, e.g. the kidney, liver, intestine, heart, and lungs. However, there are still barriers which prevent a lifelong survival of the donor graft in the recipient. Activation of the immune system is an important limiting factor in the transplantation process. As part of this pro-inflammatory environment, the complement system is triggered. Complement activation plays a key role in the transplantation process, as highlighted by the amount of studies in ischemia-reperfusion injury (IRI) and rejection. However, new insight have shown that complement is not only activated in the later stages of transplantation, but already commences in the donor. In deceased donors, complement activation is associated with deteriorated quality of deceased donor organs. Of importance, since most donor organs are derived from either brain-dead donors or deceased after circulatory death donors. The exact mechanisms and the role of the complement system in the pathophysiology of the deceased donor have been underexposed. This review provides an overview of the current knowledge on complement activation in the (multi-)organ donor. Targeting the complement system might be a promising therapeutic strategy to improve the quality of various donor organs. Therefore, we will discuss the complement therapeutics that already have been tested in the donor. Finally, we question whether complement therapeutics should be translated to the clinics and if all organs share the same potential complement targets, considering the physiological differences of each organ.

Keywords: complement system, complement therapeutics, donor management, organ donor, deceased after brain death, deceased after circulatory death

INTRODUCTION

Donor Condition

Organ transplantation is the gold standard treatment for end-stage diseases in various organs, including the kidney, liver, intestine, heart, and lungs (1). The field of organ transplantation has made enormous progress over the last decades. From immunosuppression and tissue matching to organ procurement and preservation, all these developments significantly contributed to the

progress made in the field of transplantation (2). Nevertheless, donor availability and quality are still important limitations. Organs are mostly retrieved from deceased donors, and to much lesser extent from living donors (**Figure 1**). Deceased donors include donation after brain death (DBD) and donation after circulatory death (DCD). The DCD donor can be divided into "expected" and "unexpected". Expected DCD donation takes place after planned withdrawal of life-sustaining ventilator support. In contrast, unexpected death refers to a donor who had an unanticipated cardiac arrest, without successful resuscitation. Due to the seizure of circulation, DCD-derived organs have a variable period of warm ischemia time prior to retrieval (3). Since the past few years, the number of DCD donors is increasing, in particular in Belgium, Spain, The United Kingdom and The Netherlands. However, within Europe, most organs are still retrieved from DBD donors (**Figure 2**).

Brain death is the irreversible, total loss of brain function. Due to artificial ventilation and cardiovascular support, the circulation remains intact. Therefore, DBD donors do not have a nominable time of warm ischemia. The occurrence of brain death is an important risk factor for organ quality since brain death is associated with a cascade of hemodynamic, hormonal and immunologic events that become operational after brain injury, or/and brain death (6). Intracranial events leading to brain death promote the disruption of the blood-brain barrier integrity and the neurovascular system, which results in vascular leakage, edema, and hemorrhage. With these changes an immediate rise in intracranial pressure occurs, which causes hypoperfusion of the brain and brainstem. Ischemia of the medulla leads to sympathetic hyperactivation which causes a catecholamine storm, and subsequently a rise of the mean arterial pressure and peripheral vasoconstriction. Activation of the sympathetic nervous system activates the parasympathetic nervous system as well, resulting in bradycardia. This physiological nervous system response is also known as the Cushing response. In addition, brain ischemia results into damage of the hypothalamus-pituitary-axis, causing hormonal depletion. As a consequence, plasma levels of all hormones are lower in DBD donors. Depletion of the antidiuretic hormone increases diuresis as well as the risk for hypovolemia in the DBD donor. Last but not least, the immune system responds quickly to brain injury with both a sterile and non-sterile immune reaction. Concerning the DCD donor, only a few experimental studies have investigated the pathophysiological processes following circulatory arrest in the target organs. These studies show that apoptosis is one of the most important pathways of injury in DCD donors, in contrast to the inflammatory pathways in DBD donors. Whole genome microarray analyses performed by Damman et al. support these findings, which observed enriched NOD-like receptor pathways in DCD reperfusion biopsies. These results suggest that tissue hypoxia in deceased organs leads to cell necrosis and the release of damage-associated molecular pattern molecules (DAMPs). In addition, the study demonstrates that the multiple hypoxia-related pathways found in DCD reperfusion biopsies are related with delayed graft function (DGF) (7).

Currently, deceased donors represent the primary source for transplanted organs. However, the increasing demand for organ transplants mandates for expansion of the donor pool. Therefore, alternative strategies are deployed, such as an increase of the number of living donors, extension of the criteria for deceased donation and improvements in donor management and procurement (8). Still, continuing efforts to improve and preserve

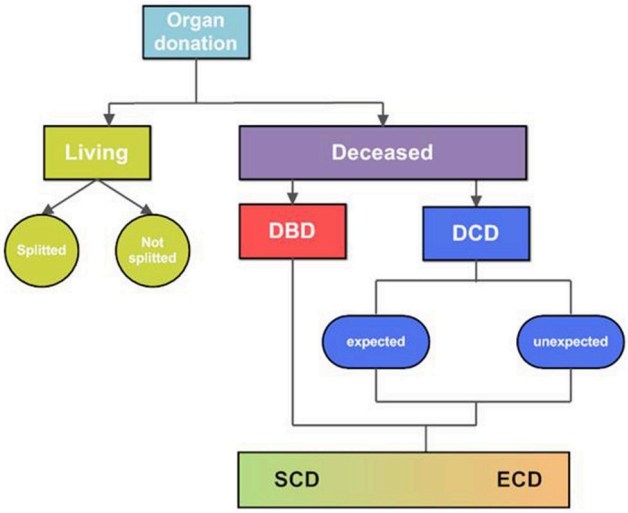

FIGURE 1 | Overview of donor types. Organs are retrieved from living and deceased organ donors. Organs from living donors can be donated partially, so-called "splitted" organ donation, or as a whole organ. The rest of the organs are retrieved from deceased donors, either from brain death (DBD) donors or circulatory death (DCD) donors. The DBD donor includes brain death with an intact circulation and preserved respiration. DCD donation refers to a donor with a cardiac arrest or loss of cardiac function, occurred before procurement of the organs. DCD donation can be divided in both expected and unexpected donation. Expected DCD donation refers to the procurement of organs after a planned withdrawal of life-sustaining treatments. Unexpected DCD donation refers to a donor with unexpected cardiac arrest, from which this donor could not be resuscitated. The quality of organs retrieved from deceased donors are variable, therefore deceased organs are classified into two groups: standard-criteria donors or extended-criteria donors (ECD). This subdivision is introduced to reflect the quality of the organ, of which ECD include potential donor organs that do not match standard donor criteria. DBD, donation after brain death; DCD, donation after circulatory death; SCD, standard criteria donor; ECD, extended criteria donor.

Abbreviations: ALAT, Alanine aminotransferase; AP, Alternative pathway; AR, Acute rejection; ARDS, Acute respiratory distress syndrome; ASAT, Aspartate aminotransferase; C1-INH, C1-inhibitor; C3aR, C3a receptor; C5aR1, C5a receptor 1; C5aR2, C5a receptor 2; CF, Cystic fibrosis; CP, Classical pathway; COPD, Chronic obstructive pulmonary disease; CRIg, Complement receptor immunoglobulins; DAMPs, Damage-associated molecular pattern molecules; DBD, Donation after brain death; DCD, Donation after circulatory death; DGF, Delayed graft function; ECD, Extended-criteria donor; HTx, Heart transplantation; IgM, Immunoglobulin; ITx, Intestinal transplantation; IPF, Idiopathic pulmonary fibrosis; IRI, Ischemia-reperfusion injury; LiTx, Liver transplantation; LP, Lectin pathway; LPS, Lipopolysaccharides; LuTx, Lung transplantation; LVAD, Left ventricular assist device; MAC, Membrane attack complex; MASP, Mannan-binding lectin-associated serine protease; MBL, Mannose-binding lectin; RTx, Renal transplantation; PAMPs, Pathogen-associated molecular pattern molecules; PPAH, Primary pulmonary arterial hypertension; sC5b-9, Soluble C5b-9; SCD, Standard-criteria donor; sCR1, Soluble complement receptor-1; SNP, Single nucleotide polymorphism; UW, University of Wisconsin.

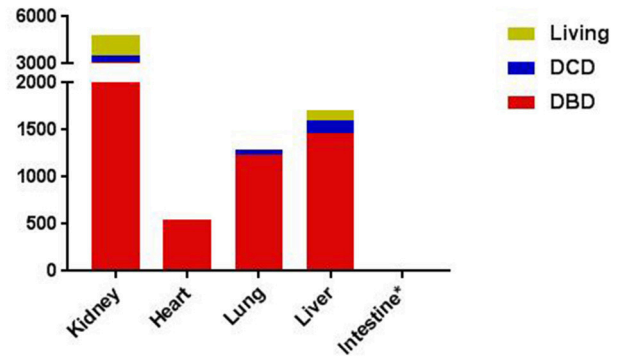

FIGURE 2 | The number of donor organs transplanted in 2017, as documented by Eurotransplant, per type of donor, per organ (4, 5). *In 2017, the number of intestines donated was six. DBD, donation after brain death; DCD, donation after cardiac death. The data was consulted on the 25th of October 2018, permission for publication was obtained.

donor organs are important, for which interventions in the donor seem a promising strategy. During the last decade, several donor pretreatment interventions have been explored in both animal and human studies, like the effect of thyroid hormone treatment and steroid treatment (9–11). However, no consensus has been reached regarding these donor treatment strategies, due to inconsistent results (12). Nevertheless, (pre)treatment of the deceased donor or the separate donor organs provides us a window of opportunity to improve graft function and graft survival. In the search for the optimal target in the donor, the complement system might play an important role.

THE COMPLEMENT SYSTEM

When the complement system was discovered more than a century ago, it was described as "heat-labile components in serum, complementing antibodies in eliminating bacteria." Nowadays, the complement system is known as a part of the innate immune system that consists of over 50 proteins in plasma and on cell surfaces. In brief, the complement system contains three activation pathways: the classical pathway (CP), lectin pathway (LP), and the alternative pathway (AP). The CP was the first pathway discovered and is activated by antigen-antibody complexes. These complexes are recognized by C1q, inducing a cascade via C2 and C4, leading to the production of CP C3 convertase (C4b2b). The same result is achieved when pattern recognition molecules of the LP [mannose-binding lectin (MBL), ficolins or collectin-11] bind to their MBL-associated serine proteases (MASP) to cleave C2 and C4. The AP is spontaneously activated by hydrolysis of C3 to C3(H_2O). Factor B is recruited and cleaved by factor D to form the AP C3 convertase (C3bBb). These convertases are intrinsically unstable, but their half-life is lengthened by interaction with factor properdin (P), a positive stabilizing regulator of the AP (13). The C3 convertases formed by the different pathways are responsible for a low grade cleavage of C3, thereby forming C3b. C3b mediates opsonization or binds to the C3 convertase to form the C5 convertase. The C5-convertases cleave C5 into C5a and C5b after which the membrane attack complex (MAC) is formed, via attachment of C5b to C6, C7, C8, and C9. The MAC complex induces the formation of lipophilic complexes in cell membranes which finally leads to cell lysis. Furthermore, C5b-9 induces tissue injury via intra-cellular pro-inflammatory signaling pathways (14). Finally, injury can be amplified by the formed split products C3a and C5a, acting as anaphylatoxins that provoke influx and activation of inflammatory cells (15, 16).

The Complement System in the Organ Donor

Under normal circumstances, the complement system is strictly controlled by complement regulators to prevent the destruction of healthy cells and tissues (15). However, when this fine balance is disturbed, the activated complement system may result in tissue injury. Damman et al. demonstrated the involvement of complement activation in deceased organ donors (17). In both DBD and DCD donors, increased systemic complement levels of C5b-9 were found in plasma, compared to C5b-9 levels of living donors. These higher complement levels were associated with increased local tissue injury in deceased donors compared to the living counterparts. Deceased donors also have a higher incidence of acute rejection in renal allografts (17). Van Werkhoven et al. showed an additional upregulation of C5a in plasma from DBD donors compared to living controls (18). Furthermore, De Vries et al. demonstrated that soluble C5b-9 (sC5b-9) release is detected in both DBD and DCD donors before reperfusion, but not in living donors (19). These higher levels of sC5b-9 in deceased donors are associated with inferior renal allograft function after transplantation (20). In accordance, systemic C4d and Bb levels were significantly higher in the deceased donor than in the living counterpart, with both complement proteins being associated with sC5b-9 levels in the DBD donor. Interestingly, there was no association seen between MBL and sC5b-9, which suggests that both the CP and AP are involved in brain death injury, but not the LP. However, with the recent findings of the potential bypasses in the complement system, the LP might still play a role in complement activation in the deceased donor (21, 22). Not only in kidneys, but also in lungs it has been demonstrated that plasma complement levels correlate with tissue injury after transplantation. Shah et al. measured plasma complement levels in recipients before transplantation and found an association with risk for acute lung injury and a higher incidence of mortality in lung transplant recipients (23). These studies indicate that activation of the complement system already commences in the donor, and that systemic complement activation may lead to local inflammation of the potential donor graft. As a result of inflammation, the (donor) organs are damaged. For DBD donors it is hypothesized that brain death initiates a sterile immune response through the release of endogenous DAMPS. Those danger signals are

TABLE 1 | Overview of the complement targets or therapeutics tested in the organ donor per organ, per type of donor.

Organ	Species	Donor type	KO/treatment	Study	Graft injury
Kidney	Rat	DBD	sCR1	(42)	↓
		DBD	C1-INH	(38)	↓
	Non-human primate	ECD	C1-INH	(41)	Unknown
	Human	DBD	C1-INH	NCT: 02435732	Unknown
Liver	None				
Intestine	None				
Heart	Mouse	DBD	$C3^{-/-}$	(79)	↓
		DBD	CR2-Crry	(78)	↓
Lung	Mouse	DBD	C3aRA	(100)	↓

Inclusion criteria were: (1) All animal and human models, testing (2) complement targets or therapeutics applied in the organ donor or during preservation, with (3) the donor injury mechanism as a target of interest (e.g., DBD, DCD). Excluded are studies in which complement targets or therapeutics were tested in IRI or rejection as mechanisms of injury. ↓ The treatment resulted in less graft injury.

released by cells under conditions of cellular stress or tissue injury. In DBD donors, these endogenous DAMPs are either actively secreted via stressed immune cells or passively released from dying brain cells or damaged extracellular matrix, which contributes to CP activation. Sterile inflammation can also cause further tissue destruction by the release of excessive amounts of DAMPs via necrotic cells. As a result, an extension of the local inflammation to the systemic circulation occurs, in a similar manner as is seen during microbial invasion. C1q and properdin will bind to these necrotic cells, which triggers the activation of the complement system. In addition, brain death causes an increase in intestinal permeability, that results in the release of pathogen-associated molecular pattern molecules (PAMPs) like LPS. In this way, the complement system is activated via the AP. In contrast, data referring to the pathogenic mechanisms in DCD-induced organ injury are scarce (24, 25). Taken together, despite the mentioned hypotheses of injury mechanisms, large knowledge gaps exist with regards to this topic. The exact role of complement activation and the involved complement components have not been fully elucidated in different donor organs and different donor types, especially in DCD donors. Of importance, since the physiological differences between organs and donor types might lead to various routes of immunological activation and therefore require different therapeutic approaches. The purpose of this review is to provide an overview of the current knowledge on the complement system in the donor (**Table 1**), the current existing knowledge gaps and future perspectives on this topic. Furthermore, we will answer the question if complement therapeutic should be clinically applied in the multi-organ donor.

RENAL TRANSPLANTATION

Challenges in Renal Transplantation

The kidney was the first organ successfully transplanted, in 1954 (26). Nowadays, renal transplantation (RTx) is the optimal treatment for patients with end-stage renal disease. Besides experience in years, the number of performed kidney transplants exceeds the number of all other solid organ transplants. In 2017, 4.419 renal transplants were performed in Europe, as registered by the Eurotransplant International Foundation (**Figure 2**). However, the number of patients waiting for a renal transplant is extensive and still increasing (27). The growing shortage of donor kidneys led to exploration of alternative strategies. First, the number of living donations increased significantly over the years. Living kidney donation is constantly evolving and goes nowadays beyond relatives, like individuals who donate a kidney to an anonymous recipient, so-called altruistic or Samaritan donation. Besides, special programs are developed, such as the Old for Old program or domino transplantation. In the Old for Old program kidneys of donors over 65 years or older are donated to recipients of the same age, without taking tissue-matching characteristics into account (28). A domino transplant occurs when the removed organ from first recipient is transplanted in a second recipient (29). Furthermore, the donor pool consisting of both DBD and DCD donors is expanded by the use of kidneys from extended-criteria donors (ECD), which refers to older donors and donors with comorbidities (3). Compared to standard-criteria donor (SCD) organs, kidneys from ECD are associated with up to a 2-fold increased risk of DGF, acute rejection, and graft loss (30). Kidneys from older donors are generally more immunogenic than kidneys from young donors, which makes immunomodulatory approaches in organs from ECDs an interesting topic for future research (30). With the increased utility of kidneys from ECDs in the clinics, more randomized controlled trials should be performed, with ECD kidneys included (31). Potentially, these ECD kidneys form a subgroup who can benefit from treatment already introduced in the donor.

The Complement System in Renal Transplantation

Most of the evidence for activation of the complement system in deceased donors is known from studies which focus on the kidney. Early studies performed by Kusaka et al. detected local C3 deposition in kidney isografts from DBD rats 1 h post-transplantation, while no C3 deposition was seen in living donor controls. C3 deposition was located on the endothelial cells and

glomeruli of DBD kidneys and could still be detected at day 5 after RTx (32). In accordance, Damman et al. showed higher renal C3 gene expression rates in DBD rats before transplantation than in living donors (33). No additional C3 gene expression was found after RTx, so renal C3 is deposited as a direct result of brain death. These results were confirmed in the human setting, in which significantly more C3 gene expression was found in kidney biopsies taken from DBD donors than in biopsies from living donors. The results on transcriptional level were supported by immunohistochemistry. C3d deposition was seen in renal grafts from human DBD donors, but not in living donors. Again, no additional C3d deposition was found after ischemia and reperfusion (33). The functional importance of local C3 synthesis is demonstrated by multiple studies. Pratt et al. showed in a murine model that renal allografts lacking C3 production survive more than five times as long as renal allografts that produce C3 (34). In addition, Brown et al. demonstrated that expression of C3 alleles by renal cells in the deceased human donor significantly affect graft survival (35). However, the association between the C3 allotypes and graft survival could not be replicated by others. Varagunam et al. detected no significant differences between the C3 alleles on long-term renal allograft survival in patients (36). These results are in accordance with the study of Damman et al. which observed that donor C3F allotypes are not associated with renal allograft outcome after RTx. Only subgroup analysis within the DCD group revealed a protective effect of the donor C3F allotype for primary non-function. These divergent results could possibly be explained by the differences in sample size and post-transplantation follow-up data (37).

The importance of the complement system is underlined by the in-depth analysis of the gene expression differences between human renal allograft biopsies from living and deceased donors. Significant renal overexpression of many complement components were seen in deceased donor kidneys before reperfusion (38). Primarily complement-related genes of the CP were involved, namely C1q, C1s, C1r, C2, and C4. Factor B, an component of the AP, was upregulated in deceased kidneys. Similar results were seen in the whole genome microarray study performed by Damman et al. This study shows enrichment of both the hypoxia and complement coagulation pathways in DBD kidneys. The same pathways were involved in the DCD kidney, but in a later phase of transplantation, namely during ischemia (7). Focusing on the downstream complement components, the C5a-C5aR-axis seems to play an important role. C5a is not only systemically upregulated in the deceased donor, but Van Werkhoven et al. showed an increased renal tubular expression of the C5a receptor 1 (C5aR1) (18). Altogether, these studies suggest that the local immune activation in the deceased renal allograft is important for the outcome after RTx. Thus, targeted therapy interfering with (local) complement activation before organ recovery or during organ storage is an attractive therapeutic approach. However, assessing local immune activation requires invasive techniques. For that reason, using complement deposition as an indicator for organ damage might not be preferred. A recent study by Schröppel et al. investigated the potential for less invasive markers by evaluating C3a and C5a levels in donor urine. They found that donor urinary C5a levels were correlated with DGF after kidney transplantation. However, no correlation was seen between urinary C3a and post-transplant DGF, despite higher levels of C3a in urine from deceased donors than in urine from healthy controls. Whether other complement proteins measured in urine correlate with graft function, potentially serving as biomarkers, has not yet been elucidated (39).

Already a few complement therapeutics were tested in the deceased kidney donor or during kidney preservation. One of the most potent complement inhibitors is C1-esterase-inhibitor (C1-INH). As a serine protease inhibitor, *in-vitro* data demonstrated that C1 inhibitor modulates activation the classical- and lectin pathway (40–42). Pre-clinical studies with C1-INH in the deceased donor showed promising results. Poppelaars et al. tested a high-dose and low-dose C1-INH in a rat model of brain death in which C1-INH was administered 30 min after confirmation of brain death. High-dose C1-INH treatment of the DBD donor resulted in significantly lower renal pro-inflammatory gene expressions and decreased serum levels of IL-6. In addition, C1-INH led to an improved renal function reflected by lower serum creatinine levels, and less renal injury as demonstrated by lower kidney injury molecule-1 gene expression levels (40). C1-INH is currently tested as a treatment strategy in human DBD donors to improve outcome after RTx (NCT02435732). At this moment, this study is in the phase of recruiting patients. In ECD donors C1-INH treatment might be of potential therapeutic use as well, which is currently being investigated by Fernandez et al. in a non-human primate model (43).

Besides C1-INH, more complement therapeutics are already tested in the deceased donor in experimental setting. Soluble complement receptor 1 (sCR1) was given to DBD rats and treatment with sCR1 before and after confirmation of brain death led in both cases to significantly improved renal allograft function. In addition, treatment with sCR1 led to reduced renal gene expression of IL-6, IL-1β, and TGF-β. These results provide proof that complement inhibition in the donor is effective, even after the confirmation of brain death (44).

Next to the use of complement therapeutics in the donor, already a few studies tested the effect of complement therapeutics during renal preservation. Patel et al. were the first, and evaluated the effect of APT070, also known as Mirococept (45). Mirococept is a membrane-localizing complement regulator, which is a derivate from complement receptor 1. Rat donor kidneys were perfused with Mirococept and subsequently subjected to 16 h of cold storage. After 16 h of cold storage, the kidneys were transplanted into syngeneic recipients. APT070 perfused renal grafts had survival rates of 64% compared to a survival rate of 26% in control-treated renal allografts. Currently, Mirococept is tested in a multicenter randomized controlled trial, in which Mirococept is administered *ex vivo* to deceased donor kidneys. The trial, called EMPIRIKAL, is still ongoing and aims to evaluate the efficacy of Mirococept in reducing the incidence of DGF in renal transplants from deceased donors (46).

Furthermore, Lewis et al. demonstrated that pharmacological targeting the C5aR is also of potential benefit. In this study a C5aR antagonist named $A8^{\Delta 71-773}$ was used, which targets both the C5aR1 and C5aR2 (47). Donor kidneys were flushed and stored for 2 h with UW or UW + C5aR antagonist. Kidneys treated with

the C5aR antagonist had significantly improved renal function and increased graft survival compared to untreated kidneys. In addition, the C5aR antagonist prevented renal injury, reflected by lower gene expression levels of TNF-α and macrophage inflammatory protein-2/CXCL2. C5 was also targeted in a recent study, in which a monoclonal antibody against C5 was used (48). Rat donor kidneys were cold stored for 28 h with or without anti-C5. Treatment with anti-C5 significantly increased the survival rate of the renal allografts from 22% to 100% after 21 days. Another C5 complement inhibitor is the recently generated recombinant anti-C5 antibody called Ergidina, which is coupled to a cyclic-arginylglycylaspartic (RGD) acid-peptide. The RGD peptide has the property to migrate to the ischemic endothelial cell. Thus, when bound to anti-C5 it will not only be able to migrate, but also be able to control ischemia tissue injury. In a study of Durigutto et al. rat donor kidneys were procured and cold stored for 24 h. Thereafter, kidneys were *ex vivo* infused with Ergidina, and stored for either 15 min or 30 min. Results showed that Ergidina was bound to the vascular endothelium of the kidney and already reached a plateau after 15 min. Next, the efficacy of Ergidina was evaluated in a rat model of IRI. Rats received Ergidina 45 min before ischemia and were sacrificed at day 1 or day 4. Ergidina preserved renal function, prevented tissue injury at glomerular and tubular level and prevented C9 deposition in the kidneys at both day 1 and day 4 (49).

Besides anti-C5, Yu et al. also evaluated the effect of AP inhibitor TT30. TT30 is a complement receptor 2/factor H fusion protein. *Ex vivo* preservation with TT30 for 28 h significantly improved renal function and renal graft survival compared to control-treated kidneys. The 21-day graft survival rate was 66% in the TT30 treated group compared to the 100% in the anti-C5 treated group (48). These survival rates did not significantly differ, but imply that next to the AP, activation of the CP or LP might play an role in in ischemia-induced injury.

Based on the studies already performed, it can be hypothesized that the renal allograft is already primed for complement activation in the deceased donor. Therapeutics interfering with complement activation before organ recovery would be an attractive therapeutic approach that deserves further investigation. Most importantly, studies using complement therapeutics in order to prevent renal injury seem to be most effective when the therapeutics are specifically delivered to the site of complement activation. Therefore, with the increased availability of new complement therapeutics, it is crucial to unravel the role of complement system in the deceased donor. Especially, it is essential to learn whether complement therapeutics can be administered in the donor before organ retrieval or if treatment after organ procurement is preferred.

LIVER TRANSPLANTATION

Challenges in Liver Transplantation

Currently, more than 1.500 liver transplantations (LiTx) are performed in Europe per year (**Figure 2**). In general, LiTx is considered for patients which suffer from acute liver failure, end-stage liver disease and primary hepatic malignancy. However, after a rapid growth, the annual number of LiTx has stopped increasing over the last 10 years. An important limitation for the stagnant number of LiTx is donor shortage (50). Therefore, alternatives to DBD donation of liver transplants are more frequently implemented. First, the concept of "split liver transplantation" is used, which enables surgeons to transplant one donor liver into two recipients. However, this technique is only feasible with ideal livers, mostly derived from young DBD donors. Second, there is an increase in living-related liver transplantation. Living donation for adults is still associated with major complications and a substantial risk for the living donor (51). Therefore, both splitting and living LiTx have not gained widespread acceptance. Finally, more extended criteria are introduced such as advanced age, steatosis and DCD liver grafts. Controversy exists about DCD liver transplants, since studies that compare graft outcome from DCD donors with standard DBD donors have been variable. Studies performed so far suggest decreased graft survival in the first year following DCD LiTx (52). Although ECD donation reduces the gap between supply and demand, managing the risk for the recipient is a critical factor in ECD donor livers. ECD livers are vulnerable to hypoxia and tissue injury associated with DGF or graft survival. In order to improve the quality of these suboptimal liver grafts, several studies evaluated the effect of intervention during donor management, such as the administration of steroids, dopamine, and hormone replacement (10, 11, 53). Taken these data into account, the current strategies applied in LiTx have mainly been focusing on treatments to stabilize hemodynamic disorders associated with deceased donation. Yet, their effects on liver graft function and survival remain unknown. Therefore, it could be beneficial to consider other molecular pathways involved, including the complement system.

Complement System in Liver Transplantation

The number of studies that investigated the role of the complement system in deceased donation for LiTx is scarce. Of interest, since the liver is responsible for the biosynthesis of 90% of plasma complement components and soluble complement regulators (54). Other type of cells including immune cells and endothelial cells produce complement components as well, but their contribution to plasma levels appear to be minor compared to hepatocytes. Moreover, several complement receptors are expressed in the liver; e.g., C5aR, CR1, CR3, CR4, and complement receptor immunoglobulins (CRIg). These complement receptors have multiple functions on different cells in the liver, from inducing the acute phase response to the clearance of C3-opsonized immune complexes. Thus, the complement system is involved in multiple liver diseases, such as transplant-induced injury (55).

The role of the complement system in the deceased liver is mainly investigated in the DBD donor. Rebolledo et al. were the first to show complement activation in DBD donor livers. In this study, rats were subjected to brain death for a period of 4 h. Results showed that DBD donor livers had a significant increase in C3 mRNA levels compared to the control group. Next, rats were pretreated with prednisolone 30 min before

induction of brain death as a proof of principle. Pretreatment with prednisolone resulted in a reduced pro-inflammatory state, reflected by lower mRNA levels of IL-6, IL-1β, TNF-α, and MCP-1. In contrast, mRNA levels of C3 were upregulated in the prednisolone treated rats (9). In an additional study, Rebolledo et al. administered prednisolone after confirmation of brain death to investigate treatment potential. Again, prednisolone reduced the levels of pro-inflammatory cytokine gene expression, but did not increase nor decrease the C3 expression compared to untreated DBD rats. These divergent C3 expressions after administration prednisolone are not fully understood, but could be explained by the fact that complement is involved in liver regeneration (11).

So far, only one study investigated the effect of complement inhibitors on the donor liver before transplantation. Bergamaschini et al. studied the potential of C1-inhibitor (C1-INH) treatment during preservation. Porcine livers were removed from donors and perfused with University of Wisconsin (UW), with or without addition of C1-INH, and stored statically at 4°C for 8 h. To assess liver function, livers were subsequently reperfused for 2 h with pig blood on an extracorporeal circuit. Results demonstrated less complement activation, reflected by normal levels of complement haemolytic activity and absence of C3 activation production in both plasma and tissue at time of reperfusion. Morphological analysis of the livers showed significantly decreased inflammation as shown by only a mild increase in portal and lobular inflammatory infiltration, compared to necrotic lesions in the untreated group (56). Taken together, C1-INH treatment during preservation seems to protect the liver against cell injury and inflammation during the preservation phase. Therefore, complement therapeutics in the donor, but also the preservation phase, might be beneficial for the liver.

Important to take into account in LiTx is the process of liver regeneration. Liver regeneration is a process in the liver that allows mature hepatocytes to re-enter the cell cycle, proliferate, and eventually replace lost or damaged hepatocytes (57). Liver regeneration is important for both donors and recipients of liver transplants, especially in the case of a failing remnant liver after "splitted" liver donation or transplantation of a small-for-size liver in the recipient (58). So far, no therapy exists for these patients, so there is a significant need for strategies that stimulate the regenerative capacity of livers. The complement system seems to have a key role in the liver regeneration, primarily during the early phases, were the hepatocyte re-enters the cell cycle and proliferates. Recently performed studies demonstrated an important role for the complement effector proteins, C3a and C5a (59, 60). Strey et al. subjected C3- and C5-deficient mice to a 70% partial hepatectomy model. Deficiency of C3 or C5 led to significantly less liver regeneration, as reflected by fatal liver failure. Reconstitution of effector molecules C3a and C5a resulted in hepatocyte proliferation, which indicates that C3 and C5 are key factors in regeneration of hepatocytes. The precise role of complement in the deceased donor still needs to be elucidated, but ischemia-reperfusion injury (IRI) studies showed an important role for both C5a and C5b-9 in the induction of injury (61, 62). These contradictive results with regards to the role of complement in hepatic IRI vs. liver regeneration emphasize the need for a fine balance between complement activation and inhibition. Therefore, it is important to have a good understanding of the two processes and test potential complement inhibitors in both disease models. A study performed by He et al. evaluated the effect of CR2-Crry in a combined mouse model of total IRI and 70% partial hepatectomy. CR2-Crry is a fusion protein that specifically targets the sites of C3 activation. This study shows that CR2-Crry is able to protect against hepatic IRI alone, however a combination of IRI and partial hepatectomy resulted in significant liver damage and a failure to regenerate compared to WT mice. The failure to regenerate is probably a result of the inability to generate sufficient levels of C3a, C5a, and C5b-9, complement effector molecules important for liver regeneration (63). Given these observations, CR2-CD59 might be a potential complement therapeutic. CR2-CD59 is a fusion protein that migrates to sites of complement activation and specifically inhibits the MAC, without the blockage of other complement components. CR2-CD59 was tested by Marshall et al. in the same mouse model of total IRI and 70% partial hepatectomy as described by He et al. The study performed by Marshall et al. showed that livers treated with CR2-CD59 have less injury, and more hepatic regeneration than control-treated mice. CR2-CD59 mice had a 100% 7-day survival rate, whereas in the CR2-Crry treated group only 40% of the mice survived (64). These results imply that in MAC-induced injury, the regenerative response of the liver is impaired. Therefore, these studies highlight the need for a tailor-made approach to protect the liver against ischemia injury and enhance the regenerative capacity. Although only a few studies are performed, they all point towards the involvement of the complement system in the deceased donor liver. The exact role of complement in the deceased donor liver and its consequences on liver transplant viability and survival remains unknown. Therefore, more studies, especially focused on the complement-dependent balance between injury and regeneration in the liver, need to be conducted.

INTESTINAL TRANSPLANTATION

Challenges in Intestinal Transplantation

Intestinal transplantation (ITx) is the least common form of organ transplantation. The field of ITx is small with only 6 ITx reported in 2017 by Eurotransplant (**Figure 2**). ITx is indicated for patients with intestinal failure who suffer from life-threatening complications when using parenteral nutrition. Despite the advances, ITx is still a challenging procedure due to multiple factors. First, the intestine is highly immunogenic because it consists of a large amount of lymphoid tissue, including the patches of Peyer and the mesenteric lymph nodes (65). Second, the intestine carries an enormous bacterial load. These characteristics create a fine balance between the maintenance of tolerance to healthy self-tissue and eliminating invading pathogens. Moreover, the intestinal mucosa is extremely vulnerable to injury, especially hypoxia injury, which is negatively associated with graft outcome (66). As a result of the susceptibility for hypoxia, DCD donor intestinal grafts are

not yet accepted for ITx (67). This makes DBD donors the sole source for intestinal grafts. Despite major achievements in intestinal grafts retrieved from DBD donors, such as improved immunosuppression, the physiological abnormal state in the DBD donor still significantly compromises the viability of the intestine. Therefore, it is concerned that ITx is underutilized due to complex pathophysiological processes and difficulties to identify markers for intestinal injury (68).

The Complement System in Intestinal Transplantation

Hardly any studies are performed to unravel the pathophysiological processes in the intestine of a deceased donor, probably since the number of ITx performed per year is low. However, based on the few studies there are, it is likely that the deceased donor state causes significant alterations in the intestine and affects intestinal barrier function. The degree of permeability already changes in response to a low level of pro-inflammatory cytokines, which results in the translocation of intestinal bacteria. The intestine contains microbial LPS, which is thought to be one of the most potent activators of the AP of the complement system. Although studies showed that LPS from different bacterial strains interact in qualitatively different ways with complement, LPS from gram-negative bacteria indeed induce consumption of complement (69). A study performed by Koudstaal et al. confirmed the involvement of LPS in the DBD donor by using a brain death model for rats. Rats subjected to 4 h of brain death had higher serum levels of LPS and LPS-binding protein (LBP), as evidence of endotoxemia. Besides, mRNA gene expression levels of LPB were significantly higher in DBD rats than in living controls. DBD rats had a high inflammatory state, reflected by the strongly elevated levels of IL-6 and MCP-1 (70). The study shows enhanced intestinal permeability in DBD rats which results in a high immunological response. The observation that brain injury can rapidly induce significant damages to the intestine is demonstrated by a study of Hang et al. This study investigated the histopathological alterations of the intestinal mucosa in rats after 3–72 h following brain injury. The intestinal mucosa was already severely damaged after 3 h, reflected by shedding and apoptosis of epithelial cells, mucosal atrophy, and loss of increase in intestinal permeability. The level of plasma endotoxin was positively related to the degree of intestinal permeability. Compared with the control group, serum endotoxin levels were significantly increased at 3, 12, and 24 h with a maximal peak at 72 h. The first peak of endotoxin levels, at 3 h, might be the result of acute gut mucosal damage due to ischemia-induced sympathetic hyperactivation. The second peak of serum endotoxin might be induced by mucosal damage and increased epithelial necrosis, which occurs at 72 h (71). Whether complement is activated in the intestine of both the DBD and DCD donor remains to be elucidated. However, the current findings indicate that protection of the intestine in the multi-organ donor is necessary, since translocation of intestinal bacteria and endotoxin lead to a systemic inflammatory response syndrome and sepsis with subsequent multi-organ failure (72). Further research needs to focus on the exact role of complement activation in the deceased intestinal donor. This could not only create a new window of opportunity for immunosuppressive strategies, but also improve the clinical success of ITx.

HEART TRANSPLANTATION

Challenges in Heart Transplantation

The technique for heart transplantation (HTx) was already developed in 1967. Nevertheless, it took more than a decade before immunosuppressive treatment strategies improved this technique to such an extent, that HTx became the gold standard treatment for end-stage heart diseases (73). In 2017, 548 heart transplants were performed in Europe, as registered by the Eurotransplant International Foundation (**Figure 2**). Both non-ischemic and ischemic cardiomyopathy are the underlying diagnoses responsible for over 80% of heart transplants (74). The increased need for HTx over the years led to the inevitable gap between donor demand and supply, analogous to other donor organs. Despite the attempt to minimize this gap by the introduction of techniques such as left ventricular assist devices (LVAD), these therapies mainly serve as a short-term, "bridge-to-transplant" solution.

Unfortunately, the majority of potential heart donors is not procured and transplanted, for several reasons. First, most countries are limited to the use of DBD hearts, and do not utilize the DCD donor pool. Anxieties exist concerning warm ischemic injury to the myocardium after circulatory death, together with the inability to assess heart function. However, new techniques are being developed to tackle these issues. In an attempt to limit warm ischemia times, implementation of techniques like *in situ* normothermic regional perfusion for thoracic organs are explored in order to convert from a DCD to a DBD-type procurement (75). In addition, normothermic regional perfusion provides the opportunity to functionally assess the donor heart. Furthermore, implementation of techniques such as *ex situ* heart perfusion are being studied (76). Despite its experimental nature it is suggested that those techniques lead to usage of more donor hearts, with comparable outcomes to the current gold standard of DBD HTx (77). A second reason for low procurement rates of potential donor hearts is the relatively strict cardiac donor selection criteria. Age < 55 years old, appropriate hemodynamics and limited inotropic support are examples of selection criteria that impede suitability (78). Godino et al. showed that hemodynamic dysfunction represented the major cause for unsuitability of heart donors, a complication that occurs frequently in DBD donors (79). In terms of immunology, experimental transplantation models have shown that hearts have differences in rejection patterns compared to abdominal organs such as kidneys and livers, leading to higher rejection rates (73). These organ-specific differences in immunology might contribute to the differences seen in graft-survival rates between organs in human transplantation. Of importance, since the immunologically active state of the organ already commences in the donor. The DBD donor has shown to exacerbate post-transplantation cardiac IRI that reduces

allograft survival, in which the complement system might play a key role (80).

The Complement System in Heart Transplantation

The role of the complement system in the deceased heart donor has only been studied in DBD donation, which raises questions for involvement in DCD donation. An experimental mouse study performed by Atkinson et al. showed increased local complement C3d deposition in the heart after brain death. C3d deposition was primarily seen in the vascular endothelium and surrounding myocytes, in a significantly higher amount than in grafts from sham-operated mice. In addition, the study demonstrated that absence of C3 reduced cardiac damage, reflected by less endothelial swelling and lower serum levels of cardiac troponin I. Also, significantly less leukocytes infiltrated the heart tissue and gene expression of P-selectin, ICAM-1, VCAM-1, TNF-α, and IL-1β were reduced upon brain death. In order to investigate whether therapeutically targeting C3 would diminish DBD-induced cardiac damage as well, mice were treated with CR2-Crry after receiving a living or DBD heart. CR2-Crry is a complement inhibitor, that targets C3 split products by binding local C3b deposits. Upon treatment with CR2-Crry, recipients who received DBD donor hearts showed reduced cardiac troponin I levels and histological injury scores, similar to levels of living transplanted hearts. Thereby, CR2-Crry treatment diminished neutrophil and macrophage infiltration and prolonged allograft survival of treated DBD donor hearts compared to untreated controls (81).

Based on these studies, it is suggested that complement inhibitory strategies applied to the deceased donor may provide protection of the heart graft. To see whether the results seen in rodent models are clinically relevant, Atkinson et al. analyzed complement deposition in human DBD heart biopsies and living donors. The human biopsies taken from DBD donors before implantation showed C3d complement deposition and inflammation in all grafts, compared to minimal C3d deposition in biopsies from living donor hearts. The complement staining patterns in human DBD hearts demonstrate that complement activation already occurs in the DBD heart, independently from the ischemia-reperfusion phase (80). However, the contribution of each activation pathway of the complement system in DBD heart injury is not fully known. The CP might be involved, since IgM complexes show similar distribution patterns as seen for C3d staining in murine DBD hearts (81). In human heart biopsies from both DBD and living donors stained for C4d, 50% of the cases showed C4d deposition in DBD biopsies before implantation into the recipient. In contrast, living donor hearts showed no C4d deposition at all, which suggests a potential role for the CP in DBD-induced heart injury (80). Whether the other complement activation pathways are involved as well-needs to be further elucidated. Furthermore, the role of the complement system in DCD heart donation requires additional attention. Zhang et al. investigated the role of natural immunoglobulins in a model for myocardial warm IRI and revealed that pre-existing IgM's, which recognize "ischemic antigens," are the main initiator of pathology through activation of the complement system (82). Regarding variable warm-ischemia times in DCD donors, this might be an interesting field of future research.

LUNG TRANSPLANTATION

Challenges in Lung Transplantation

In 2017, 1.233 lung transplants have been performed in Europe, as registered by the Eurotransplant International Foundation (**Figure 2**). The most important indications for bilateral lung transplantation (LuTx) are Chronic Obstructive Pulmonary Disease (COPD), Cystic Fibrosis (CF), Interstitial Pulmonary Fibrosis (IPF), and Primary Pulmonary Arterial Hypertension (PPAH) (83). Although the number of LuTx is much lower than the numbers of abdominal organs transplanted, donor shortage is an important issue in LuTx as well. This observation is mainly the result of a lower utilization rate of lungs than of abdominal organs. More than 70% of the donor livers and kidneys are procured and used for transplantation, while lungs are only suitable for transplantation in around 20% of the cases (84). Lung injury is an important reason for excluding donor lungs for transplantation and is caused by the process of donor death and complications at the intensive-care unit (85).

Most donor lungs are procured from DBD donors, in which the process of brain death leads to inflammation and pericapillary leakage, resulting in pulmonary edema (86). Those mechanisms of injury make the lung more susceptible to IRI and lead to poor graft survival rates of only 54% after 5 years (87). The last years attempts have been made to enlarge the donor pool. Examples are transplantation of lungs from DCD 3 donors (88), usage of ECD (89), living-donor lobar lung transplantation (90), and application of the technique of *ex vivo* lung perfusion (EVLP) in an attempt to test and repair discarded donor lungs (91, 92). So far, those attempts have demonstrated similar outcomes on graft survival compared to standard DBD lungs (93, 94). However, those efforts have not yet closed the gap between supply and demand in LuTx and did not lead to improved graft survival. In an immunological point of view the lung is an interesting organ, because of continuous exposition to the outside environment, serving as a first line barrier to infection. As a result, the immunomodulation regimen in lung transplant recipients makes those patients more prone to fungal infections (95). This issue emphasizes the challenge to create a balance between infection and rejection. In order to do so, the exact immunological pathways involved in donor lung injury need to be further elucidated before development of novel immunosuppressive strategies, which tackle the issues of donor shortage and graft survival in the field of LuTx.

The Complement System in Lung Transplantation

Complement in LuTx has mostly been studied in recipients, focused on the role of the complement system on lung IRI and rejection. However, Budding et al. demonstrated that the complement system is already involved from the first step of the lung transplantation process. The study showed

that recipients who received a donor lung with a CD59 protein single nucleotide polymorphism (SNP) configuration, had a higher risk for chronic rejection after LuTx. Under normal circumstances CD59 acts as a regulatory protein, which suppresses MAC formation by binding C9 to C5b-C8 complexes, which results into inhibited cell lysis. The presence of a CD59 SNP configuration affects CD59 expression and sensitivity to complement-mediated cell lysis, which increases the risk for rejection in recipients who receive a CD59 SNP donor lung (96). These results suggest that C5b-9 is involved in donor-related lung injury. However, considering the direct effects of complement split-products like C3a and C5a produced in the earlier steps of the complement system, the question is raised whether regulatory proteins of the terminal pathway should be the target of interest. In lungs, C5a, but also C3a, have shown to induce acute pulmonary injury by constriction of smooth muscle walls in bronchioles and pulmonary arteries, and cause focal atelectasis (97). C3a levels in plasma have additionally been described to be associated with later development of acute respiratory distress syndrome (ARDS) in polytrauma patients (98). Furthermore, C3a and C5a are described to attract and activate neutrophils (99, 100). Of importance, since the amount of recruited and infiltrated neutrophils are associated with graft survival after LuTx (101). The beneficial effect of targeting the donor lung on the level of C3a has been demonstrated by Cheng et al. in a mouse model for LuTx. First, the study confirmed that the process of brain death induces donor lung injury. Pathology lung injury scores examining congestion, hemorrhage and inflammation, were significantly increased. Secondly, the amount of infiltrated neutrophils and macrophages were elevated in DBD mice compared to both sham-operated mice and living donor mice. Furthermore, a significantly elevated expression of complement receptor C3a (C3aR) in DBD donor lungs was found. The C3aR was mainly expressed on bronchial and epithelial cells and lung endothelium. Thereafter, targeting the C3aR with a nebulized complement C3a receptor antagonist was tested in a mouse model of LuTx. Recipients of untreated DBD donor lungs showed aggravated IRI and acute rejection (AR) grades, which was ameliorated after treatment with the C3aR antagonist. IRI and AR grades were even returned to levels as seen after living-donor LuTx (102).

Despite the limited amount of research performed on the topic of DBD-related lung injury, it is suggested that the complement system is already involved from the first step of the process, namely the donor. Of particular interest, given the observation that lungs locally produce complement proteins. Pulmonary alveolar type II epithelial cells generate proteins of the CP and AP, in particular C2, C3, C4, C5, and factor B (103). Besides that, human bronchiolar epithelial cells are able to synthesize C3 (104). However, literature on the mechanisms of the complement system in the lung donor and the contribution of local complement production in this pathophysiology is scarce. Which specific pathway should be the target of interest, and if treatment focused on those components will lead to improved graft survival without compromised defense mechanisms against pathogens, needs to be further investigated.

FUTURE PERSPECTIVES

The complement system regained new interest in the field of transplantation and the amount of acquired knowledge is increasing. The complement system was first studied in RTx, revealing its potential role in reperfusion injury and survival. However, new studies elucidated that the complement system already plays a role from the first step of the transplantation process, in the donor (33). Other studies showed that this damage accumulates throughout the rest of the transplantation process (81). Striving to restore an immunologically active organ before implementation in the recipient may have advantages, in favor of transplantation outcomes in patients. The complement system might be a potential therapeutic target for this purpose, and not only in the field of RTx. However, multiple knowledge gaps exist with regards to the role of the complement system throughout the transplantation process. Those need to be elucidated before therapeutics can be implemented in the clinical setting. For some organs, the pace of research developments can be complicated by the small number of transplants performed. The intestines, for example, has only been transplanted 6 times in 2017, as registered by the Eurotransplant International Foundation (**Figure 2**).

One of the questions that remains unanswered regarding complement-targeted interventions is the optimal timing of drug delivery to the donor graft. Different timing possibilities are (1) treatment in the organ donor, (2) during preservation, or (3) after implementation in the recipient. The main benefit of treating the organ donor is the opportunity to target the immunologically active state of the organs, nearly directly after the damage has occurred. However, it should be considered that all organs will be subjected to the same type and dose of treatment. An important disadvantage of this strategy is the risk that not all donor organs benefit from the same treatment. One organ might benefit from certain therapy, while the other organ might be even negatively affected. This is of particular importance for the liver, given the fine balance between injury and regeneration in this organ (63, 64).

The preservation state, however, provides a window of opportunity to treat the organ in an isolated manner. Various approaches are herein possible, which depends on the method of preservation. Cold static storage is the preservation technique with the longest history and is mostly used (1). Therapeutics can be added to the storage solution or organs can be infused just before cold static storage. The latter approach was demonstrated by Durigutto et al. who infused renal allografts with a targeted complement inhibitor, with beneficial results after reperfusion (49). However, the technique of cold storage is increasingly taken over by *ex vivo* perfusion systems. Various strategies in *ex vivo* perfusion are being practiced such as different perfusion solutions and perfusion temperatures (1). Nevertheless, in all *ex vivo* perfusion strategies blood flow through the organ is mimicked, thereby reducing ischemia times. Besides, oxygen and other additives can be supplemented to the perfusate solution in order to preserve or improve the quality of the organ, which provides opportunities for complement therapeutics as well. In the lungs, even *ex vivo* inhalation of therapeutics can be

considered as route of administration. An important benefit of treating the organ in an isolated manner is that a lower treatment dose might be required, especially in in organs with little metabolic activity. This might lower costs of complement-targeted therapies. However, little is known about the approach of treatment during preservation, especially with regards to the effect of treatment on donor-related injury. The few experimental studies that have been performed have mainly focused on preventing or diminishing IRI, by treating unharmed, "healthy" organs with complement inhibitors during the preservation phase (48, 56, 105). Therefore, the question whether inhibiting complement during the preservation phase has an effect on deceased donor-related injury as well, should still be investigated. Furthermore, it should be considered that the complement system might be activated by interaction with foreign materials (106).

Finally, treatment of the organ after implementation in the recipient should be considered as a possible timing of drug delivery. It should be emphasized that donor-related graft injury is amplified by the inevitable event of IRI (81). Beneficial effects of treatment applied in the organ donor or the isolated graft might not cover this second-hit of injury, occurring in the latter phase of the transplantation process. Application of complement-therapeutics in the recipient might tackle this issue, as demonstrated by Ferraresso et al. in a rat model for HTx. They showed that treatment of the recipient with anti-C5 therapy prior to reperfusion, prevented IRI-induced graft injury (107). Nevertheless, possible adverse side effects of complement therapeutics in the recipient need to be elucidated before implementation in the clinics. Finally, it can be considered to combine multiple time points of drug delivery, of which the net effects need to be further studied.

Another unanswered question is the translatability of results from one organ, to other organ systems. Especially since most knowledge on the involvement of complement is gained from research in deceased donor kidneys, it is questioned whether those findings apply to other organs as well. In order to answer this, more studies are needed that focus on unraveling the mechanisms of complement activation, specifically per organ and donor type. Especially, given the physiological dissimilarities between organs and the differences in mechanisms of pathophysiology between DBD and DCD donors. The role of the complement system has been underexposed mostly in DCD donation. Yet this will be of growing importance, since DCD donors are increasingly deployed in an attempt to expand the donor pool.

CONCLUSION

In conclusion, it has become evident that the complement system plays an important role in the donor, affecting all potential donor organs. To answer the question whether complement therapeutics should be clinically applied in the multi-organ donor, several uncertainties need to be elucidated first. These uncertainties include the timing, route of drug delivery and optimal target of complement therapeutics, complicated by dissimilarities in the pathophysiology of organs and differences between donor types. A tailor-made approach for each donor organ is pursued, aiming to improve the quality of donor grafts that possibly leads to an enlargement of the donor pool and improved outcomes after transplantation.

AUTHOR CONTRIBUTIONS

JvZ and NJ contributed to the conception and design of the work, drafting the work, and agree to be accountable for all the aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. MD, ME, HL, and MS revised the work critically for important intellectual content and provide approval for publication of the content.

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REVIEW
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Complement in Thrombotic Microangiopathies: Unraveling Ariadne's Thread Into the Labyrinth of Complement Therapeutics

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Thrombotic microangiopathies (TMAs) are a heterogeneous group of syndromes presenting with a distinct clinical triad: microangiopathic hemolytic anemia, thrombocytopenia, and organ damage. We currently recognize two major entities with distinct pathophysiology: thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). Beyond them, differential diagnosis also includes TMAs associated with underlying conditions, such as drugs, malignancy, infections, scleroderma-associated renal crisis, systemic lupus erythematosus (SLE), malignant hypertension, transplantation, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), and disseminated intravascular coagulation (DIC). Since clinical presentation alone is not sufficient to differentiate between these entities, robust pathophysiological features need to be used for early diagnosis and appropriate treatment. Over the last decades, our understanding of the complement system has evolved rapidly leading to the characterization of diseases which are fueled by complement dysregulation. Among TMAs, complement-mediated HUS (CM-HUS) has long served as a disease model, in which mutations of complement-related genes represent the first hit of the disease and complement inhibition is an effective and safe strategy. Based on this knowledge, clinical conditions resembling CM-HUS in terms of phenotype and genotype have been recognized. As a result, the role of complement in TMAs is rapidly expanding in recent years based on genetic and functional studies. Herein we provide an updated overview of key pathophysiological processes underpinning complement activation and dysregulation in TMAs. We also discuss emerging clinical challenges in streamlining diagnostic algorithms and stratifying TMA patients that could benefit more from complement modulation. With the advent of next-generation complement therapeutics and suitable disease models, these translational perspectives could guide a more comprehensive, disease- and target-tailored complement intervention in these disorders.

Keywords: thrombotic microangiopathy, complement inhibitors, hemolytic uremic syndrome, HELLP syndrome, transplant-associated thrombotic microangiopathy

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INTRODUCTION

Thrombotic microangiopathies (TMAs) represent a heterogeneous group of syndromes with the same phenotype: a clinical triad of microangiopathic hemolytic anemia (MAHA), thrombocytopenia and organ damage. This heterogeneous group of syndromes with considerable clinical overlap includes two major entities with distinct pathophysiology: thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (1). Besides these two well-defined clinical conditions, the TMA spectrum also includes pathologies associated with underlying conditions, such as drugs, malignancy, scleroderma-associated renal crisis, systemic lupus erythematosus (SLE), malignant hypertension, transplantation, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), and disseminated intravascular coagulation (DIC).

Since clinical presentation alone is not sufficient to differentiate between these entities, pathophysiological features need to be used for early diagnosis and appropriate treatment. Over the last decades, our understanding of the complement system has evolved rapidly leading to the characterization of diseases fueled by complement dysregulation that are also referred to as "complementopathies" (2). These are disorders in which activation of the complement system is a driving factor in disease pathophysiology and with evidence of effective complement inhibition in the disorder.

Among TMAs, atypical HUS has long served as an archetypal disease model of complement dysregulation, in which mutations of complement-related proteins represent the first hit of the disease and complement inhibition is an effective and safe strategy. Based on this knowledge, conditions resembling atypical HUS in terms of phenotype and genotype have emerged. As a result, the role of complement in TMAs is rapidly expanding in recent years due to genetic and functional studies (3). In an effort to facilitate early diagnosis and treatment, two recently published consensus documents have changed the terminology of these syndromes from an underlying disease-based model to a pathophysiology-driven model (4, 5). This review is based on the standardization of terminology proposed for TMAs by Scully et al. (5). Among others, this consensus has introduced the term complement-mediated HUS (CM-HUS) to describe discrete and also overlapping clinical entities with pronounced microangiopathic and thrombophilic manifestations which are shared by HUS and are likely underpinned by genetic alterations and/or functional derailment of the complement system leading to inflammatory damage of the glomerular endothelium (5).

Realizing the unmet needs of better understanding TMAs in this complex setting, this review aims to summarize current knowledge regarding complement activation in TMAs focusing on (a) complement-mediated HUS (b) infection-associated HUS, (c) HELLP syndrome, and (d) transplant-associated TMA. Emphasis will be placed on defining the clinical features that will enable complement modulation using new and emerging therapeutic options.

Box 1 | Differential diagnosis of TMAs.

1. ADAMTS13 deficiency
Thrombotic thrombocytopenic purpura
2. Complement dysregulation
Complement-mediated hemolytic uremic syndrome
3. Infection associated
Shiga-toxin
Campylobacter jejuni
Streptococcus pneumonia
Human immunodeficiency virus
Cytomegalovirus
Epstein–Barr virus
Parvovirus B19
BK virus
Influenza
4. Disseminated intravascular coagulation
5. Systemic lupus erythematosus
6. Antiphospholipid antibody syndrome
7. Scleroderma
8. Vasculitis/glomerulonephritis
9. Pregnancy
10. Malignant hypertension
11. Drugs
Calcineurin or mTOR inhibitors
Quinine
Estrogen/progesterone
Gemcitabine/mitomycin C
Interferon
Vascular endothelial growth factor inhibitors/tyrosine kinase inhibitors
Cocaine
12. Metabolic/cell signaling
Cobalamin responsive methylmalonic acidemia
Diacylglycerolkinase epsilon mutation
13. Malignancy
14. Transplantation

Pathophysiology of Complement Activation

Complement-Mediated Hemolytic Uremic Syndrome (CM-HUS)

Clinical features

Diagnosis of complement-mediated HUS (CM-HUS) remains a clinical diagnosis of exclusion. Although it has been traditionally considered a pediatric disease, onset occurs in adulthood for the majority of patients (6). The syndrome manifests with signs and symptoms of anemia, thrombocytopenia and acute kidney injury. Other complications have been also reported, including neurologic, pulmonary and gastrointestinal disorder, peripheral gangrene, arterial stenosis, dilated cardiomyopathy and cardiorespiratory arrest.

Differential diagnosis of CM-HUS requires exclusion of secondary causes of TMAs, such as DIC, drugs, malignancy, scleroderma-associated renal crisis, SLE. **Box 1** summarizes differential diagnosis in CM-HUS. In addition, shiga-toxin

testing is necessary to exclude infection-associated or typical HUS that will be further discussed. Then, the differential diagnosis lies between TTP and HUS, with ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13) activity being the only reliable clinical diagnostic tool. In patients with ADAMTS13 activity less than 10%, diagnosis of TTP is established. TTP is a TMA caused by impaired processing of ultra large von Willebrand factor multimers due to severe deficiency of ADAMTS13. Severe ADAMTS13 deficiency is either inherited (Upshaw-Schulman syndrome) (7, 8) or acquired and immune-mediated, resulting from autoantibodies directed against ADAMTS13 (9–11). Detection of ADAMTS13 inhibitors suggests that the disorder has an immune-mediated background. Due to disease pathophysiology, plasma exchange is an effective treatment of immune-mediated TTP that needs to be employed immediately (12, 13).

Based on this knowledge, common clinical criteria for CM-HUS include: (1) a serum creatinine level at or above the upper limit of the normal range, (2) microangiopathic hemolytic anemia, (3) thrombocytopenia, (4) ADAMTS13 activity of 5% or more, (5) and negative stool tests for Shiga toxin–producing infection (14). The majority of clinical studies in the field have adjusted these criteria to certain cut-off values to define HUS (15).

Functional evidence of complement activation

The wide appreciation that deregulated AP-mediated complement activation, both in the fluid phase and on the glomerular endothelial cell surface, has a definitive pathogenic role in CM-HUS and likely in other HUS-like TMAs, has been supported by a series of genetic studies and mechanistic investigations that have revealed fascinating roles of complement-derived effectors, as either disease drivers or exacerbators, in HUS-related pathologies.

The pathogenic role of complement in driving HUS-related pathologies is tightly linked to the distinct anatomical blueprint of the glomerular capillary network and the unique complement-activating properties and AP regulatory potential of the endothelial surface in the kidney, as compared to the microvasculature of other organs such as the brain, heart, or lung (16). The ability of endothelial surfaces to withstand autologous complement attack is also associated with the integrity of the sugar-rich glycocalyx that overlays these cells and its ability to recruit endogenous complement regulators, such as complement factor H (CFH), through their binding to polyanionic glycosaminoglycans comprising this specialized layer. In diseases such as CM-HUS where vascular endothelial/platelet activation disturb the homeostatic control of the endothelium, gradual deterioration of the glycocalyx through a sequence of thrombogenic and inflammatory insults exposes the underlying endothelial cell layer to a derailed intravascular complement system. This is particularly relevant to CM-HUS pathology as approximately two thirds of patients harbor mutations in complement genes, including loss-of-function variants in complement regulators such as membrane cofactor protein (MCP), CFH, and complement factor I (CFI) (17, 18). Loss of surface expression of MCP on endothelial cells, in conjunction with complement-activating insults such as the release of heme during mechanical hemolysis in the occluded vessels, appear to drive a vicious cycle of AP amplification and C3b deposition on the endothelial surface. The prevalent concept is that genetic defects in complement regulation in these patients shape a predisposing phenotype toward complement overactivation. This "at-risk" complement phenotype is then coupled to a secondary "hit" that can propagate complement activation in the kidney microvasculature (e.g., heme release), thus exacerbating the disease and fueling a vicious cycle that culminates in complement exhaustion and glomerular damage (19, 20). Notably, the clinical overlap between TTP and CM-HUS has nurtured the hypothesis that the susceptibility of the glomerular endothelium to complement attack might also be modulated by intrinsic determinants, such as the expression of soluble factors that promote platelet aggregation and microthrombi formation in the vasculature. In this direction, it has been suggested that the increased susceptibility of glomerular endothelial cells to complement activation might partly be attributed to the reduced release of von Willebrand Factor (vWF) which promotes complement deposition on the glomerular endothelial cells (21).

The strong penetrance of complement gene variants in CM-HUS is supported by genetic studies that have revealed a pathogenic role for certain complement haplotypes harboring mutations in C3, MCP, complement factor B (CFB) and CFH (22–28). Interestingly, certain C3 mutations in CM-HUS patients have been shown to code for gain-of-function C3 variants that form stable, decay-resistant C3 convertases, through high-affinity binding to CFB and conversely, through reduced binding to regulators that mediate their breakdown, such as MCP, CFH or complement receptor 1 (CR1), e.g., R139W/C3 mutant (26, 28). Of note, such disease-associated C3 variants are thought to drive HUS pathology, in the context of an overall compromised complement regulatory landscape (i.e., presence of at-risk CFH and MCP haplotypes), and consequent to a priming event that makes the renal endothelium more susceptible to C3 deposition and inflammatory damage (26, 29). Consistent to this notion, recent functional and *in silico* prediction studies have identified a number of gain-of-function CFB genetic variants that predispose for an overactive AP though stabilization of the C3 convertase, C3bBb, and increased resistance to decay by regulators such as FH (30). However, these findings cannot be generalized to all complement–related HUS/ TMA cases and caution should be exercised when attempting to classify such rare variants as disease-causing factors.

Several *in vitro* models have been utilized to demonstrate effects of complement activation in experimental studies. Endothelial cells play the central role in these models as the basic target cells of complement-induced damage in HUS. To be more specific, the effects of complement-induced damage have been demonstrated in glomerular, primary human umbilical vein, human microvascular and blood outgrowth endothelial cells (21, 26, 28, 30, 31). Although these assays are extremely useful in discerning the various cellular and molecular determinants of CM-HUS pathophysiology, their use as functional assays

in the daily routine of a diagnostic laboratory should only be considered in a broader context that also embraces a wide spectrum of genetic analyses and serological or other biochemical assays. Thus, selecting the appropriate functional assays to aid or refine the clinical diagnosis of CM-HUS remains a subject of intense investigation. In this respect, reliable functional assays of APC activation have long been sought after in the field of TMAs. Traditional markers used in clinical complement laboratories, such as hemolytic assays for measuring classical and alternative pathway activity (CH-50 and AP-50, respectively) and Wieslab ELISA for measuring C3 concentration or alternative pathway activity (Wieslab Complement System; Euro Diagnostica, Malmo, Sweden), may yield normal values and thus cannot confirm a diagnosis of CM-HUS (32).

Recently, terminal complement activation products C5a and soluble C5b-9 or membrane attack complex (MAC) were compared in CM-HUS and TTP. In spite of increased plasma C5a and C5b-9 levels in CM-HUS, there was a significant overlap of values between syndromes (33). Other studies have reported urine C5b-9 as a more reliable marker compared to plasma C5b-9 (34, 35). Translational studies have also found increased C5b-9 deposition on human microvascular endothelial cells (HMEC) by confocal microscopy in acute phase and remission of CM-HUS patients compared to controls (36). A most recent study has utilized C5b-9 deposition on HMEC to detect evidence of complement activation in patients with recurrent TMA after transplant (37).

In an effort to develop a rapid and reliable *in vitro* diagnostic assay for CM-HUS, the modified Ham test was introduced based on the principle of the Ham test traditionally used for paroxysmal nocturnal hemoglobinuria (PNH) diagnosis (38). As our understanding of complement-mediated disorders evolves, it seems that cell-based assays may better reflect complement activation *in vitro*. Interestingly, recent studies of CM-HUS associated with mutations in complement-related serum factors reveal that serum complement is not activated *per se*, but activation is caused as a result of defective interaction of complement regulatory proteins, such as factor H, with cell surfaces (39). The modified Ham test utilizes PNH-like cells that are susceptible to complement-mediated cell death to detect complement activated serum, like CM-HUS serum. It has been documented that the modified Ham test distinguishes complement-mediated TMAs from other TMAs (38, 40, 41). Further improvements on the positive control of the modified Ham test and the addition of a confirmatory assay with a strong correlation with the modified Ham test are expected to further improve its accuracy and applicability (42). However, it is a rather cumbersome assay for clinical laboratories and cannot be recommended for clinical routine yet.

Unfortunately, no direct comparison among assays has been performed and therefore, no safe conclusions can be drawn for the usefulness of each assay. Soluble C5b-9 levels, C5b-9 deposits on endothelial cells and the modified Ham test have shown evidence of complement activation in patients with or without mutations or autoantibodies (36, 38, 43). The modified Ham test has also shown a 100% positive predictive value for response to eculizumab (43). However, the modified Ham test and C5b-9 depositions require cell culture techniques, while measurement of soluble C5b-9 has a wider applicability. Standardization is another important issue, especially for cell-based assays. Although the modified Ham test offers a clear cut-off value to differentiate CM-HUS from TTP, soluble C5b-9 levels have a significant overlap between HUS and TTP (33); while no comparison exists for surface C5b-9 deposition (36). In addition, soluble C5b-9 and the modified Ham test have limited applicability on discriminating patients in acute phase vs. remission, although more data on C5b-9 deposition might prove useful in this field. As suggested by initial reports, monitoring and guiding eculizumab treatment may be feasible by measuring C5b-9 deposition on endothelial cells (36) and soluble C5b-9 in the vasculature (35). Lastly, testing the effects of novel complement inhibitors is feasible by employing the modified Ham test (44) and measuring C5b-9 deposition (36).

Genetic evidence of complement activation

Genetic testing for CM-HUS is also difficult to utilize in clinical practice. Its major limitations include the high cost and the time consuming process in an urgent life-threatening situation that requires immediate treatment (45). In addition, genetic testing needs to be performed by an expert complement-focused laboratory able to analyze the results of next-generation sequencing (NGS). NGS analysis produces a large number of variants in each patient that needs to be carefully interpreted. Current methodology reports only rare variants, since these are considered to be pathophysiologically linked to the development of rare syndromes such as TMAs. In an effort to better understand the clinical significance of rare variants, helpful databases are created, such as the Database of Complement Gene Variants, that could be an integrated part of bioinformatics analysis in the future (46). However, this approach does not take into account the majority of detected variants, whose functional and clinical significance remains to be studied. Even in this case, genetic testing is useful for about half of the patients with CM-HUS that are expected to harbor a mutation in any of the complement-related genes already implicated in aHUS (27, 47).

Pathophysiology of complement activation

CM-HUS is characterized by excessive activation of the alternative pathway of complement (APC) and its pathophysiology is currently described by a two-hit disease model. The first hit results from inherited mutations in APC genes or from acquired alterations in APC activity, such as autoantibodies to APC proteins (anti-CFH antibodies) (5, 48). Commonly associated triggers of the second hit are considered crucial for the manifestation of the disease and include pregnancy, inflammation, surgery or autoimmunity (29, 49, 50).

Mutations cause either loss of function of complement regulatory proteins, including complement factor H (CFH), complement factor I (CFI), thrombomodulin (THBD), or CD46/membrane cofactor protein (MCP), or gain of function of complement activating proteins, including complement factor B (CFB) and C3 (51). Although THBD may also act as a

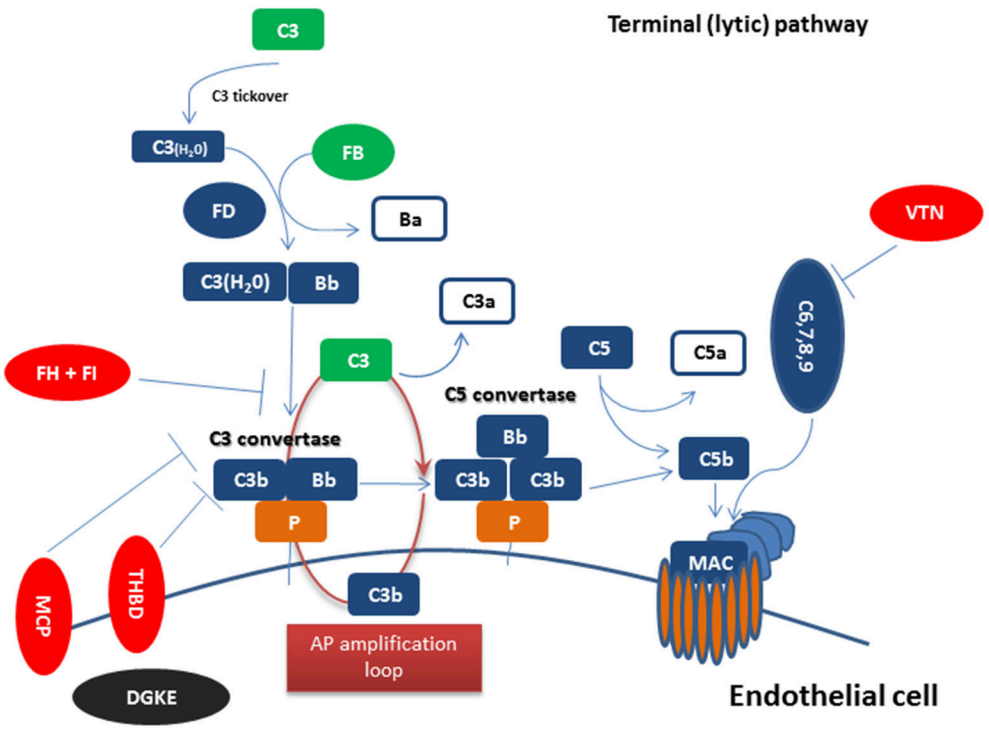

FIGURE 1 | Complement dysregulation in complement-mediated HUS Complement activation initiated by any of the three pathways (classical, alternative, or lectin pathway) leads to C3 activation and C3 convertase formation on C3-opsonized surfaces. C3 activation through the alternative pathway of complement (APC) amplifies this response (APC amplification loop), culminating in pronounced C3 fragment deposition on complement-targeted surfaces (proximal complement). In the presence of increased surface density of deposited C3b, the terminal (lytic) pathway is triggered, leading to membrane attack complex (MAC) formation on the surface of target cells. Dysregulated or excessive complement activation mainly affects renal endothelial cells which show increased susceptibility to complement attack due to a deteriorating glycocalyx in pathologies such as CM-HUS (terminal complement). Complement alternative pathway dysregulation results from loss-of-function mutations in regulatory factors (Factor H, I, THBD/thrombomodulin, and vitronectin/VTN in aHUS) shown in red, gain-of-function mutations (C3 and Factor B) shown in green, and DGKE mutations shown in black, indicating the unknown effect on complement cascade.

complement regulator (52), further studies are needed to confirm the role of proteins involved in the coagulation pathway (48). A most recent study has also revealed mutations in *VTN*, which encodes the terminal complement inhibitor vitronectin, in CM-HUS patients (17). The only mutations not associated with complement dysregulationre found in diacylglycerol kinase-e (DGKE) (53, 54). Therefore, HUS associated with DGKE mutation is not classified under the terms complement-mediated HUS or HUS with activation of the complement alternative pathway (5, 48).

Figure 1 summarizes complement dysregulation observed in CM-HUS. Except for rare germline mutations predisposing to CM-HUS, common genetic variants in CFH, CD46, and the CFHRs have been also reported as risk factors for CM-HUS (25). Mutations in CFH related genes occur in up to 5% of the general population (55). Interestingly, genetic mutations are found in 50% of patients diagnosed with CM-HUS, while a number of these mutations has uncertain clinical significance as discussed above.

INFECTION-ASSOCIATED HEMOLYTIC UREMIC SYNDROME (IA-HUS)

Clinical Features

Infection-associated (IA) or typical or Shiga-toxin-secreting *Escherichia coli* (STEC) HUS represents a TMA of infectious etiology presenting mainly in children infected with Shiga-toxin-secreting *Escherichia coli* 0157:H7. Other subtypes of *E. coli*, *Salmonella*, *Shigella,* and *Campylobacter* have been also detected in IA-HUS patients (56). Diagnosis of IA-HUS is confirmed by the presence of an enterohemorrhagic strain of E. coli and/or identification of *Stx1* or *Stx2* genes in the stool sample or rectal swab. Two recent case reports have also identified Bordetella pertussis infection as a trigger of IA-HUS (57, 58).

Clinical manifestations span a wide spectrum from uncomplicated diarrhea to hemorrhagic colitis and post diarrheal HUS. HUS manifestations include MAHA, thrombocytopenia and acute kidney injury, while neurological and cardiac involvement may be also be present in severe forms. Long-term

renal involvement has been documented in 30% of surviving patients (59, 60), with mortality rates up to 5% in patients developing HUS (61). Neurologic involvement, anemia, and hyponatremia have been recently described as predictors of mortality in IA-HUS (62).

Functional and Genetic Evidence of Complement Activation

Evidence from human (63–66) and animal (67–69) studies have suggested that complement activation may play a role in the course of IA-HUS. However, the epidemic nature of the disease hinders functional studies and the role of complement has not been fully characterized. In addition, the largest genetic analysis so far implicates variants mostly in several non–complement-related genes involved in iron transport, cytokine signaling, platelet function, pathogen recognition, and endothelial function and less in complement-related genes (70). However, this analysis focuses on single nucleotide polymorphisms (SNPs) and not on rare variants found in TMAs.

HELLP SYNDROME

Clinical Features

Preeclampsia is characterized by hypertension and proteinuria, with or without end organ damage occurring in 3–5% of pregnant women (71). HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) manifests as a severe form of preeclampsia. Since 1982, HELLP syndrome has been reported in up to 0.8% of all pregnancies (72). Diagnostic classifications take into account platelet count, lactate dehydrogenase (LDH) levels, bilirubin and aspartate aminotransferase (AST) with or without alanine aminotransferase (ALT) levels to establish the diagnosis (73, 74). Clinical manifestations resemble CM-HUS, including MAHA, thrombocytopenia, hypertension and renal or neurological dysfunction. Due to these similarities, HELLP syndrome might be often characterized as pregnancy-associated HUS, according to the traditional terminology, and vice versa (75). Of note, HELLP syndrome is a cause of severe morbidity and mortality for both the mother and fetus (76).

Functional and Genetic Evidence of Complement Activation

The pathophysiology of HELLP syndrome has not been fully elucidated yet, although it has been traditionally considered to be part of the spectrum of preeclampsia (77). Since 1990, Haeger et al. have suggested increased complement activation in HELLP syndrome with increased C5b-9 levels (78). Urine C5b-9 levels have been more recently considered a more robust marker in patients with preeclampsia (34). The modified Ham test has also provided data of increased APC activation in severe preeclampsia and HELLP syndrome. In addition, the modified Ham test was also used as an *in vitro* model suggesting that eculizumab effectively blocks complement-mediated effects in HELLP serum (40). APC-related mutations have been reported in up to 20% of HELLP patients, suggesting that APC dysregulation might be a distinct contributor to HELLP pathology (79). A more recent study has confirmed these findings reporting APC-related rare germline mutations in 46% of patients with HELLP syndrome. The authors were also able to show that combined complement-related phenotypes and genotypes were highly predictive of HELLP syndrome (41).

TRANSPLANT-ASSOCIATED TMA

Clinical Features

TA-TMA is a potentially life-threatening complication of allogeneic hematopoietic cell transplantation (HCT) observed in 7–39% of HCT recipients (80–85). It manifests with the clinical triad of a TMA including MAHA, thrombocytopenia and often renal or neurologic dysfunction. Its diagnosis is largely hindered by the high incidence of cytopenias and organ dysfunction in HCT recipients. Currently used diagnostic criteria include both the Bone Marrow Transplant Clinical Trials Network (BMT-CTN) and the International Working Group (IWG) criteria (80, 86). Both criteria have been criticized for limitations in their diagnostic sensitivity (87).

Functional and Genetic Evidence of Complement Activation

Our understanding of TA-TMA pathophysiology is rapidly evolving. Initially, the syndrome was considered and treated as a form of TTP. However, plasma exchange had limited efficacy in these patients (88, 89). In line with these findings, studies have shown that ADAMTS13 is not deficient in TA-TMA and therefore, cannot be used as a disease marker (90, 91). Better understanding of CM-HUS has helped researchers and clinicians understand that TA-TMA resembles more CM-HUS than TTP both in pathophysiological and clinical features (92). This notion is supported by genetic and functional evidence of complement activation in TA-TMA. Jodele et al have first described APC-related mutations in pediatric HCT recipients (93), with additional data of poor prognosis in patients harboring APC-related mutations (94). Data of genetic susceptibility support the idea of the two-hit hypothesis being true also for TA-TMA.

The second hit may result from several clinical factors that have been associated with TA-TMA, including age, donor type, conditioning regimen, calcineurin or mTOR inhibitors, graft-vs. host disease or infections (81, 82, 95–100). Although several cross-sectional associations have been reported, a few reports have investigated underlying mechanisms. Of note, complement dysregulation has been implicated in GVHD regulation of mice (101–103) and humans. Additional links between GVHD and complement activation have been shown in human cutaneous tissues, where C3 inhibition by compstatin reduced $CD4^+$ T-cell proliferation and Th1/Th17 polarization (104). A recent *in vitro* study has also implicated the C5a/C5aR IL-17A axis in chronic GVHD (105). Furthermore, C3 levels have been associated with sclerotic cutaneous GVHD patients (106) and patients with sclerotic GVHD have shown abnormalities in complement factor H and APC functional assays (107). Complement activation has been also linked with thrombin generation in patients treated with antithymocyte globulins (ATG) (108).

IMPACT OF THE PRODUCTS OF C3 VS. C5 CONVERTASE ACTIVITY IN THE CONTEXT OF CM-HUS AND OTHER TMAs

Complement dysregulation at the level of the formation of the C3 and C5 convertases can exacerbate the pathological process by exerting a plethora of detrimental effects that altogether contribute to TMA pathology. For instance, AP amplification leads to pronounced C3 fragment deposition (opsonization) on the endothelial cell wall thus facilitating the recruitment of innate immune cells such as macrophages or monocytes bearing CR3/CR4 phagocytic receptors (109, 110). Additionally, upregulation of P–selectin on endothelial cells in CM-HUS kidneys, may provide a tether for focusing AP convertases and further fueling the AP amplification loop on C3-opsonized endothelial cells (68). Notably C3 can serve as direct binding target for heme released from erythrocytes by mechanical hemolysis within the renal microvascular network (29, 111). Heme has been shown to intercalate into C3 molecules promoting homophilic C3 interactions and subsequent formation of overactive C3/C5 convertases (29). Moreover, the release of C3a in the vicinity of the opsonized endothelium may stimulate the C3aR-dependent activation and recruitment of neutrophils, basophils or mast cells that can cause endothelial damage through degranulation and release of free radicals and proinflammatory mediators (112, 113). Furthermore, C3aR stimulation on the renal endothelium in a murine model of STEC-HUS has been linked to increased thrombogenic responses that can facilitate microthrombi formation and vaso-occlusion (68). Additionally, glomerular endothelial cells appear to be particularly sensitive to complement C3a responses via upregulation of C3aR expression in response to various inflammatory stimuli, thus providing an alternate route for C3aR-dependent endothelial cell activation and damage (16). On the other hand, an overactive C5 convertase may lead to massive release of C5a, a highly proinflammatory mediator that, through neutrophil activation and chemotactic recruitment, can exacerbate endothelial damage (114). Notably, C5a can also promote a thrombogenic response via tissue factor (TF) upregulation and release from either infiltrating monocytes/neutrophils or the renal endothelium itself (26, 115). Furthermore, C5a-mediated stimulation of microvascular endothelial cells may also result in increased cell retraction, promoting paracellular permeability (116). The terminal product of the lytic pathway, C5b-9 (MAC) is known for its capacity to trigger endothelial activation, promoting neutrophil adhesion via P-selectin and ICAM-1 upregulation, and it also enhances vascular permeability, via endothelial cell contraction and gap formation (117, 118). Targeting the complement cascade at the level of the C3 convertase may attenuate endothelial C3b opsonization and thus prevent the adhesion of damaged red blood cells and activated platelets to the endothelium via tethering to neutrophil-expressed CD11b/CD18 (119). Therapeutic C3 inhibition may also reduce the release of C3a, thus blunting its procoagulant activities in the microvascular network of the kidney. Of note, C3 blockade can also prevent the downstream generating of C5-derived inflammatory effectors (C5a, C5b-9), thereby offering a much broader inhibitory strategy for ameliorating pathological changes in the context of CM-HUS and other complement-related TMAs (120).

COMPLEMENT THERAPEUTICS

CM-HUS

Current Treatment

CM-HUS is an urgent life-threatening syndrome requiring prompt initiation of therapy. Plasma exchange should be initiated at presentation, often before the results of differential diagnosis are available due to the aggressiveness of the syndrome. Although plasma exchange is effective in some patients underlying complement-mediated damage to kidneys and central nervous system often persists (121). Within 1 year from diagnosis, more than 50% of patients treated with plasma exchange or plasma infusion develop permanent renal damage, progress to end-stage renal disease or die (122). Affected patients may suffer from lifelong systemic complications causing multiple organ damage of (renal, gastrointestinal, central nervous system, cardiac) and death.

In recent years, clinical complement intervention has revolutionized the field, with a diverse array of complement-targeted drug candidates currently being evaluated in clinical trials as treatment options for several complement-mediated indications (123). The first-in-class complement inhibitor, eculizumab, a complement C5-targeting monoclonal antibody that blocks generation of C5a and prevents the assembly of the pore-forming MAC, marked a milestone in therapeutic complement inhibition, showing efficacy and safety in two prospective clinical trials of primary adult CM-HUS patients (15, 124). These studies led to the FDA approval of eculizumab for the treatment of atypical HUS in 2011. Eculizumab has shown high efficacy with sustained benefits in 2-year follow-up data and good safety profile in both children and adults (124–126). More recent open label studies have also shown high efficacy rates of approximately 70% and no eculizumab related death (127–129). Although experience with eculizumab raised confidence in the approach, its clinical use in aHUS has revealed limitations that warrant further investigation.

Given the lack of a confirmatory diagnostic assay and the high cost of the drug, therapy is often delayed or not administered. Commonly used criteria of plasma exchange failure include: (1) failure to achieve hematologic response (improvement in platelet count and decrease in the LDH) over the first 4–5 days, (2) progressive end organ injury (renal and/or neurologic) over the first 4–5 days of PEX therapy, and (3) ADAMTS13 activity higher than 10% (130, 131). However, clinical application of these criteria is not always straightforward. Even in CM-HUS patients, mild hematologic response is often observed after 4–5 plasma exchange sessions, leading to clinical dilemmas for treating physicians. The fact that response to eculizumab is often used to confirm CM-HUS diagnosis is indicative of the diagnostic difficulties in the

disease (132). Another important issue for clinicians is the risk of Neisserial infections due to terminal complement blockade. Thus, all patients treated with eculizumab should receive anti-meningococcal vaccination at least 2 weeks before initiation of treatment. In severe life-threatening syndromes, such as TMAs, experience from PNH patients has shown that vaccination and eculizumab can be administered the same day along with 2 weeks of prophylactic treatment with ciprofloxacin (133). In immunocompromised patients, such as those with transplant-associated TMA, administration of eculizumab has been safe and effective without anti-meningococcal vaccination (134).

Last, accumulating evidence suggests that cessation of treatment is feasible in the majority of patients. Studies of eculizumab cessation have enrolled 91 patients. Among them, 27 (approximately 30%) relapsed (135–139). The majority of patients had a known mutation. Recurrences have been also reported in individual patients: 1 with complement factor H mutation (140), 1 with C3 mutation (141), and 1 after kidney transplantation (142). In all cases, close patient monitoring led to prompt re-initiation of treatment and complete recoveries. An alternative strategy toward restrictive use of eculizumab includes prolongation of time intervals between dosages (143). However, this requires either therapeutic drug monitoring or measuring of the pharmacodynamics effect that are not available in all centers. Further studies are needed to determine the high-risk patient population prone to relapse or a widely applicable assay for monitoring patients. Until then, close surveillance for signs and symptoms of recurrent TMA is recommended if physicians and patients decide to discontinue eculizumab.

Next-Generation Complement Therapeutics

A plethora of novel complement inhibitors is on the horizon, with some being in late clinical development (123, 144, 145). **Table 1** summarizes novel complement inhibitors currently being evaluated in pre-clinical and clinical phases. The choice of the appropriate target and drug has to be carefully considered taking into account the limitations of eculizumab treatment. To accomplish that, an important question needs to be answered. Is there a need for novel complement inhibitors in CM-HUS?

Unlike PNH, the medical community has failed to recognize a specific sub-group of CM-HUS patients that are likely to benefit from novel complement inhibitors. Eculizumab's limitations include the requirement of life-long intravenous infusions every 2 weeks and the substantial economic burden associated with chronic anti-C5 treatment. To overcome these limitations, a number of novel C5 inhibitors have been introduced that are either long-lasting or administered in a subcutaneous or oral form. Such inhibitors are currently investigated in CM-HUS patients: Cemdisiran (an investigational RNAi therapeutic targeting complement component C5 administered subcutaneously every 4 weeks), ALXN1210 (a longer-lasting intravenously administered C5 inhibitor) and Avacopan (an orally-administered small molecule inhibitor of C5a receptor 1), as highlighted in **Table 1**.

From the pathophysiological point of view however, C5 inhibition with alternative agents is not expected to lead to significant advantages in efficacy and safety compared to eculizumab. Therefore, alternative targets in proximal complement pathways that efficiently inhibit the alternative complement pathway involved in HUS may provide advantages in HUS patients not only in terms of higher efficacy but also in terms of potentially less infectious complications. Although the effects on infectious complications need to be further studied, early data suggest that inhibitors of the alternative pathway leave the classical and lectin pathways active against invasive pathogens (146). Furthermore, it should be noted that the opsonophagocytic killing of meningococci in whole blood from vaccinated volunteers was recently shown to be increased in the presence of an alternative pathway-directed inhibitor (anti-FD agent, ACH-4471) as compared to anti-C5 treatment, thereby suggesting that vaccination may provide better protection against meningococcal disease in patients treated with an AP-specific inhibitor (147). Targets of the alternative pathway may include factors B, D, and the alternative pathway C3 convertase. Inhibitors of these agents under development for complement-mediated indications are summarized in **Table 1**. Both factor B inhibitor LPN023 and factor D inhibitor ACH-4471 have the additional benefit of oral administration which is particularly useful for lifelong administration. Both inhibitors are currently under phase II clinical trials: factor B inhibitor in IgA nephropathy and PNH, factor D inhibitor in C3G nephropathy and PNH.

Recently, the clinical advancement of C3-targeted inhibitors of the compstatin family has opened up new avenues for exploring viable anti-complement therapies in various clinical indications, including complement-mediated TMAs (148). Of note, the C3-targeted peptide therapeutics APL-2 and AMY-101, both developed on the same compstatin scaffold with various modifications aimed at increasing plasma residence and target affinity, are being evaluated in Phase Ib-III trials in clinical indications ranging from PNH and autoimmune hemolytic anemias to geographic atrophy and C3 glomerulopathies (123, 145). Thus, far, C3-targeted therapeutic agents have displayed safety, tolerability, preliminary biological efficacy and a capacity to saturate plasma C3 levels during prolonged dosing in humans, thus supporting the investigational use of C3 inhibitors in human clinical trials. By affording broader and comprehensive inhibition of the complement cascade, regardless of initiating trigger or pathway, C3 inhibitors can simultaneously intercept multiple pathogenic drivers in complement-mediated TMAs. Of note, the contextual nature of C3's involvement in pathogen immunosurveillance and bacterial outgrowth (149), the ability to swiftly recover C3 activity in plasma after interrupting treatment with small-sized C3 inhibitors (as compared to the slower plasma clearance of larger biologics) and the likely auxiliary role of C3 in immunosurveillance during adulthood, as supported by observations in younger patients with primary complement deficiencies (150), all argue against the long-held assertion that prolonged pharmacological C3 intervention might increase the risk of infections in treated patients. Furthermore, the stable immune and blood biochemical profile of non-human primates (NHP) subjected to prolonged, systemic C3 inhibition, together with the faster skin wound healing and absence of skin infections in NHPs treated with the C3 inhibitor Cp40, further attest to the safety of this targeting approach (151).

TABLE 1 | Complement-targeted therapeutics in various stages of clinical development for complement-mediated indications.

Complement therapeutic	Entity	Target	Mechanism of action	Mode of administration	Pharmaceutical sponsor	Stage of development
Eculizumab*	mAb	C5	Inhibition of C5 activation	IV infusions	Alexion Pharmaceuticals	In the clinic
ALXN1210/ ravulizumab*	mAb	C5	C5 inhibition/same epitope as ecu	Bimonthly IV infusions	Alexion Pharmaceuticals	Phase II/III
ABP959	mAb	C5	Inhibition of C5 activation/biosimilar of ecu	IV infusions	Amgen	Phase III
SKY59/RO7112689	mAb	C5	Inhibition of C5 activation/different epitope from ecu	IV and SC injections	Hoffmann-La Roche	Phase I/II
LFG-316/ tesidolumab	mAb	C5	Inhibition of C5 activation/different epitope from ecu	IV infusions	Novartis	Phase II
REGN3918	mAb	C5	n.a.	IV and SC	Regeneron	Phase I
Mubodina	Minibody (Fab-based)	C5	C5 inhibition/ different epitope from ecu	n.a.	Adienne	Preclinical stage
Coversin (OmCI)	Recomb. protein	C5	Inhibition of C5 activation	SC injections	Akari Therapeutics	Phase II
RA101495	Peptide	C5	Allosteric inhibition of C5 activation	Daily SC injections	Ra Pharmaceuticals	Phase II
Cemdisiran (ALN-CC5)*	siRNA	C5	Silencing of hepatic C5 production	SC injections	Alnylam	Phase II
AMY-101	Peptide	C3	C3 inhibition/ blockage of C3 convertase activity	Daily SC injections	Amyndas Pharmaceuticals	Phase I completed/Phase II announced
APL-2	PEGylated peptide	C3	C3 inhibition/ blockage of C3 convertase activity	Daily SC injections	Apellis Pharmaceuticals	Phase II, (Phase III announced)
Mini-FH/AMY-201	Recomb protein	AP C3 convertase	Surface directed inhibition of AP	n.a.	Amyndas Pharmaceuticals	Preclinical stage
LNP023	Small Molecule	Factor B	Inhibition of AP C3 convertase formation	Orally	Novartis	Phase II
IONIS-FB-LRx	Antisense oligonucleotide	Factor B	Inhibition of AP C3 convertase formation	SC injections	Ionis Pharmaceuticals/ Roche	Phase II (announced)
ACH-4471/ACH-0144471	Small molecule	Factor D	Inhibition of AP C3 convertase formation	Orally	Achillion Pharmaceuticals	Phase II
Lampalizumab	mAb (Fab)	Factor D	Inhibition of AP C3 convertase	Intravitreal inj.	Genentech/Roche	Phase III (discontinued)
CLG561 (Novartis)	mAb	Properdin	Inhibition of Alternative pathway	Intravitreal injections	Novartis	Phase II
TNT009/BIVV009/ Sutimlimab	mAb	C1s	CP inhibition/inhibition of C1s protease activity	IV infusions	True North Therapeutics/ Bioverativ/Sanofi	Phase II/III (CAD patients)
OMS721	mAb	MASP-2	Inhibition of Lectin P activation	IV	Omeros corporation	Phase III
Mirococept (APT070)	Recomb. protein	C3/C5 convertases	Inhibition of both CP and AP convertases	IV	MRC (UK)	Phase III
Avacopan (CCX168)*	Small molecule	C5aR1	Inhibition of C5aR1 signaling	Orally	Chemocentryx	Phase III
IFX-1	mAb	C5a	Blocks biological activity of C5a	IV	(InflaRx)	Phase II

*IV, intravenous; SC, subcutaneous; CP, classical pathway; AP, alternative pathway; siRNA, small interfering RNA; n.a., not available; *Components in clinical development for complement-mediated TMAs.*

In conclusion, whether inhibitors of the alternative pathway of complement can offer an effective and safe treatment in complement-mediated TMAs will be ultimately determined in future clinical trials. To prove the efficacy of novel complement inhibitors, experimental models of complement activation might be a useful step before clinical application (44).

IA-HUS

The care of children with IA-HUS remains supportive, with no established targeted therapies. Debated approaches include plasma exchange, plasma infusions, immunoadsorption and antibiotics. Terminal complement inhibition by eculizumab has also been used in IA-HUS with controversial results and reports suggesting some benefit (152, 153), while others stating no benefit (56, 154). A large multicenter retrospective study has recently tried to shed light on the role of terminal complement inhibition (155). Despite limitations linked to the nature of the syndrome and methodology, this study suggested that eculizumab is effective in patients with neurological dysfunction and patients with sustained complement inhibition. In addition, experimental evidence of the driving role of C3-mediated thrombosis in IA-HUS suggest that proximal complement inhibition at the level of C3 might provide additional advantages in these patients (68). Indeed, the beneficial effect of C3aR antagonism on attenuating thrombogenic responses in a rodent model of STEC-HUS indicates that upstream complement intervention at the level of C3 might offer broader therapeutic coverage in IA-HUS. C3 inhibitors could likely interfere with multiple pathogenic drivers in IA-HUS by simultaneously blocking the generation of C3-derived proinflammatory effectors (i.e., C3a) and also by attenuating the generation of terminal (lytic) pathway effectors that contribute to microvascular endothelial injury and inflammation (i.e., C5a, C5b-9) (68). It is noteworthy that a broader role for C3a-C3aR signaling in modulating thrombogenic responses (i.e., NETosis-driven hypercoagulation) has been documented in diverse pathologies including intestinal cancer (156). Taking into account these considerations, prospective controlled studies are expected to provide more insight into the role of complement inhibition in IA-HUS (**Table 1**).

HELLP

Current treatment strategy remains supportive in HELLP syndrome, consisting of steroid and magnesium administration and proper hypertension management (71, 157, 158). The treatment of choice is delivery, taking into account that neonatal morbidity and death are mainly associated with gestational age (158). The first successful use of targeted anti-complement treatment (i.e., eculizumab) was reported in 2013, suggesting that eculizumab permits safe prolongation of pregnancy and successful outcomes for both the mother and the fetus (159). Indeed, eculizumab's use in PNH patients has proven safety and efficacy during pregnancy (160). Nevertheless, further studies are needed to explore complement inhibition in HELLP syndrome. Of note, the frequent presence of germline APC gene mutations in HELLP patients, along with a pronounced activation of the APC in patient sera (41), both suggest that therapeutic modulation of the AP, or targeted inhibition of its central protein C3, might be a promising new avenue to alleviate pathology in these patients.

TA-TMA

The majority of TA-TMA patients are refractory to conventional treatment, leading to high mortality rates (up to 100%, median 75%) (161). Depending on each clinical center's policy conventional treatment includes withdrawal of calcineurin inhibitors, corticosteroids, plasma exchange or rituximab. Novel approaches have investigated eculizumab treatment in both adult and pediatric patients with TA-TMA (134, 162–164). Although results are encouraging compared to mortality rates in the pre-eculizumab era, timing of initiation, proper patient selection, dosing and duration of therapy remain to be further investigated in this complex field of transplanted patients. In light of the emerging correlation of TA-TMA pathology with distinct genetic aberrations in the APC, and the central role of C3 in amplifying APC activation, it is intriguing to speculate that therapeutic strategies targeting upstream complement components, or centrally the C3 protein, may elicit more beneficial therapeutic outcomes in TA-TA patients. Therefore, the clinical evaluation of novel complement-based drug candidates that are registered in the pipelines of several biopharmaceutical companies (see **Table 1**), is highly anticipated in patients with TA-TMA. Despite lack of pathophysiological evidence suggesting activation of the lectin pathway, an inhibitor of the lectin pathway, the MASP-2 inhibitor OMS721, has shown encouraging results in a preliminary analysis of a phase II study in TA-TMA patients (**Table 1**). However, these results were compared to dramatic outcomes of a historical control group treated only by conventional treatment.

CONCLUSION

Complement-mediated TMAs represent a rapidly evolving field aiming to provide better outcomes for patients with benign, yet life-threatening syndromes. Unraveling Ariadne's thread into the labyrinth of complement therapeutics is challenging, especially when novel agents are continuously studied and novel patient groups are identified. Beyond TMAs analyzed in this review, preliminary data suggest complement dysregulation in more entities, such as TMAs associated with lupus nephritis (165). Better understanding of the role of complement dysregulation in these entities will facilitate diagnosis, promote patient stratification into cohorts that may optimally respond to therapeutic modulation of the complement system and provide effective therapeutic options for treating physicians.

AUTHOR CONTRIBUTIONS

EG and DM conceived the review topic, wrote the outline, and the manuscript. AA edited and approved the manuscript.

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REVIEW
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Therapeutic Inhibition of the Complement System in Diseases of the Central Nervous System

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The complement system plays critical roles in development, homeostasis, and regeneration in the central nervous system (CNS) throughout life; however, complement dysregulation in the CNS can lead to damage and disease. Complement proteins, regulators, and receptors are widely expressed throughout the CNS and, in many cases, are upregulated in disease. Genetic and epidemiological studies, cerebrospinal fluid (CSF) and plasma biomarker measurements and pathological analysis of post-mortem tissues have all implicated complement in multiple CNS diseases including multiple sclerosis (MS), neuromyelitis optica (NMO), neurotrauma, stroke, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). Given this body of evidence implicating complement in diverse brain diseases, manipulating complement in the brain is an attractive prospect; however, the blood-brain barrier (BBB), critical to protect the brain from potentially harmful agents in the circulation, is also impermeable to current complement-targeting therapeutics, making drug design much more challenging. For example, antibody therapeutics administered systemically are essentially excluded from the brain. Recent protocols have utilized "Trojan horse" techniques to transport therapeutics across the BBB or used osmotic shock or ultrasound to temporarily disrupt the BBB. Most research to date exploring the impact of complement inhibition on CNS diseases has been in animal models, and some of these studies have generated convincing data; for example, in models of MS, NMO, and stroke. There have been a few recent clinical trials of available anti-complement drugs in CNS diseases associated with BBB impairment, for example the use of the anti-C5 monoclonal antibody (mAb) eculizumab in NMO, but for most CNS diseases there have been no human trials of anti-complement therapies. Here we will review the evidence implicating complement in diverse CNS disorders, from acute, such as traumatic brain or spine injury, to chronic, including demyelinating, neuroinflammatory, and neurodegenerative diseases. We will discuss the particular problems of drug access into the CNS and explore ways in which anti-complement therapies might be tailored for CNS disease.

Keywords: complement, therapeutics, CNS, neurodegeneration, injury

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INTRODUCTION

The Central Nervous System (CNS) as a Distinct Environment

The CNS was, for a long time, considered an immunologically privileged organ because the brain and spinal cord are protected from circulating inflammagens by the BBB. The BBB is a specialized membrane comprised of endothelial cells with tight junctions, vascular pericytes and perivascular glia (**Figure 1A**), which cooperate to form a selectively permeable barrier, protecting the CNS from fluctuating ion concentrations and circulating neurotransmitters, macromolecules, large proteins such as complement, and pathogens (1). However, isolation of the CNS is not absolute and there are a number of pathways by which systemic inflammation can be communicated to the CNS [reviewed; (2)]. Indeed, the recent demonstration of a CNS lymphatic system further undermines the concept of brain immunological privilege (3).

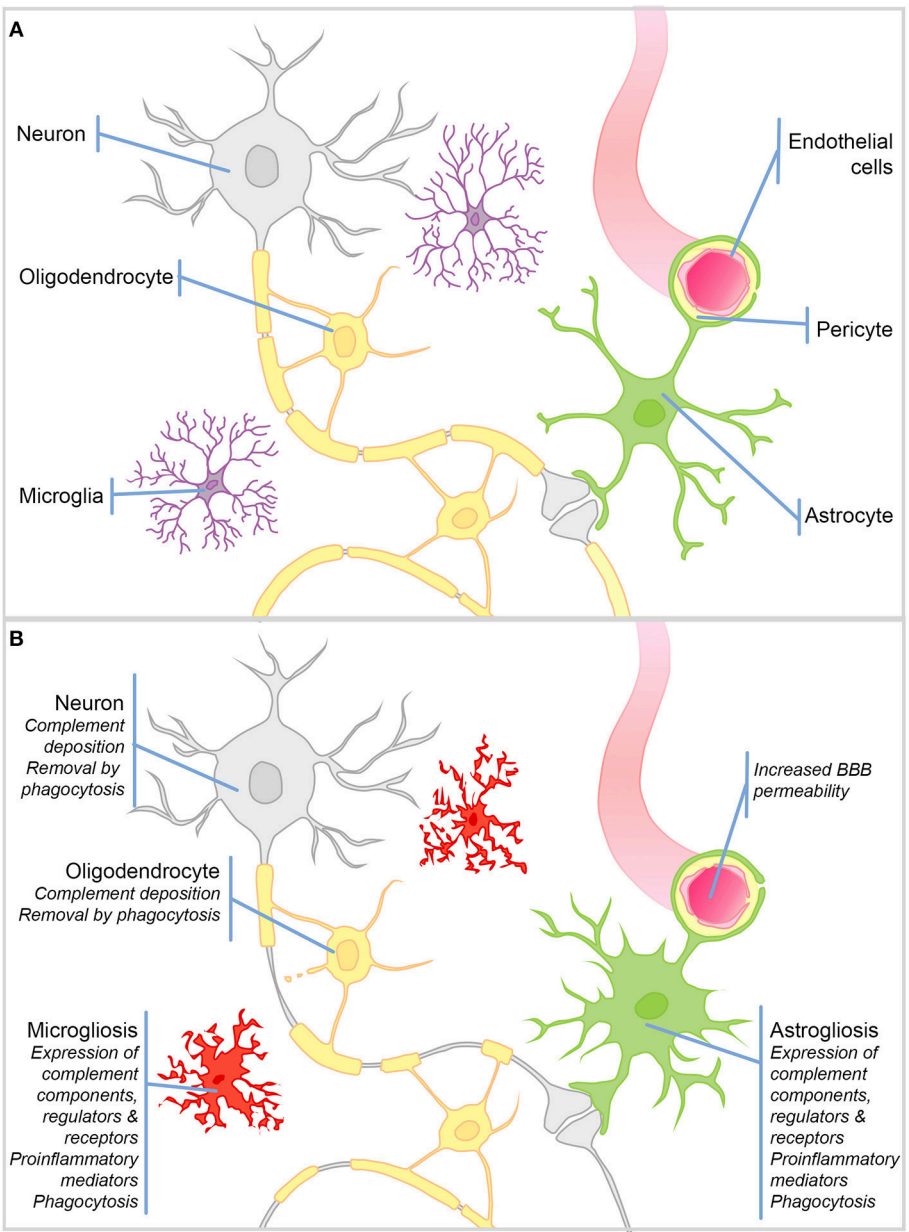

FIGURE 1 | Schematic representation of cell types in the brain and their responses to injury. **(A)** Schematic representation of the cell types in the healthy brain. **(B)** During CNS injury and disease the BBB is compromised. There is significant microgliosis and astrogliosis, characterized by glial cell proliferation, upregulation of complement components, regulators and receptors, proinflammatory mediators, and active phagocytosis. Complement protein expression/deposition are increased on neurons and oligodendrocytes tagging them for removal by phagocytosis and driving neurodegeneration and demyelination.

The healthy BBB forms early in development and restricts the infiltration of circulating immune cells into the brain parenchyma; hence, the dominant immune cells of the brain are the resident macrophage population—microglia (**Figure 1B**). This self-renewing (4), yolk sac-derived population develops within the CNS (5–7) and differs in many respects from macrophage populations found in the periphery (8–10). Compared to tissue macrophages, microglia are relatively "immune suppressed" due to expression of receptors for soluble signals in the extracellular milieu, for example, β2 adrenergic receptor binding of noradrenaline (11, 12), and signals delivered through direct contact with surrounding neurons, including CD200R, CX3CR1 (13, 14). The downstream signaling of such receptors suppresses the production of proinflammatory mediators and encourages a neuroprotective microglial phenotype. Resting microglia are relatively sessile, ramified cells; their numerous highly motile protrusions sample the entire brain every few hours (15, 16). Upon stimulation, these protrusions are withdrawn to create ameboid microglia that are migratory and upregulate expression of proinflammatory mediators and activating receptors involved in pattern recognition and phagocytosis (**Figure 1B**). This activated phenotype, if not kept in check, can cause havoc in the vulnerable CNS. More recently, it has been recognized that there are brain region-specific subpopulations of microglia with different responses to triggers and varying degrees of immune-vigilance (17, 18). There also exist resident non-microglial populations in the healthy brain including perivascular, meningeal and choroid plexus macrophages, which are capable of responding to noxious stimuli. In addition, during pathology blood borne macrophages and other immune cells are recruited to the injured brain as a result of increased BBB permeability. Astrocytes are a neglected cell type, despite the fact that they comprise ∼70% of the cells in the brain, where they form syncytial networks around neurons. During health their primary role is homeostatic; they provide neurons with energy and neurotrophic support, and buffer ion and neurotransmitter concentrations [Reviewed elsewhere by Sofroniew and Vinters (19)—**Figure 1A**]. During inflammation, astrocytes demonstrate their immune-competence; they produce proinflammatory cytokines, are capable of phagocytosis, and can even present antigens to adaptive immune cells; however, acquisition of these immune roles is often associated with loss of homeostatic functions [(19)—**Figure 1B**].

Importantly for the subject matter of this review, there is compelling evidence that, during inflammation, not only microglia and astrocytes, but also neurons, oligodendrocytes, and endothelial cells in the brain, can express complement components, receptors, and regulators.

The Complement System

Complement is recognized as an important branch of the innate immune system, providing the first line of defense against microorganisms. As complement is the subject of this issue, we will confine ourselves to a brief summary (represented in **Figure 2**). Complement comprises multiple recognition molecules that detect and bind target surfaces and recruit a cascade of protease enzymes and substrates, resulting in: (1) production of potent anaphylatoxins that attract and activate phagocytes; (2) formation of the lytic membrane attack complex (MAC); (3) target opsonization for phagocytosis and destruction (**Figure 2**). Three activation pathways, classical, lectin and alternative, converge on a common final pathway. The classical pathway is initiated by the C1 complex binding to antibody/antigen aggregates; the lectin pathway is triggered by binding of mannose-binding lectin (MBL) or ficolins to carbohydrate epitopes on targets; the alternative pathway is better considered as an amplification loop that is engaged regardless of the initial trigger. The activation pathways converge at the central C3 and C5 convertases, which generate potent anaphylatoxins C3a and C5a, C3b to opsonize surfaces facilitating phagocytosis and C5b to initiate MAC formation. The complement pathway mediates many of its effects through specific receptors on cells and is tightly controlled by regulators present on cells and in plasma, as discussed below (20).

The majority of complement proteins are predominantly synthesized in the liver (21, 22); however, it is becoming increasingly clear that complement proteins and their cognate receptors and regulators are expressed throughout the CNS. Most studies to date have utilized primary brain cell cultures and relevant cell lines and have identified complement expression at messenger RNA (mRNA) and/or protein level. Human primary oligodendrocytes expressed mRNA for all the components of the classical and terminal pathways and protein was detected for most of these (23). Human oligodendrocyte cells (HOG cell line) expressed the membrane complement regulators CD59, decay accelerating factor (DAF) and membrane cofactor protein (MCP) and secreted C1-inhibitor (C1INH), Vitronectin and Clusterin, whereas human astrocyte-derived cell lines expressed the same membrane regulators and the important C3/C5 fragment receptors complement receptor 1 (CR1) and C5a receptor (C5aR) (24, 25). Cultured microglia from human post-mortem brain (normal and Shy-Drager's syndrome) constitutively expressed mRNA for C1qB and C3 while C4 was expressed upon interferon (IFN)-γ stimulation (26). C4, C9, C1q, FH, C1INH, C3, C6, and Factor B (FB) were expressed in human neuronal cells *in vitro* whereas primary rat cerebellar granule cells expressed mRNA for C4, C1q, and C3 upon differentiation (27). Additionally, complement expression can be upregulated in disease; for example, C3, C1r and C1s expression was increased in primary microglia and astrocyte cultures from post-mortem brain upon exposure to cytokines associated with AD (28). Expression of C1INH, C1s, C1q and C3 mRNA was detected in AD and control brain extracts (29). Additionally, C1q, C3 and C4 gene expression was reported in primary microglia from AD patients (30). Cumulatively, the evidence suggests that nearly all complement proteins, regulators, and receptors are expressed in the CNS, and many are upregulated by inflammatory signals; it is therefore likely that a functional complement system is present in the CNS independent of peripheral complement.

Roles of Complement in CNS Development

Complement proteins are involved in both prenatal and postnatal development of the healthy brain. In Xenopus embryos, morpholino knockdown of C3a receptor (C3aR) or blocking

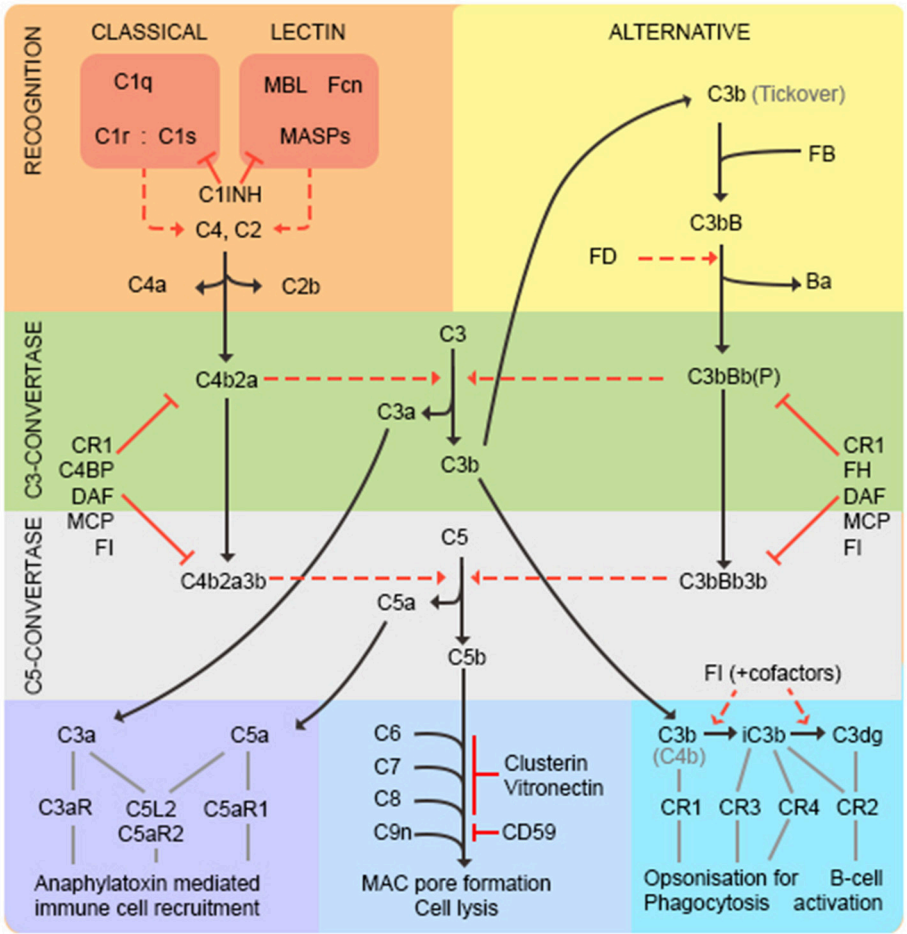

FIGURE 2 | The complement pathway. The classical pathway is activated through antibody/antigen recognition by C1q in complex with C1r and C1s. The proteases C1r and C1s cleave C4 and C2 to generate the C3 convertase C4b2a regulated by complement receptor 1 (CR1), C4 binding protein (C4BP), decay accelerating factor (DAF), membrane cofactor protein (MCP), and factor I (FI). The lectin pathway is triggered by binding of mannose-binding lectin (MBL) or ficolins (FCN) to carbohydrate epitopes on targets. The MBL-associated serine proteases (MASPs) then cleave C4 and C2 to generate the C3-convertase as in the classical pathway. C1-inhibitor (C1INH) functions as a regulator to prevent excessive activation of both classical and lectin pathways. The alternative pathway is better considered as an amplification loop. C3b binds factor B (FB) to form C3bB. FB is cleaved by Factor D (FD) to form the C3bBb C3-convertase stabilized by properdin (P). This process is regulated by CR1, FI, factor H (FH), DAF and MCP. At this point the pathways converge—both C3-convertases cleave C3 to generate the anaphylatoxin C3a, and more C3b that binds to form the C5-convertases (C4b2a3b and C3bBb3b) that cleave C5 into C5a and C5b. C3a and C5a are potent anaphylatoxins that act through their respective receptors (C3aR, C5aR1, C5L2, and C5aR2) to recruit immune cells. C5b binds C6, C7, C8 (inhibited by vitronectin and clusterin) and multiple copies of C9 (inhibited by CD59) to form the lytic membrane attack complex (MAC). C3b opsonizes targets for phagocytosis and B-cell activation; C3b decays to iC3b then C3dg catalyzed by FI in the presence of cofactors (CR1, MCP, FH, C4BP).

antibody against C3a administered during neural tube formation cause loss of neural crest cell organization, demonstrating a role for C3a and its receptor in the migration of neural crest cells (31). Central lectin pathway components Mannan-binding lectin associated serine protease (MASP)-1 and MASP-2 are highly expressed in the developing mouse brain with MASP (and C3) knockout mice showing defects in neuronal migration suggesting critical roles for complement activation in CNS development (32).

Complement also plays key roles in postnatal brain development. In humans and rodents, removal of redundant connections by synaptic pruning during childhood and early adult life is crucial for optimal brain function (33, 34). Using the developing rodent visual system as a model for synaptic pruning, it was shown that C1q and C3 (likely C3b/iC3b) localize to, and tag, specific synapses in the dorsal lateral geniculate nucleus (dLGN) for removal during development (35). C3 deficient ($^{-/-}$) mice had improved hippocampal-dependent learning and memory (36), and failed to show the age-associated synapse loss observed in wild type animals (37), suggesting that complement is detrimental to synapse health. However, in a finding illustrating the dual nature of complement, $C1q^{-/-}$, $C3^{-/-}$, and $C4^{-/-}$ mice all showed defects in synaptic pruning in the CNS that, in the former, associated with increased susceptibility to epilepsy (35, 38, 39). Furthermore, the C3b/iC3b receptor complement receptor 3 (CR3) is expressed on the

surface of microglia and CR3$^{-/-}$ mice showed defects in microglial engulfment of synapses, suggesting a collaboration between complement and microglia in synapse elimination (40). Taken together, these data highlight a critical involvement of the classical pathway in refinement of synapse networks during normal development.

Complement and Neuroinflammation in CNS Disorders—Identifying Druggable Targets

As has been thoroughly reviewed elsewhere (41), inflammation in general and neuroinflammation in particular, is a double-edged sword, evolved to fight infection and restore or maintain homeostasis but, when uncontrolled, capable of wreaking havoc. The aim is therefore not to stop inflammation but encourage a protective rather than destructive profile and prompt resolution of inflammation. Given the important roles of complement in the developing brain, in defense against infection and in maintaining homeostasis, there may be situations where enhancing complement activity may be of benefit; however, in the context of CNS pathology, complement dysregulation leading to over-activation, has deleterious consequences and contributes to neuroinflammation.

Thus, the complement system offers an attractive drug target for these diseases that are currently without effective therapies. Drugging the complement pathway in the periphery is well researched and this knowledge could potentially be extrapolated to the CNS.

CNS disorders can be divided into acute, for example traumatic injury and stroke, and chronic, for example, demyelinating and neurodegenerative disorders, dependent on the causation, severity and duration. In the sections below, we will present evidence for the role of complement in both acute and chronic neurological disorders. We will not include stroke because this is discussed elsewhere in this issue and will also omit detailed discussion of the demyelinating diseases MS and NMO for brevity and because these have been well reviewed elsewhere (42, 43). Our overall aim is to provide insight into how complement therapeutics might impact these problematic diseases.

COMPLEMENT PROTEINS IN ACUTE NEUROLOGICAL DISORDERS—TRAUMATIC BRAIN AND SPINAL CORD INJURY

Traumatic Brain Injury (TBI)

TBI is classified as an injury to the brain due to trauma to the head via an external force; this can occur as a result of road traffic accidents, falls, sporting injuries or assaults; consequently, TBI is the major cause of brain injury and death in young adults in the Western world. TBI can cause diffuse or focal damage to the brain tissue and blood vessels depending on the type of injury. Subsequent to this primary injury, the BBB becomes compromised and there is a huge influx of cells, inflammatory mediators and plasma proteins, including complement proteins, that drive the delayed secondary inflammation, which is the major determinant of clinical outcome and thus recovery and survival (44). Human post-mortem TBI studies have shown increased expression of C3 and FB in brain and CSF (45) and both axonal and astrocytic expression of Clusterin (46). Increased soluble C5b-9 (terminal complement complex; TCC) levels were found in CSF after TBI, positively correlating with the degree of BBB damage (47), and further increased in response to secondary insults (oxygen deprivation/seizures) (48).

A wide array of TBI models are utilized in animal research, including cryoinjury, controlled cortical impact and standardized weight drop [models are reviewed elsewhere; (44)]. Increased C3 deposition, Clusterin and MAC deposition were observed alongside increased microglial and astrocytic activation markers after cortical contusion in the rat (49). Serum proteomics in Sprague-Dawley rats after "severe" deep cortical impact reported increased C9 and FB within the first few days after TBI (50). Complement deficient mouse models have been used to identify the impact of complement on neuropathology after TBI (**Table 1**). C3$^{-/-}$ and C5$^{-/-}$ mice showed reduced neutrophil extravasation upon traumatic brain cryoinjury (51); C4$^{-/-}$ mice, but not C3$^{-/-}$ or C1q$^{-/-}$ mice showed reduced motor deficits and tissue damage following controlled cortical impact (52). In the same TBI model, CR2/CR1$^{-/-}$ mice showed improved outcome with decreased mortality, neuronal cell death, C3 deposition, astrogliosis, and microgliosis (53). FB$^{-/-}$ mice also showed reduced cell death in TBI with increased anti-apoptotic and decreased pro-apoptotic markers (54). In one study, MBL$^{-/-}$ mice were protected from neurological injury following TBI (70); in contrast, another reported that MBL$^{-/-}$ mice showed increased levels of degenerating neurons in the hippocampus CA3 region and impaired performance in non-spatial learning tasks (71). Despite several such inconsistencies, the studies to date suggest that deficiencies of individual complement proteins of the classical, alternative or terminal pathway improves outcome after TBI. However, all these studies used rodent models where the relevant protein is knocked out systemically from embryogenesis. Thus, it is important to identify whether anti-complement therapeutics administered immediately post-injury can have a similar beneficial effect, and to define the "therapeutic window of opportunity" for intervention post-injury.

The majority of therapeutic studies in TBI models have focused on targeting the C3 convertase as a central player of all three activation pathways (**Table 2**). Administration of soluble CR1 (sCR1) pre- and post-TBI in rats reduced neutrophil accumulation in the injured brain (72). Administration of vaccinia virus complement control protein (VCP), an inhibitor of C3 activation, improved performance in spatial memory tasks after TBI (77). In mice, systemic inhibition of C3 via administration of Crry-Ig (recombinant chimeric Complement receptor 1-related protein Y (Crry) fused to mouse IgG1 Fc) 1 and 24 h post-TBI ameliorated neuronal damage in hippocampus and improved neurological outcome (87). Transgenic mice expressing soluble Crry (sCrry) specifically in astrocytes were protected in closed head injury TBI, with reduced C3 deposition, decreased BBB damage and improved neurological scores (88).

TABLE 1 | Consequence of complement deficiency on outcome of neurodegenerative disease.

	Model	Deficiency	Consequence	Reference
TBI	Traumatic brain cryoinjury	C3	Reduced pathology	(51)
	Controlled cortical impact	C3	No effect	(52)
	Controlled cortical impact	C4	Improved function	(52)
	Traumatic brain cryoinjury	C5	Reduced pathology	(51)
	Closed head injury	CR2	Improved function	(53)
	Controlled cortical impact	C1q	No effect	(52)
	Closed head injury	FB	Reduced pathology	(54)
SCI	T9 contusion	C1q	Improved function	(55)
	Contusion induced injury	FB	Improved function	(56)
	Weight drop	C3	Improved function	(57)
	Contusion injury	C5a	Improved function	(58)
	Contusion induced injury	CD59	Impaired recovery, increased injury	(56)
AD	Tg2576	C1q	Ameliorates synapse loss	(59)
	oAβ injection	C1q	Ameliorates synapse loss	(60)
	APP/PS1	C3	Ameliorates synapse loss	(60)
	J20 APP	C3	Exacerbated pathology	(61)
	oAβ injection	CR3	Ameliorates synapse loss	(60)
	APP/PS1	C3	Improved function	(62)
ALS	$SOD1^{G37R}$	C1q	No effect	(63)
	$SOD1^{G37R}$	C3	No effect	(63)
	$SOD1^{G37R}$	C4	No effect	(64)
	$SOD1^{G37R}$	C5aR1	Extended survival	(65)
HD	R6/2	C3	No effect	(66)
PD	MPTP induction of PD	C3	No effect	(67)
	MPTP induction of PD	C1q	No effect	(68)
	Paraquat/maneb induction of PD	CR3	Reduced dopaminergic neurodegeneration	(69)

TBI, Traumatic brain injury; SCI, spinal cord injury; AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; PD, Parkinson's disease; HD, Huntington's disease.

Intravenous (iv) administration of C1INH 10 min post TBI reduced cognitive deficits and brain lesion size (89) and, in a separate study, improved motor scores, reduced cognitive dysfunction and reduced injury volume (90). Alternative pathway inhibition with systemically administered anti-FB reduced neuronal damage after TBI in mice (73). Lectin pathway inhibition using a multivalent MBL ligand improved functional and pathological outcome measures in a mouse TBI model (76). Terminal pathway inhibition using either the tick-derived C5 inhibitor OmCI or C6 antisense oligonucleotide decreased neuropathology and promoted recovery in severe closed head injury (74), and targeted inhibition of the terminal pathway using a CD59-CRIg hybrid that localized to areas of C3b/iC3b deposition in the injured brain was strongly neuroprotective in the same model (75). Recently, to determine which component or pathway of complement should be targeted for most efficient protection in TBI, three hybrid proteins, all containing CR2 to target to areas of complement activation, CR2-CD59 (inhibition of MAC), CR2-Crry (all complement pathways) and CR2-FH (alternative pathway), were compared; the latter two were most effective, demonstrating important roles for early activation products, both opsonins and anaphylatoxins (91).

Spinal Cord Injury (SCI)

SCI can be caused by sudden traumatic insults that crush or sever the cord, or non-traumatic injuries, for instance, triggered by cancer, arthritis or infection and usually compressing sections of cord; here we will restrict discussion to traumatic causes. SCI results in dysfunction and sometimes complete loss of function below the lesion site. Symptoms are often life-long and, since SCI is most common in under-30s, is associated with huge personal and health-care costs (https://www.spinal-research.org/). In traumatic SCI the primary pathology is caused by a mechanical force directly damaging the neural tissue—this primary insult is difficult to protect against. However, post-injury inflammation, with infiltration of immune cells and production of pro-inflammatory mediators, results in secondary pathology in adjacent areas characterized by oedema, ischemia, and excitotoxicity (92). There is considerable blood-spinal cord barrier (BSCB) damage and resultant inflammation in this secondary phase; despite this, studies of complement in SCI are scarce. Early human studies showed elevated C3, C4, and C5 levels in plasma of patients post-SCI suggestive of an acute phase response (93). As with TBI, rodent models of SCI vary widely; many different approaches to inducing injury have been taken, including weight drop, contusion, compression, laceration, and chemical injection. Complement proteins were deposited at sites of SCI in rodents; C1q, FB, C4, and TCC expression all increased at and around the injury within 24-h post-SCI and remained high up to 6 weeks (94, 95), and FH and Clusterin were elevated in lesioned neurons and oligodendrocytes (96). In a less severe weight-drop contusion SCI mouse model, C3 (likely C3b/iC3b) was deposited in white matter at the site of injury at 4-h and more widely in adjacent white and grey matter at 12- and 24-h, returning to baseline by 3 days post-injury (57).

$C3^{-/-}$ mice were significantly protected in contusion-induced SCI with reduced lesion size, necrosis, demyelination, and neutrophil infiltration, improved locomotor score and accelerated recovery (57); $C1q^{-/-}$ mice showed decreased lesion volume and improvements in locomotion and fine motor control compared to controls in the same model (55). Mice deficient in FB subjected to contusion SCI showed accelerated recovery of locomotion, marked improvements in macroscopic tissue integrity, and reduced demyelination, C3 and C9 deposition and infiltration of neutrophils and macrophages compared to controls (56), while mice deficient in the terminal pathway inhibitor CD59 showed increased pathology with loss of myelin structure, scarring and vacuolation, hemorrhage, neutrophil and macrophage infiltration, and TCC

TABLE 2 | Consequence of pharmacological complement inhibition on outcome of neurodegenerative disease.

	Model	Drug	Timing	Consequence	References
TBI	Weight drop	sCR1	2 h and 2 min prior and 2 h post	Decreased neutrophil accumulation	(72)
	Weight drop	Anti-FB	1 and 24 h post	Decreased tissue damage	(73)
	Closed head	OmCI	Immediately prior, 15 and 30 min post	Improved function, reduced pathology	(74)
	Closed head	C6 α-sense oligoNT	6 days prior for 4 days	Improved function	(74)
	Cryoinjury	AcF	Immediately prior	Decreased neutrophil extravasation	(51)
	Closed head	CD59-2a-CRIg	30 min and 24 h post	Improved function	(75)
	Cortical impact	Polyman9	10 min post	Improved function, reduced pathology	(76)
	Lateral fluid percussion	VCP	15 min post	Improved function but not neuropathology	(77)
SCI	Weight drop	sCR1	1 h post and daily	Reduced degeneration	(78)
	Mild impact	VCP	Immediately post	Improved function and reduced pathology	(79)
	Pneumatic impact	C1inh	2 h post injury	Improved function	(80)
	Contusion	Anti-FB	1 and 12 h post injury	Improved function	(56)
	Compression	PMX53	45 min pre and 24 h post	Improved function	(58)
	Contusion	PMX205	14 days post	Detrimental for functional recovery	(81)
	Weight drop	CR2-Crry	1 h post	Improved function	(57)
AD	Tg2576, 3xTg	PMX205	After plaques for 2–3 mo 2x weekly	Reduction in fAβ deposits and activated glia	(82)
	APP/TTA	SB290157	3x week for 5 weeks from 7.25 mo	Reduction in Aβ deposits	(83)
	Oligo Aβ	ANX-M1	17 and 2 min pre and 24 and 48 h post	Prevented synapse loss and impairment of LTP	(60)
ALS	$SOD1^{G93A}$ rat	PMX205	P28 and P70	Improved function	(84)
	$hSOD1^{G93A}$ ms	PMX205	P35 (pre) and P31 (post)	Improved function	(85)
HD	3-NP rats	PMX53	2 days prior	Improved function	(86)
	3-NP rats	PMX205	2 days prior and 2 days post	Improved function	(86)

TBI, Traumatic brain injury; SCI, spinal cord injury; AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; PD, Parkinson's disease; HD, Huntington's disease.

deposition (56). $C5aR^{-/-}$ mice showed acute but not long-term improvements in functional recovery (97). *In vitro* studies showed that C1q-treatment increased cortical neurite length on myelin by inhibition of growth cone repulsion by myelin associated glycoprotein (MAG); however, comparison of C1q KO and C1q WT mice in a peripheral conditioning lesion model of SCI showed no differences in axon length, lesion volume or scarring, although C1q deficiency was associated with increased axonal turning (98).

There are currently no proven therapies for SCI, unsurprising given the many obstacles in promoting re-wiring of axons and remyelination. Preventing or reducing the inflammation-driven secondary phase offers opportunity; indeed, methylprednisolone is the only currently available treatment though its effectiveness is unclear (99–101). There have been a few studies of anti-complement agents in SCI rodent models and the majority of these have utilized iv administration, possible because of BSCB disruption post-injury. Injection of the C3 convertase inhibitor VCP into the injured spinal cord in a rat SCI model, restored spinal cord tissue integrity, reduced macrophage and microglial activation and improved acute motor deficits (79, 102); iv administration of recombinant sCR1 in mice 1 h post-SCI and daily thereafter reduced neuron swelling, degeneration, necrosis and neutrophil infiltration and improved recovery (78); iv C1INH 2-h post-SCI in rats improved motor recovery, reduced lesion volume and leukocyte infiltration (80). Alternative pathway inhibition with iv anti-FB mAb accelerated recovery and reduced lesion size in the same model (56). In a contusion SCI mouse model, iv administered CR2-Crry localized to the lesion site, improved locomotor deficits and reduced necrosis, demyelination, and neutrophil infiltration (57); because CR2-Crry targets specifically areas of pathology, it is bioavailable in the SCI when given at a dose that does not influence circulating complement activity, reducing the risk of infections and other undesirable effects of systemic complement inhibition. Another strategy to reduce infection risk is to target C5a, a potent chemoattractant, or its receptor C5aR1 (CD88), a G protein-coupled receptor (GPCR) expressed on granulocytes monocytes/macrophages peripherally and on astrocytes and microglia (and at low level neurons and oligodendrocytes) in CNS (103, 104). Two small cyclic peptide C5aR1 antagonists PMX53 and PMX205 (86, 105) have been tested in SCI models; iv administration of PMX53 improved functional recovery, and reduced macrophage/microglial numbers, expression of pro-inflammatory cytokines IL-1β and TNF-α and astrogliosis in mice compared with controls (58). In a rat SCI model, the impact of PMX205 administration was dependent on timing post-injury and was linked to the sequence of immune cell recruitment to the site; whereas early administration accelerated recovery, late administration inhibited the macrophage/microglial response and slowed functional recovery and re-myelination following injury, further emphasizing the importance of timing interventions (81). Mice treated with C5aR antagonist [hydrocinnamate—(OpdChaWR)] showed acute but not long term improvements in functional recovery (97). Additionally, bone marrow chimeric mice lacking peripheral but not central

C5aR showed no differences from control mice. Together these data suggest an initially detrimental role of C5aR followed by a delayed neuroprotective role, likely mediated by CNS resident cells.

Taken together, these studies indicate that inhibition of classical/lectin and/or alternative pathways or specific effectors like C5a can be efficacious in SCI. Timing of interventions may be crucial to avoid impacting beneficial clearance roles of complement. Terminal pathway inhibition has not been tested in the models but the impact of CD59 deficiency noted above suggests that this is a viable target.

COMPLEMENT PROTEINS IN CHRONIC NEUROLOGICAL DISORDERS—DEMYELINATION AND NEURODEGENERATION

Inflammation was noted early as a feature of chronic brain disease; indeed, astrogliosis and microgliosis were included in the original descriptions of AD by Alois Alzheimer over a century ago. Despite this, a classification divide emerged with diseases like MS considered inflammatory while diseases like AD were considered degenerative. The artificial nature of this divide has become clear in recent years with the realization that there are many shared features. The evidence implicating inflammation as a driver of pathology in chronic neurodegenerative diseases is now substantial and includes genetic studies identifying inflammatory risk genes (106), clinical studies demonstrating that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) is protective in humans (107) and mouse models (108) and the observation that systemic infections and inflammation increase the risk and/or rate of progression of dementia (109–111). Complement, the focus of this review, goes hand-in-hand with inflammation and represents a potential driver of chronic CNS diseases.

Alzheimer's Disease (AD)

AD is the leading cause of dementia affecting almost 50 million people worldwide, a number projected to increase to 150 million by 2050 (https://www.alz.co.uk/research/statistics). AD is characterized by two hallmark pathologies; amyloid-β (Aβ) plaques and neurofibrillary tangles comprising hyperphosphorylated tau. Recent studies have implicated complement in AD pathogenesis. Genome wide association studies identified single nucleotide polymorphisms (SNPs) associated with risk of late-onset AD in genes encoding complement proteins Clusterin (*CLU*) and CR1 (*CR1)* (106, 112, 113). Biomarker studies have identified complement proteins and activation products in plasma and/or CSF that distinguish AD from controls and predict risk of progression to AD (114–117). Immunohistochemistry (IHC) of post-mortem AD brain revealed complement proteins and activation products decorating plaques and tangles. In particular, classical pathway proteins C1q, C3, and C4 co-localized with amyloid fibrils, Aβ deposits and neurofibrillary tangles, notably in temporal cortex, amygdala, and hippocampus, in AD brain (118–120). The terminal pathway activation marker TCC was abundant in AD cortex in association with aggregated Aβ, neurofibrillary tangles and neuropil threads (121). Cells expressing C5a receptors C5aR1 and C5L2 were associated with neurofibrillary tangles, neuropil threads, and dystrophic neurites in AD plaques in hippocampus and frontal cortex (122). A weakness of these IHC studies is that they are performed on post-mortem brain, inevitably end-stage disease, and do not provide insight into early disease or disease progression. A large-scale microarray study of young, healthy old and AD brains identified marked changes in complement expression with ageing, and elevated expression of *C4A, C4B, C3aR1, C5aR1, CFHR1,* and *CLU* in AD compared to age-matched controls; C1q binding protein (C1qBP) expression decreased in AD (123). Increased C1q expression in brain with ageing (healthy or AD) has been robustly replicated (59, 124, 125).

Mechanisms of complement activation in the AD brain have been studied *in vitro* and in animal models. Aβ fibrils activate and consume complement classical and alternative pathways *in vitro* and generate C3a, C5a, and TCC (119, 126). C5a administration resulted in death of primary mouse neurons in culture; this could be blocked by addition of C5aR1 antagonist PMX53, demonstrating that C5a (acting via C5aR1) is sufficient to induce neuronal cell death *in vitro* (127). Animal models have underpinned the majority of research into roles and mechanisms of complement in AD. Most mouse models mimic the rare early-onset forms of AD in which single gene mutations have been identified rather than the common polygenic late onset AD, and thus individually mimic only certain aspects of the disease; it is therefore unsurprising that different models yield different and often contradictory results. Despite these reservations, these mouse models have aided understanding of AD pathology. Broadly, models can be divided into three groups: Aβ pathology; Tau pathology; both. These mouse models recapitulate many of the pathologies found in AD brain; for example, in the PS/APP model fibrillar Aβ plaques form and C1q localizes to these plaques (128). Back-crossing AD mouse models onto complement deficiencies has been used to determine the role of complement in the pathophysiology of AD (**Table 1**). Deficiency of C1q (classical pathway) in Tg2576 (Aβ pathology) mice reduced glial activation and synaptic loss without influencing Aβ load compared with controls (59). Genetic deletion of C1q, C3 or CR3, all of which are required for effective opsonization and phagocytosis of synapses, reduced microglial numbers and synapse loss when crossed to two different Aβ models (J20 and APP/PS1); further, when Aβ fibrils were injected directly into brain, C1q deficiency protected from toxicity (60). In contrast, C1q deficiency in the 3xTG model exacerbated neurodegeneration because of a loss of C1q-triggered expression of neuronal survival pathways (129). Others showed that C3 deficiency improved performance on learning and memory tests and decreased microglia and astrocyte number associated with plaques in an Aβ model (APP/PS1) (62); in contrast, C3 deficiency was associated with increased amyloid burden, decreased neuronal staining and activated glia in the J20 (Aβ) model (61). Despite the evidence noted above that Tau pathology is associated with

complement, there was a dearth of studies in Tau models; two recent publications have changed this. Administration of blocking anti-C1q antibodies in a mouse Tau model (P301S) inhibited microglial synapse loss and rescued synapse density (130), while C3aR deletion attenuated neuroinflammation and reduced synaptic deficits and neurodegeneration in the PS19 Tau model (131).

As is clear from the anti-C1q experiment described above, mouse models also offer a way to test *in vivo* the impact of complement therapeutics on disease (**Table 2**). The C5aR1 antagonist PMX205 decreased amyloid and tau deposits, reduced activated glia and improved cognition in two Aβ models (Tg2576 and 3xTg) (82). Levels of C1q and C3 were unchanged upon PMX205 treatment, suggesting that their physiological functions are preserved. As noted above, blocking antibody against C1q [(ANX-M1/ANX005); Annexon Biosciences] protected from synapse loss in Aβ models and reduced toxicity of Aβ fibrils injected into the lateral ventricles (60); this agent showed no toxicity, even at high doses (200 mg/kg) and has proceeded to clinical trials (132). A note of caution in the use of anti-complement agents comes from a study of C3 inhibitor sCrry administered to an Aβ model (hAPP × TGFβ1) which resulted in increased Aβ deposition and neuronal degeneration (133).

The evidence—genetic, clinical, and from models—implicating complement as a driver of pathology in AD is compelling. A complicating factor is that complement may also have protective roles in clearing debris in early disease. Improved understanding of the time course of complement involvement may identify therapeutic windows where complement inhibitors will improve outcome.

Amyotrophic Lateral Sclerosis (ALS)

ALS, also known as Lou Ghering's disease, is an adult onset neurodegenerative disease, usually fatal within 2–5 years of onset (134). ALS is caused by progressive loss of upper and lower (α) motor neurons (135), resulting in denervation of neuromuscular junctions in the peripheral nervous system, progressive muscle weakness, atrophy, spasticity, respiratory failure, and ultimately paralysis and death. Approximately 90% of ALS cases are sporadic and 10% familial. Causative missense point mutations have been identified in superoxide dismutase (*SOD1*), TAR DNA binding protein (*TDP-43*), fused-in-sarcoma-protein (*FUS*), and chromosome 9 open reading frame 72 (*C9orf72*). The only currently available treatment for ALS is Riluzole, an ion channel blocker and inhibitor of glutamate release which modestly increases survival (136, 137).

Neuroinflammation is a consistent feature of ALS with abundant reactive microglia and astrocytes and T-cell infiltration observed (138). IHC identified increased C1q protein in motor cortex and spinal cord of ALS post-mortem tissue; C3 activation fragments and TCC were also demonstrated in areas of pathology (139, 140). C3c labeled astrocyte-like cells in the former study whereas C1q and C3d co-localized with neurons, astrocytes and microglia, and TCC primarily microglia, in the latter. Others described C4d and TCC staining of degenerating neurons and glia in ALS motor cortex and spinal cord (141) and C5aR1 upregulation in areas of pathology (142). C3d and C4d were also found on oligodendroglia and degenerating neurites, surrounded by CR4-positive microglia, in spinal cord and motor cortex (141, 143). C1q, C3, and TCC were present on motor end-plates in intercostal muscles in ALS donors even early in the disease process (144); DAF and CD59 were upregulated at the end-plates, perhaps reflecting a response to complement activation and TCC/MAC deposition. TCC immunoreactivity at end-plates negatively correlated with α-bungarotoxin staining, implicating TCC/MAC in loss of end-plates (144). In myasthenia gravis, end-plate destruction is dependent on complement activation and MAC formation (145), supporting a causative role in ALS.

The source of complement in ALS pathology is unclear; the BBB is disrupted in the disease (146); however, local biosynthesis likely also contributes. *In situ* hybridization demonstrated upregulated *C1qb* and *CLU* mRNA in areas most affected by neurodegeneration (147); more recently, increased C1q and C4 expression by glial cells was demonstrated in ALS cord white matter (140) indicating a local source of complement. Complement expression positively associated with increased infiltration of dendritic cells and CD8+ T-lymphocytes from the periphery (140, 141). Biomarkers also implicate complement. Complement activation products C3c and C4d were present in CSF and correlated with disease severity scores (148–150). Levels of C5a and TCC were significantly elevated in ALS plasma, and leukocytes from ALS patients had increased surface (C5aR1-bound) C5a (151). These biomarker findings strongly support a role for complement dysregulation in ALS patients; however, the nature and location of complement protein deposition in different studies was contradictory, perhaps due to differences in disease stage or comorbidities.

Numerous rodent models of ALS have been generated based on known causative mutations in SOD1, responsible for ∼10% of familial ALS. Rodents over-expressing human mutant $SOD1^{G93A}$ recapitulate key neuropathological and functional hallmarks of ALS, characterized by lumbar motor neuron loss which correlates with progressive motor deficits and ultimately paralysis, and by inflammatory changes including robust astrogliosis, microgliosis, and BBB-disruption (152–154). Complement dysregulation is apparent from increased expression and deposition of C1q, C4, FB, C3 activation products and TCC, increased expression of C5aR1, and reduced expression of complement regulators DAF and CD59 (64, 84, 144, 154–156). Complement deposition has also been observed in sciatic nerves (64) and at the neuromuscular junction (156) in ALS models, consistent with the concept that complement contributes to nerve terminal destruction in ALS. In the $TDP43^{Q331K}$ mouse model, progressive motor deficits, astrogliosis, and microgliosis correlated with complement dysregulation in the spinal cord; expression of C1qB, C4 and C3 was elevated and DAF mRNA reduced in the lumbar spinal cord and in tibialis anterior muscle of $TDP43^{Q331K}$ mice compared with controls (157). Immunofluorescence confirmed markedly increased C1q and C5aR1 in motor neurons and microglia.

Surprisingly, C1q deletion in $SOD1^{G37R}$ ALS mice exacerbated synaptic loss at end-stage and it was implied that this was a consequence of increased microglial phagocytosis; however, C1q deletion did not significantly affect disease onset, progression, or

survival and had no effect on global astrogliosis, microgliosis, or neuronal loss (63) (**Table 1**). Deletion of the gene encoding C4, which is necessary for activation of both the classical and lectin pathways, significantly reduced the number of activated macrophages found in sciatic nerves of mSOD1^{G93A} mice but again failed to influence the disease course (64). C3 deletion also failed to affect overall survival or motor neuron loss in SOD1^{G93A} ALS mice (63); the finding that deletion of C3, central to all complement pathways, fails to rescue disease has provoked the suggestion that complement does not contribute to ALS disease progression (at least in this model). The demonstration that anti-complement drugs ameliorate disease in a similar model contradicts this suggestion (**Table 2**). Oral administration of C5aR1 antagonist PMX205, even when given in established disease, reduced weight loss and motor deficit scores, slowed disease progression and enhanced survival times in SOD1^{G93A} rats and mice (84, 85). These functional improvements were associated with reduced astrocyte proliferation, reduced influx of proinflammatory monocytes and granulocytes and an increase in the CD4+: CD8+ T-cell ratio, consistent with the reported neuroprotective role of CD4+-T cells in ALS (158). The same authors showed that deficiency of C5aR1 (upregulated in human and rodent ALS) extended survival in SOD1^{G93A} mice (65). Taken together, these data strongly implicate the C5a/C5aR1 axis in disease and identify it as a target for therapy in ALS.

Huntington's Disease (HD)

HD is an autosomal dominant, inherited neurodegenerative disease characterized by progressive motor symptoms, psychiatric disturbances, and dementia. It is caused by expansion of a three-base-pair (CAG) repeat (39–121 repeats vs. normal range 8–39 repeats) in exon 1 of the *HTT* gene that translates into a polyglutamine tract at the N-terminus of the protein. This results in a polyglutamine length-dependent misfolding and accumulation of huntingtin protein in the striatum and cortex (layers 3, 5, and 6) followed by neuronal loss in these areas which spreads to the hippocampus (159, 160). Neuropathology of HD is graded based on Vonsattel staging (161) dependent on the severity of neuronal loss, astrogliosis, and brain atrophy. Precisely how the huntingtin trinucleotide expansions result in neuronal death and associated gliosis remain unclear. Microglial activation can be demonstrated by PET scanning even in early disease and correlates with disease severity (11C-raclopride binding) (162); indeed, even in pre-symptomatic gene carriers, microglial activation was present and correlated with striatal neuronal dysfunction and with risk of developing HD within 5 years (163).

HD post-mortem tissue showed abundant reactive astrogliosis and microgliosis and intranuclear ubiquitin positive inclusions in the caudate and temporal lobes (164). IHC showed that neurons, astrocytes and myelin sheaths in the HD caudate and striatum were immunoreactive for C1q, C4, C3 and neo-epitopes in iC3b and TCC (164). Expression of mRNA encoding early complement components C1q (c-chain), C1r, C3, and C4, complement regulators C1INH, Clusterin, MCP, DAF and CD59, and complement receptors C3a and C5a was upregulated in the HD striatum. Early disease stages did not stain for complement suggesting that early neuronal damage precedes local complement synthesis and activation. Microarray analysis in HD post-mortem tissue demonstrated increased expression of complement components C4A, C4B and C3, most significantly in the most affected areas, caudate nucleus, and motor cortex (165). Unbiased proteomic profiling revealed 18 proteins that were differentially expressed in HD plasma, several of which are involved in the innate immune system; Clusterin, C7 and C9 increased with disease severity (166).

Early animal models of HD utilized toxin-mediated striatal lesions; for example, Lewis rats given intracerebral 3-nitropropionic acid (3-NP), an inhibitor of the mitochondrial citric acid cycle, developed striatal lesions, weight loss, gait disturbances, dystonia and ataxia, thus reproducing some of the pathological and clinical characteristics of HD (86). Oral administration of C5aR antagonist (PMX53 or PMX205) reduced weight loss and motor deficits, even when given post-toxin administration, whereas NSAID, ibuprofen, and a TNF-α inhibitor (infliximab) had no significant functional impact, suggesting that ability to rescue these deficits hinged on the complement pathway *per se* rather than neuroinflammation in general (**Table 2**). 3-NP treatment caused lesions with robust neuronal death and neutrophil infiltration and surrounded by C5aR-, C3-, and C9-positive glia. C5aR blockade reduced lesion volume and number; lesions contained fewer apoptotic cells and astrocytes and were no longer surrounded by complement-positive glia. While these data were a helpful proof of concept (and this was the first paper demonstrating that PMX53 and PMX205 cross the BBB), the model used is extremely artificial, acute and invasive, unlike the chronic, cumulative dysfunction seen in HD.

R6/2 transgenic mice provide a more realistic HD model; these mice express exon 1 of the human *huntingtin* gene, including a pathological trinucleotide repeat; they develop progressive behavioral and neuropathological deficits, including synaptic loss, but do not develop neuronal loss and fail to demonstrate upregulation of complement proteins (66). It is, therefore, unsurprising that C3 deficiency did not alter disease progression in this model. C5aR was the only complement molecule upregulated in the model and it remains undetermined whether targeting the C5a-C5aR1 axis would be beneficial.

Parkinson's Disease (PD)

PD is characterized by loss of dopaminergic neurons in the substantia nigra and deposits of the protein α-synuclein that form the pathological hallmarks of the disease, Lewy bodies. Patients present with resting tremor, bradykinesia, and rigidity. Complement activation has been associated with α-synuclein and Lewy bodies in Parkinson's disease; *in vitro* studies demonstrated that the disease-associated splice variant α-synuclein 112, but not the full length protein, cause activation of complement (167). *In vivo*, C3d, C4d, C7 and C9 localization in Lewy bodies was reported in one study (168), although this was not recapitulated in a separate study (169). More recently, deposition of iC3b and C9 in Lewy bodies and melanized neurons was reported; iC3b immunoreactivity increased with normal ageing

and was further elevated in PD vs. age-matched controls (170). A correlation was described between the ratios of C3/Aβ42 or FH/Aβ42 in CSF and severity of Parkinson's disease motor and cognitive symptoms, but not with absolute levels of C3 or FH (171).

Although there are many mouse models of PD, drug or neurotoxin induced, or genetic, none fully replicates the human disease (172). A few studies have explored roles of complement in these models; absence of C3 in mice did not protect against depletion of dopaminergic neurons in the toxin-induced MPTP model (67) (**Table 1**). There was an increase of C1q in relevant brain regions in this model but C1q deficiency did not protect from disease (68). A very recent study identified a role for CR3 in activation of the microglial NADPH oxidase (Nox2) and subsequent neurodegeneration in a toxin-induced PD model; CR3 knockout mice were protected from dopaminergic neuron loss and motor dysfunction, suggesting that complement opsonization and CR3 engagement contribute to the disease process (69).

TARGETING COMPLEMENT IN NEUROLOGICAL DISEASE

Getting Therapeutics Into the CNS

Having made the case above for an involvement of complement in acute neurological injuries and neurodegenerative diseases, attention naturally turns to therapeutic significance. There is a huge and growing complement therapeutics industry with a myriad of new drugs emerging; however, to date CNS targets have been largely ignored (173). Drug delivery is a major limiting factor for CNS therapies that needs to be considered when designing therapeutics for treating neurological conditions. The BBB precludes passive entry of molecules larger than ∼400 kDa thus occluding entry of macromolecules, including antibody and protein therapeutics. In TBI and SCI the BBB is impaired to some degree, enabling drugs to access the injured areas (47). Treatment options to access the CNS in diseases where the BBB remains intact include both invasive and non-invasive techniques [reviewed in (174, 175)]. Historically, access of drugs to the CNS involved disruption or damage to the BBB or the use of pharmacological agents to increase its permeability; however, in many cases this resulted in widespread neuronal damage and an associated inflammatory response. Less damaging ways of opening the BBB include the use of focused ultrasound waves of low intensity that cause local and temporary disruption to the BBB and administration of "osmotic shock" agents (176). Pharma companies have designed a host of other strategies to deliver therapies, including Trojan horse delivery, use of viral vectors, nanoparticles and chimeric peptides, expanded on below.

Trojan horse technologies involve the creation of fusion proteins that lock the drug to a delivery component that utilizes receptors in the BBB, such as the insulin receptor and transferrin receptor, to enable bidirectional transport into and out of the brain. As an example of this concept, a recombinant anti-Aβ single chain fV antibody (fragment variable region only) fused to a fAb fragment of an anti-insulin receptor mAb bound the insulin receptor at the BBB, was transcytosed across the barrier enabling it to access and recognized Aβ within the brain and was then shuttled out again with its Aβ cargo for disposal (177). Anti-complement therapeutic antibodies, of which there are many in the clinic or in development, could be similarly piggy-backed into the brain to inhibit complement.

Small, hydrophobic molecules can cross the BBB via lipid-mediated diffusion. As an example, oral administration of the small molecule NLRP3 inhibitor MCC950 in PD mouse models reduced nigrostriatal dopaminergic degeneration, motor deficits and accumulation of α-synuclein through inhibiting inflammasome activation (178). Several small-molecule complement inhibitors are in development but, with the exception of the anti-C5aR1 molecules PMX53 and PMX205 described above, these have yet to be assessed for BBB permeability.

Targeting Complement in CNS

Eculizumab, a humanized anti-C5 antibody, is the lead anti-complement drug but to date has only been approved for two rare disorders, paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) and recently for Myasthenia Gravis. In a recent small trial of eculizumab in NMO, a demyelinating disease characterized by BBB disruption and inflammation/degeneration of the optic nerve and spinal cord (www.clinicaltrials.gov NCT00904826), treatment reduced the number of neurological episodes (179). This study has raised the prospect of using eculizumab for other inflammatory CNS diseases, although the BBB is likely a much greater hurdle in these other conditions and it is unclear whether they can be treated systemically. There is an urgent need to apply rational drug design for targeting complement activation in the CNS to obtain effective treatments with low side-effects and costs; for example, there is little point in designing an anti-C1q therapeutic to be administered systemically for a CNS disease given that C1q exists throughout the body and in the circulation at micromolar levels—extremely high drug doses would be required to have any impact at the desired site in the CNS even if the drug is BBB penetrant. Given the ubiquitous expression of complement proteins throughout the body and the role of complement proteins in fighting infection and maintaining homeostasis, anti-complement therapeutics at these doses would likely have consequences throughout the body. Rather, therapies should target areas of pathology, as described above for the fusion proteins linking CR2 (localizes to C3 activation products in tissues) with a complement regulator, or target complexes, for example MAC, which exist at much lower concentrations and are found only in areas of pathology.

A common stumbling block in designing treatments for neuroinflammatory disorders is timing. Despite early conceptions about the fixed nature of post-mitotic neurons in the CNS, there is a great deal of redundancy and flexibility in neuronal circuits. Networks are hence able to compensate for a huge amount of cell loss through synaptic plasticity so that, by the time patients present at the clinic with symptoms, major neuronal death has already occurred. Inflammation as

a cause and consequence of this neuronal death occurs early and, unlike in acute conditions, fails to resolve because the primary stimulus, cell death, accumulation of toxic proteins or mitochondrial dysfunction, persists. Thus, effective treatments that aim to halt or slow disease progression must be administered early—likely before symptoms are apparent—and must prevent further neuronal cell death *and* encourage resolution of inflammation. Evidence from studies of the impact of NSAIDs on neurodegeneration support the idea that early and long-term treatment is protective but treatment post-onset fails (reviewed by 106). Early intervention requires ways of identifying those at high risk; genetic studies have identified polygenic signals that include many inflammatory genes and are highly predictive of risk of AD (106), and inflammatory biomarkers may also help predict risk (114). For large scale screening of pre-symptomatic populations expensive interventions such as brain imaging or CSF sampling are not practicable; in contrast, plasma offers an attractive source of biomarkers, although the levels of inflammatory markers in plasma may not reflect inflammation in the CNS. Simple and highly predictive plasma biomarkers are emerging and are likely to transform treatment of AD and other neurodegenerative diseases in the near future (180).

With regard to anti-complement therapies, it is likely that different approaches will be needed for different diseases; identifying for each disease when complement is activated, which pathways are activated and what the consequences are will be essential for effective interventions. Studies to date have been restricted to models and have focused on targeting the C3 convertases, central to all three activation pathways and thus a blunt tool likely to impact immune defense and other beneficial functions. The implication of C5a/C5aR in several CNS diseases offers the prospect of more targeted therapy with less risk of iatrogenic disease, although the systemic impact of long-term inhibition of the C5a/C5aR axis are uncertain. Evidence from models obtained either by complement gene deletion (**Table 1**) or anti-complement therapies (**Table 2**) has been helpful but are often contradictory, studies reporting both positive and negative impacts in similar models; this likely results from differences in timing and precise nature of the intervention and highlights once again the need for a thorough understanding of the underpinning biology prior to human studies.

CONCLUDING REMARKS

Therapy of acute neurological injury and neurodegenerative diseases represent a major therapeutic challenge. Most of the diseases described above currently have no effective therapies and new approaches are desperately needed. Although there are some common features, notably inflammation and complement activation, the described diseases are very heterogeneous, even within disease labels—AD is not a single disease! Patient stratification, for example, selecting patients with high inflammatory markers and evidence of ongoing complement activation for anti-complement drug therapy, will be necessary for successful trails in the future; this requires better biomarkers. For most of the diseases, proof of concept for new approaches to therapy is stymied by the lack of good models; critically, agents that are effective in current models usually fail in human trails (https://www.nature.com/articles/d41586-018-05722-9). For AD, models that better reflect the human disease are now available and may help overcome this issue. Switching off complement systemically will impact immune defense; while this may not be an issue for short-term therapy in acute conditions, in chronic diseases requiring life-long treatment it is a major consideration. Despite all these problems, inflammation and complement activation present tractable targets in neuroinjury and neurodegenerative disease and deserve investment into basic understanding and the development of CNS-targeting anti-inflammatory and anti-complement drugs.

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REVIEW
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Complement-Dependent Mechanisms and Interventions in Periodontal Disease

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Periodontitis is a prevalent inflammatory disease that leads to the destruction of the tooth-supporting tissues. Current therapies are not effective for all patients and this oral disease continues to be a significant public health and economic burden. Central to periodontal disease pathogenesis is a reciprocally reinforced interplay between microbial dysbiosis and destructive inflammation, suggesting the potential relevance of host-modulation therapies. This review summarizes and discusses clinical observations and pre-clinical intervention studies that collectively suggest that complement is hyperactivated in periodontitis and that its inhibition provides a therapeutic benefit. Specifically, interception of the complement cascade at its central component, C3, using a locally administered small peptidic compound (Cp40/AMY-101) protected non-human primates from induced or naturally occurring periodontitis. These studies indicate that C3-targeted intervention merits investigation as an adjunctive treatment of periodontal disease in humans.

Keywords: complement, C3, therapeutics, compstatin Cp40, AMY-101, primate models, inflammation, periodontitis

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INTRODUCTION

Complement represents an interactive network of soluble, cell surface-associated and intracellular molecules that activate, amplify, and regulate immunity and inflammation (1, 2). In addition to the classic serum proteins (C1-9), the network contains overall some 50 proteins, including pattern-recognition molecules, convertases and other proteases, receptors and regulators. Complement activation is initiated via distinct pathways, the classical, lectin, or alternative (**Figure 1**). The classical and lectin pathways are activated following the binding of complement-associated pattern-recognition molecules (e.g., C1q and mannose-binding lectin, respectively) to immune complexes (classical pathway) or to carbohydrate moieties exposed on microbial or damaged/necrotic host cells (lectin pathway). The alternative pathway is initiated by a tick-over mechanism and moreover amplifies the initial response induced by the other two complement pathways.

All three pathways converge at the central component of the complement system, C3, the activation of which leads to the generation of effectors that facilitate the ability of antibodies

and phagocytes to clear microbial pathogens (via C3b opsonization), induce chemotaxis and inflammation (via the C3a and C5a anaphylatoxins), and lyse susceptible microbial targets (via the C5b-9 membrane attack complex [MAC]) (**Figure 1**). Furthermore, in cooperation with other immune and physiological systems, complement contributes to normal tissue and organ development, integrates and coordinates innate and adaptive immunity, mediates apoptotic cell clearance, and promotes tissue repair following injury (1, 3). In recent years, it has also become increasingly appreciated that complement is not exclusively produced in the liver and its actions are not restricted in the intravascular and extracellular compartments. Indeed, it has been shown that complement components can be produced locally by resident tissue cells as well as by recruited leukocytes. Moreover, an intracellular complement system was identified and shown to have novel homeostatic and immune functions, such as regulation of $CD4^+$ T cell activation (2, 4).

Despite its importance in homeostatic immunity, complement may become dysregulated or excessively activated (e.g., due to host genetic or microbial virulence factors), thereby turning from a homeostatic to a pathological effector that can drive or exacerbate a number of disorders, such as cancer or inflammatory diseases, including periodontitis (**Figure 1**) as discussed in detail in the next section (5–8). Periodontitis is a common chronic inflammatory disease that causes destruction of the periodontium, i.e., the tissues that surround and support the teeth, namely, gingiva, periodontal ligament, and alveolar bone. If left untreated, periodontitis can lead to tooth loss and possibly impaired mastication (9). It is estimated that severe periodontitis afflicts ∼10% of adults (10, 11). In its severe form, periodontal disease is associated with elevated risk for certain systemic conditions, such as atherosclerosis (12).

Recent evidence from clinical microbiome studies and mechanistic studies in animal models have shown that periodontitis is a dysbiotic disease rather than an infection attributed to a select few species (13–15). Connective tissue damage and loss of alveolar bone is mediated by a dysregulated and excessive inflammatory response, which includes components of both innate and adaptive immunity but fails to control the dysbiotic microbial challenge that induced it (16). In fact, the destructive periodontal inflammatory response is exploited by the dysbiotic microbial communities to procure nutrients from tissue breakdown products (17, 18). The fact that the disease is predominantly mediated by the host inflammatory response and that inflammation is necessary to support dysbiotic microbial communities justifies the rationale for developing host-modulation strategies to treat periodontitis. Such novel interventions may be used as adjuncts to improve current therapies (e.g., mechanical removal of the pathogenic biofilm), which are not always sufficient to treat periodontitis (19–22), thus accentuating its significant public health and economic burden (9, 11, 23, 24). Here, we discuss clinical and preclinical studies that have collectively linked complement overactivation to periodontitis and provided a mechanistic understanding of this relationship, paving the way to complement-targeted therapies to treat this oral inflammatory disease with strong associations to increased risk for other systemic conditions.

CLINICAL STUDIES

The possible involvement of complement in human periodontitis was first recognized in the 1970s and 1980s by histological and clinical studies analyzing the gingival crevicular fluid (GCF) in periodontal health and disease. GCF represents the inflammatory exudate which bathes the gingival crevice or pocket, i.e., the space between the free gingiva and the tooth surfaces (25). GCF samples from periodontitis patients were shown to have complement-dependent hemolytic activity, suggesting that a functional complement system is present in this inflammatory exudate (26, 27). Activated complement fragments were shown to be highly abundant in the GCF from patients, but were undetectable or present in lower concentrations in GCF from healthy control individuals (28–32). Similarly, complement components and cleavage products were also readily detected in chronically inflamed gingiva but were undetected or at lower abundance in healthy tissue samples; the complement components detected in diseased gingiva (and also in GCF) were representative of the entire cascade (e.g., C1q, factor B, Bb, C3, C3a, C3b, C3c, C3d, C4, C5, C5a, C5b, C9) (26–37).

Importantly, periodontal therapy that resulted in decreased clinical indices of periodontal inflammation and tissue destruction also led to decreased C3 activation in the GCF (38). Conversely, and consistently, the progression of gingival inflammation during an experimental human gingivitis study was associated with elevated C3 cleavage in the GCF (32). Specifically, this study examined the cleavage of factor B, C3, and C4 in GCF collected during the experimental period and demonstrated, respectively, their conversion to Bb and C3c but not to C4c, thus implying selective activation of the alternative pathway (32). An immunohistochemical study showed that the complement regulator CD59 is expressed at lower levels in the gingiva of periodontitis patients as compared to healthy individuals, which might imply compromised protection of periodontitis-involved tissues against MAC-mediated autologous tissue damage (35).

More recent studies also support an association between complement and periodontitis. A case of aggressive periodontitis with severe angioedema localized to the gingiva was linked to dysregulated complement function, specifically deficiency of the C1-esterase inhibitor (C1INH) (39). A single nucleotide polymorphism affecting C5 (rs17611), which was previously linked to elevated C5 in serum and susceptibility to the complement-associated disease liver fibrosis (40), was shown to be more prevalent in patients with periodontitis than in healthy controls (41). In terms of its expression, C3 was shown to be among the top 5% genes that are most strongly downregulated following periodontal therapy (42). Another study has used integrative gene prioritization and databases from genome-wide association studies and microarray experiments, and identified C3 among the top 21 most promising candidate genes involved in periodontal disease (43). Interestingly, partial C4 gene deficiencies are significantly more frequent in periodontitis patients than in healthy individuals (44). This finding might suggest a protective function associated with C4, the activation of which occurs via the classical or the lectin pathways. However, it should be noted that C4a was recently shown

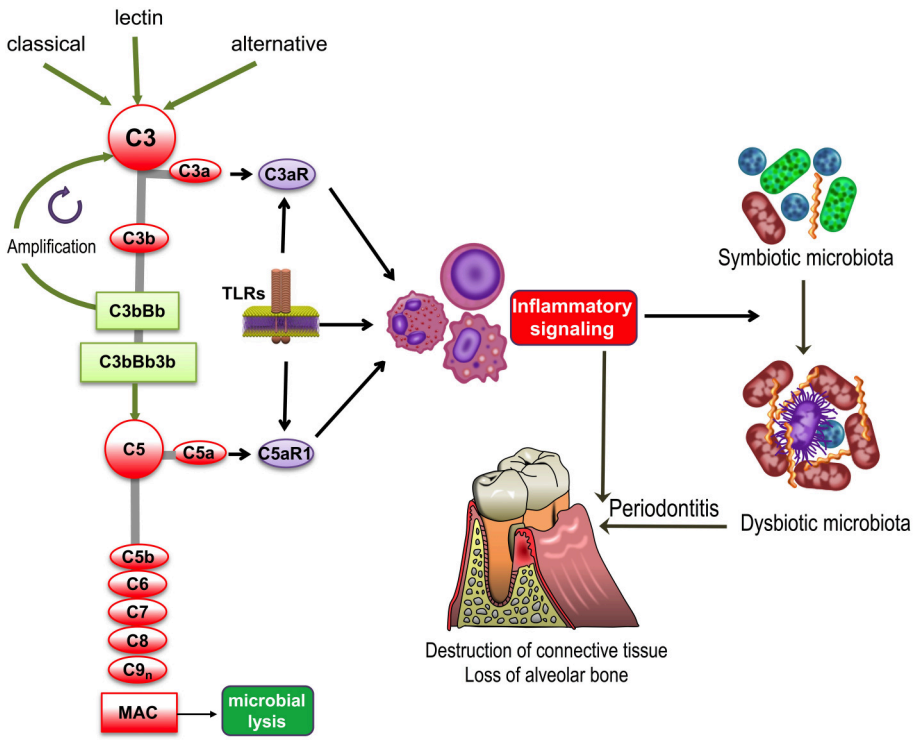

FIGURE 1 | Complement and TLR involvement in dysbiosis and inflammatory bone loss in periodontitis. The classic, lectin, and alternative pathways converge at and activate the central complement component, C3, resulting in the generation of various effectors, such as, the C3b opsonin, the inflammatory anaphylatoxins C3a and C5a, and the C5b-9 membrane attack complex (MAC). The C3a and C5a activation fragments activate respectively C3aR and C5aR1, which cross-talk with Toll-like receptors (TLRs) and synergistically activate inflammatory leukocytes. TLRs also upregulate the expression of C3aR and C5aR1. Inflammation induced by complement-TLR crosstalk interactions not only causes gingival tissue destruction and bone loss in periodontitis but also contributes to the remodeling of a symbiotic microbiota into a dysbiotic one, thereby further potentiating destructive inflammation.

to bind and activate protease-activated receptors (PAR) 1 and 4 (45) which are expressed by platelets and endothelial cells (46). Thus, C4-mediated effects may not necessarily involve downstream triggering of C3-dependent activities. Whether C4a might mediate complement crosstalk with the coagulation and/or the endothelial barrier system is currently uncertain as is the impact of such interactions on periodontitis.

The aforementioned clinical studies collectively indicate a role for complement activation in periodontal disease pathogenesis. However, the correlative nature of these human studies could not safely establish a causal relationship between complement and periodontitis and distinguish it from the alternative possibility that complement activation could simply be a marker of local periodontal inflammation. Causal evidence was derived from mechanistic animal model-based studies described below.

MECHANISTIC STUDIES IN ANIMAL MODELS

Animals models can be engaged to determine causative links between potential mechanisms and disease pathogenesis (47), thereby not only promoting knowledge on pathogenesis but also identifying therapeutic targets and paving the way to human clinical trials. As the triggering of the complement cascade is intertwined with TLR activation (3), the two systems are discussed together in the studies presented here.

In response to microbial infection or tissue damage, complement and TLRs are swiftly activated, frequently by the same agonists. In this regard, bacterial lipopolysaccharide (LPS; a TLR4 agonist), fungal zymosan (TLR2/6 agonist) and bacterial CpG DNA (TLR9 agonist) not only induce TLR signaling but also can activate complement (48). In fact, complement and TLRs are not only co-activated in response to microbial infection and other types of insult, such as tissue injury, but they also engage in signaling crosstalk interactions in several myeloid cell types (monocytes, macrophages, neutrophils, and dendritic cells) (49–54) (**Figure 1**). In a pioneering study, different TLR agonists systemically given to mice lacking a major membrane-associated complement regulator, the decay-accelerating factor, induced significantly higher plasma levels of pro-inflammatory cytokines than wild-type controls (55). Similarly, mice systemically co-injected with TLR agonists (specifically TLR2, TLR4, and TLR9 ligands) and a potent complement activator (cobra venom factor) display remarkably high plasma levels of pro-inflammatory cytokines (55). In the complement-TLR crosstalk, the activated signaling pathways converge at mitogen-activated protein kinases (extracellular signal-regulated kinase-1 and-2 and

c-Jun N-terminal kinase), which in turn activate key transcription factors, namely activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (55). Although this synergy has the potential to invigorate innate immunity against infection, it may also contribute to destructive inflammatory responses.

In line with these findings, the concomitant activation of C5aR1 and TLR2 in the mouse gingiva by local co-administration of specific ligands (C5a and Pam3Cys, respectively) resulted in the induction of significantly higher levels of IL-1β, IL-6, IL-17, and TNF than stimulation of either receptor alone (56). These data suggested that a synergy between complement and TLRs may be a major contributor to the induction of periodontal inflammation. In support of this notion, mice lacking C5aR1 are quite resistant against inflammatory bone loss regardless of the presence of TLR2 (57) and, in an analogous manner, mice lacking TLR2 are protected from inflammatory bone loss regardless of the presence of C5aR1 (58). TLR9-deficient mice are also protected against experimental periodontitis (59), which could be attributed, at least in part, to complement-TLR9 synergy (55). These studies utilized a murine model of periodontitis in which the disease is initiated by dysbiosis following oral gavage with the keystone pathogen *Porphyromonas gingivalis* (60, 61). Consistent with the importance of complement involvement in periodontal disease pathogenesis, C3-deficient mice were protected against periodontitis in three distinct models, ligature-induced periodontitis, *P. gingivalis*-induced periodontitis, and aging-associated periodontitis (62). The ligature model involves the placement of silk ligatures around molar teeth leading to massive accumulation of indigenous bacteria and induction of inflammation and alveolar bone loss in specific-pathogen-free (but not germ-free) animals (60, 63–65). In the aging-associated periodontitis model, periodontal inflammation and bone loss develops naturally as a function of old age when homeostatic mechanisms break down (66–68). Interestingly, C3-deficient mice also exhibited reduced periodontal bacterial load in *P. gingivalis*-induced periodontitis as compared to wild-type littermate controls (62). These data suggest that lack of complement activation does not lead to defective control of the periodontal microbiota and, moreover, are consistent with the concept that destructive inflammation is required to sustain a quantitatively and compositionally altered dysbiotic microbiota (18).

TRANSLATIONAL PRECLINICAL STUDIES

The studies discussed earlier suggested that complement may be a promising target for the treatment of periodontitis. Indeed, in the oral gavage model of *P. gingivalis*-induced periodontitis, intra-gingival microinjection of wild-type mice with PMX-53, a C5aR1 antagonist (69), suppressed the induction of inflammatory cytokines (IL-1β, IL-6 and IL-17, and TNF) in the gingival tissue and inhibited alveolar bone loss (56). This protective effect occurred despite the presence of intact TLR2, in other words, inflammatory bone loss can be effectively inhibited by blocking only one of the two cross-talking receptors (56). PMX-53 was also tested in the ligature-induced periodontitis model where disease can be initiated independently of *P. gingivalis* (63). Although substantial inflammatory bone loss was induced after 5 days at the ligated areas of control-treated mice, mice locally microinjected (at the ligated sites) with PMX-53 exhibited significant protection against periodontal inflammation and bone loss (56). Rats given PMX-205 [another C5aR1 antagonist (70)] via the drinking water were also protected from ligature-induced bone loss (71), although with reduced efficacy perhaps due to the different route of drug administration and/or the use of a different animal species.

It is important to note that the same inflammatory mediators (e.g., TNF, IL-1β, prostaglandin E2) have been implicated in inflammatory periodontal bone loss across different species, such as mice, rats, dogs, non-human primates, and humans (72–77). Therefore, mice appear to be a useful model for human periodontitis especially for mechanistic studies, since mice currently represent the only available species with engineered knock-in or knock-out mutations for a whole panel of key immune response genes. However, promising results obtained in higher animals, such as non-human primates, increase the possibility that candidate drugs can be protective also in humans. In this regard, the periodontal tissue anatomy and immune system of non-human primates are similar to those of humans, and periodontitis in monkeys displays clinical, microbiological, and immuno-histological features that are highly similar to those of human periodontal disease (78–82). In fact, the use of non-human primates becomes necessary for testing drugs that lack specificity for the widely used rodent models and other small animals.

In this regard, compstatin and new generation analogs are small peptidic inhibitors that have an exquisite specificity for human and non-human primate C3 (83–85). Given the absence of available C3 inhibitors in mice, the appropriateness of C3 as a therapeutic target in periodontitis could only be tested in primates. Specifically, the third-generation compstatin analog Cp40 was tested in cynomolgus monkeys (*Macaca fascicularis*) (62). Cp40 has a subnanomolar affinity for C3 ($K_D = 0.5$ nM; 6,000-fold greater than that of the original compstatin) and a plasma human half-life (48 h) that exceeds expectations for most peptidic drugs. Mechanistically, the original compstatin and new generation analogs bind C3 and block its interaction with and cleavage by the C3 convertase into its active fragments, C3a and C3b (86) (**Figure 2**). In other words, the compstatin family of C3 inhibitors protect the C3 substrate rather than interfere with the C3 convertase. As a consequence, the compstatins prevent propagation and amplification of complement activation and generation of effector molecules regardless of the mechanism that initiated complement activation (83, 84).

Periodontitis in adult cynomolgus monkeys was induced by placing silk ligatures around posterior teeth on both halves of the mandible (lower jaw) for a period of 6 weeks. Local treatment (through intra-gingival injection) with Cp40 started 3 days following ligature placement. A split-mouth experimental design was applied, where one side was treated with Cp40 and the other with an inactive control peptide, thus each animal served as its own control. The disease was monitored clinically by analyzing clinical indices that assess

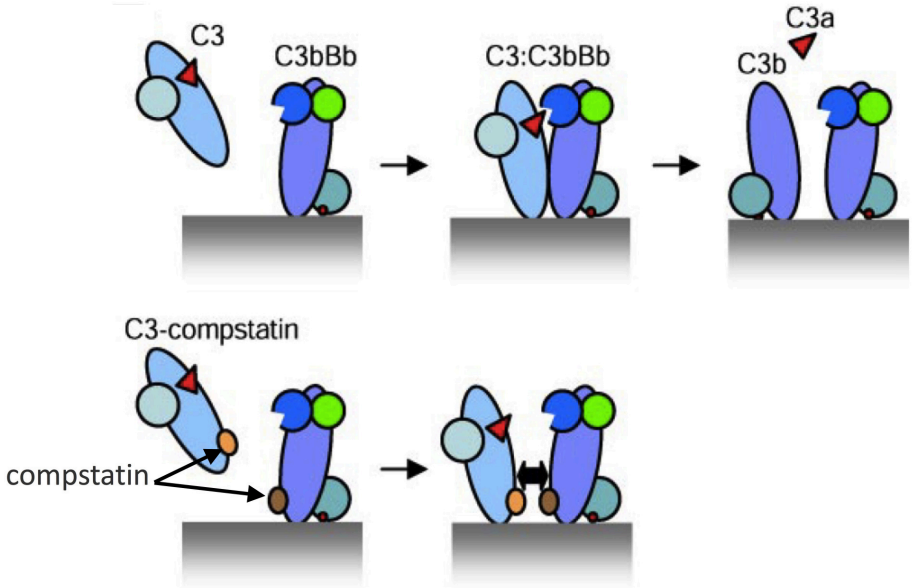

FIGURE 2 | Model of C3 activation and its inhibition by compstatin. **(Top)** Depiction of key protein interactions resulting in the formation of C3 convertase on a target surface (e.g., a microbial cell surface). Native C3 binds to the convertase (C3bBb) and is cleaved into its active fragments, C3a and C3b. **(Bottom)** Compstatin acts by blocking protein-protein interaction. Specifically, compstatin binds both native C3 and C3b and sterically hinders the binding of native C3 by C3 convertases, hence preventing C3 cleavage into its active fragments. From ref. 87. Used by permission.

periodontal inflammation and tissue destruction (87). Cp40 treatment resulted in significant decrease of gingival index and clinical attachment loss, which correlated with lower levels of proinflammatory and osteoclastogenic cytokines (e.g., TNF, IL-1β, IL-17, and RANKL) in the GCF, as well as with decreased numbers of osteoclasts in bone biopsy specimens (62). In contrast to RANKL, the GCF content of osteoprotegerin (OPG), a natural inhibitor of RANKL, was maintained at increased levels in Cp40-treated as compared to control sites. Therefore, Cp40 appeared to cause a favorable reversal of the RANKL/OPG ratio, which is a potential indicator of periodontitis (88). Consistent with these data, radiographic analysis showed that Cp40-treated sites had significantly less bone loss as compared to control-treated sites (62).

To determine the potential usefulness of Cp40 in a therapeutic setting, the drug was administered to adult cynomolgus monkeys with pre-existing, naturally-occurring chronic periodontitis (89). The animals were locally injected in the gingiva with Cp40 either once a week (group of 5 animals) or three times per week (group of 10 animals) for a 6 weeks treatment period followed by a 6 weeks follow-up period in the absence of Cp40 treatment. Clinical examinations and collections of GCF samples were conducted at baseline and throughout the study. In both groups, treatment with Cp40 led to significant decrease in clinical indices that assess periodontal inflammation (gingival index and bleeding on probing), tissue destruction (probing pocket depth and clinical attachment loss) or tooth mobility which is often linked to bone loss. The improvement of clinical disease as reflected by reduced clinical indices correlated with decreased levels of proinflammatory and osteoclastogenic mediators (e.g., IL-17 and RANKL) in the GCF and decreased osteoclast numbers in bone biopsies. The protective effects mediated by Cp40 endured, although with reduced effectiveness, for at least 6 weeks after the drug was discontinued. Cp40 could therefore reverse pre-existing chronic periodontal inflammation without additional treatments, such as scaling and root planing (SRP) (89). Proteomic analysis of GCF samples collected from that study showed involvement of both the alternative and classical pathways of complement activation in naturally occurring non-human primate periodontitis; however, the alternative pathway was the most enriched of all biological pathways identified by gene ontology analysis (90). These proteomic findings are consistent with early clinical reports indicating that the complement alternative pathway is predominantly activated in GCF samples from human periodontitis patients, although the classical pathway is also activated (28, 29, 38). Based on this consideration (and the likelihood that carbohydrate or glycoprotein components of periodontal bacteria may activate the lectin pathway) the concomitant inhibition of all three pathways (as can be done by Cp40) is likely to provide increased protection against periodontitis as compared to inhibition of individual pathways of complement activation. Another main target revealed by the proteomic fingerprinting of GCF samples from Cp40-treated NHPs was leukocyte degranulation. Neutrophils account for considerable tissue damage in human periodontitis, in great part through degranulation of tissue-degrading proteases and cytotoxic molecules (76, 91–94). In this regard, the ability of Cp40 to suppress exocytosis likely represents another host protective mechanism.

In a follow-up study in cynomolgus monkeys with naturally-occurring chronic periodontitis, it was shown that an effective therapeutic dose of locally administered Cp40 [100 μg/site; used in the study by Maekawa et al. (89)] does not cause local irritation and has long-lasting protective effects even when given as infrequently as once per 3 weeks (95). Therefore, taken together, clinical observations in humans and pre-clinical intervention studies in non-human primates suggest that complement is overactivated in periodontitis and that C3 inhibition by Cp40 is a promising host-modulatory therapy that warrants investigation as a potential treatment of human periodontitis.

Given the potential for synergism between complement and TLRs, C3 inhibition in periodontitis can also inhibit inflammation that is activated by TLR signaling either in response to microbial TLR ligands (e.g., LPS, lipoproteins, and bacterial DNA) (96, 97) or in response to endogenous TLR ligands (e.g., biglycan, hyaluronan, or heparan sulfate fragments) that are released upon tissue injury and act as danger-associated molecular patterns (DAMPs) (98, 99). The latter suggests that complement inhibition may also suppress damaged tissue-induced inflammation, thereby blocking also the progression of periodontitis. Interestingly, several TLRs (TLR2, TLR3, TLR4, and TLR9), when activated by bacterial molecules or DAMPs released from stressed/damaged tissues, were shown to induce local expression of complement components (e.g., macrophage production of factor B and C3), thereby further promoting complement activity in an inflammatory environment (100–103). For instance, LPS induces production and release of factor B through a TLR4-TRIF pathway in macrophages (100). Moreover, TLR signaling suppresses the desensitization of G-protein-coupled receptors (GPCRs) by downregulating the expression of G-protein-coupled receptor kinases, required for inducing GPCR phosphorylation and internalization (104). This suggests a mechanism by which TLRs may potentially prolong the activation of GPCRs, such as C3aR and C5aR1. Furthermore, TLR-induced cytokines, such as IL-6, promote the expression of C3aR and C5aR (105). Therefore, TLRs regulate the expression of complement factors and both the expression and activation of complement receptors, which—as alluded to earlier—in turn can amplify TLR-dependent responses. This pro-inflammatory and potentially destructive feed-forward loop can be potentially disrupted by complement inhibition (**Figure 3**), a notion that may underlie the success of Cp40 treatment in the non-human primate preclinical model. Complement inhibition at the C3 level may also inhibit inflammasome-dependent inflammation since complement pathways (C3aR signaling and sublytic membrane attack complex) were shown to promote the activation of the NLRP3 inflammasome and IL-1β release (106, 107).

SAFETY AND OTHER CONSIDERATIONS FOR CLINICAL USE

Given the participation of the dysbiotic periodontal microbiota in the pathogenesis of periodontitis, the targeting of complement may not appear as an intuitive therapeutic option for this oral disease. In general, a potential concern regarding the therapeutic use of complement inhibitors is whether complement blockade may undermine the competency of host antimicrobial defenses and thus increase the risk of infection. Although this possibility may not be an issue in acute conditions that require transient complement inhibition [e.g., in hemodialysis (108)], it has to be carefully considered in conditions that will require long-term use of complement inhibitors. In this regard, individuals with primary C3 deficiencies have increased risk of certain infections (e.g., *Neisseria meningitidis* and *Streptococcus pneumoniae*) although this enhanced susceptibility appears to subside in adulthood, presumably owing to the development of compensatory defense mechanisms (109–111). Current experience from FDA-approved anti-complement drugs, such as eculizumab that blocks C5 activation, shows that immunization against encapsulated bacteria (such as meningococci) can largely diminish infectious risks. Therefore, vaccinations as well as prophylactic use of antibiotics may be included to enable safe use of complement inhibitors in chronic settings. Importantly, in cases of complement inhibition with small-molecule inhibitors, such as compstatin, the compound can more readily phased out (than an antibody for instance) if necessary, thus enabling swift recovery of complement-dependent antimicrobial functions. Importantly, the monitoring of non-human primates under prolonged (up to 3 months) systemic treatment with Cp40 revealed no significant differences in biochemical, hematological, or immunological parameters in their blood or tissues as compared to those of vehicle alone-treated controls, despite complete inhibition of C3 in the plasma. Intriguingly, moreover, wounds inflicted in the skin of the Cp40-treated animals did not show any signs of infection but rather exhibited a trend toward faster wound healing as compared with the vehicle-treated controls (112). This finding is consistent with earlier observations in mice in which C3 deficiency resulted in faster skin wound healing as compared to C3-sufficient control mice (113).

Although a chronic condition, periodontitis is a local inflammatory disease and thus can be treated via local complement inhibition, a much safer approach than systemic administration of the same inhibitor. Systemic exposure with complement inhibitors following local injection into the periodontal tissues should be negligible and thus not impair complement activity in circulation or other tissues. This notion can be exemplified by experience with Cp40. As C3 is the most abundant protein of the complement system in blood (1.0 to 1.5 mg/ml), small amounts of locally injected Cp40 that could "escape" from the periodontal tissue should be readily bound by excess C3 in blood, hence not reaching other tissues at active (inhibitory) concentrations. In the treatment regimen used in the above-described NHP study by Maekawa et al. (89), a total of 1.5 mg Cp40 was injected (15 sites at 100 μg/site). Even if the full local dose were to be given systemically, this would only result to an amount of 0.2–0.3 mg/kg bodyweight in non-human primates (0.02–0.03 mg/kg bodyweight in humans), whereas a systemic Cp40 dose of 1–2 mg/kg bodyweight is necessary to reliably attain target-exceeding drug levels in non-human primates (114).

Even at the local level, complement inhibition is unlikely to lead to defective control of the periodontitis-associated microbiota. As discussed above, C3-deficient mice have

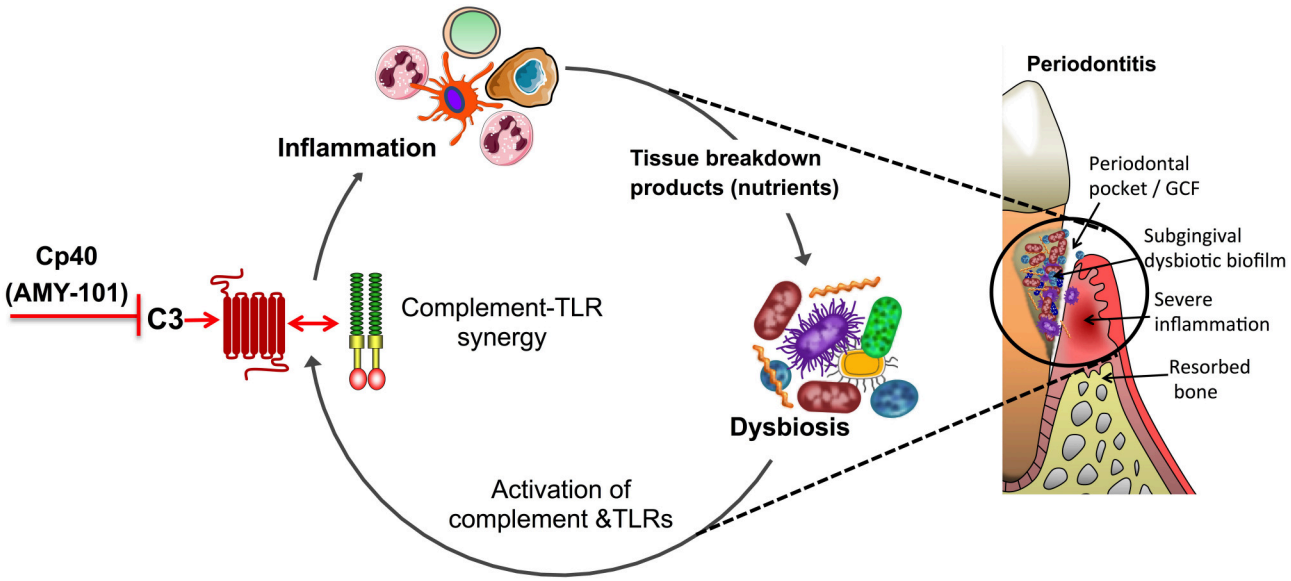

FIGURE 3 | Complement-targeted inhibition blocks a vicious cycle linking destructive inflammation and dysbiosis in periodontitis. Periodontitis is driven by a reciprocally reinforced interplay between dysbiotic microbial communities and inflammation. Whereas, dysbiosis induces inflammation, the nutritionally favorable inflammatory environment is a major ecological factor that exacerbates dysbiosis. Studies in different preclinical models showed that complement inhibition abrogates complement-TLR crosstalk signaling and resulting inflammation, thereby breaking the disease-provoking vicious cycle regardless of the presence of intact TLR signaling. For instance, blocking the central complement component C3 through the use of the peptidic compound Cp40/AMY-101 has blocked experimental or naturally occurring periodontitis in non-human primates.

decreased periodontal bacterial load compared to C3-sufficient controls during experimental periodontitis (62). These data are consistent with the notion that inflammation is an ecological driver of dysbiosis in periodontitis (**Figure 3**). Indeed, destructive periodontal inflammation causes the generation of tissue breakdown products (such as degraded collagen peptides or heme-containing compounds) that are used as a nutrient source by a subset of bacterial species associated with dysbiosis; these are mainly proteolytic/asaccharolytic organisms with iron-acquisition mechanisms and/or can thrive by utilizing other inflammatory byproducts, such as nitrate for anaerobic respiration, thereby outcompeting health-associated bacteria and exacerbating dysbiosis (18, 115, 116). Therefore, complement inhibition by Cp40 may not simply inhibit inflammation but may additionally interfere with the outgrowth of the dysbiotic microbiota (**Figure 3**). Experimental support of the notion that anti-inflammatory approaches can have indirect anti-microbial effects has been obtained in mouse and rabbit models of periodontitis, where the control of inflammation not only protected against disease but also decreased the bacterial load and reversed dysbiosis (76, 77, 117–119). Conversely, and in line with the previous statement, the bacterial biomass of biofilms associated with human periodontitis increases with increasing clinical inflammation (120).

CONCLUSIONS AND OUTLOOK

The studies discussed above suggest a clinical value of inhibiting all three main pathways of complement activation in periodontitis, which can be achieved by targeting the central component C3. C3 inhibition can directly inhibit inflammation and indirectly counteract dysbiosis. The safety and efficacy of Cp40 in non-human primate periodontitis (62, 89, 95) paves the way to clinical trials for the treatment of human periodontitis. To this end, aspects that need to be considered include questions regarding administration frequency, dosing, and selection of those patients who would most benefit from such a treatment. Even though Cp40 was successfully tested as a stand-alone treatment for both induced and naturally-occurring periodontitis in monkeys, the drug is more likely to be used as an adjunctive therapy to the management of human periodontal disease. Future clinical trials may investigate the combined potential of Cp40 and SRP to treat periodontal inflammation and suppress bone loss as compared to SRP alone. In very severe cases of periodontitis, combined Cp40 and SRP therapy could be compared to periodontal surgery, to determine whether the Cp40/SRP treatment can obviate the need for a surgical approach. It should be noted that a Cp40-based treatment (and host-modulation interventions in general) may not necessarily be applied in a therapeutic setting but also on a preventive basis (before the onset of periodontitis) to high-risk patients, such as cigarette smokers and diabetic patients (121–123).

The protective effects of Cp40 in non-human primate periodontitis are maintained for many weeks following drug withdrawal from treated monkeys (89, 95). This is an encouraging finding although the optimal frequency of Cp40 administration for long-term treatment of human periodontitis may need to be decided empirically. The unique pharmacokinetic

properties of Cp40 described earlier are consistent with a "target-driven" model, where an initial rapid clearance of excess free peptide (i.e., not bound by C3) is followed by slow clearance of C3-bound peptide. The tight binding of Cp40 to C3 thus appears to delay its clearance and, indeed, the determined half-life values of different compstatin analogs correlate with their C3-binding affinities (85). Similarly, strong binding of Cp40 to abundant C3 in the inflamed periodontal tissue could contribute to delayed clearance of the drug from the tissue, thus contributing to sustained protection from periodontal inflammation. Moreover, the ability of Cp40 to arrest inflammation and presumably to reverse dysbiosis may reset the balance toward tissue homeostasis, which on its own (despite the absence of the drug) could resiliently inhibit or delay the recurrence of pathological processes.

Amyndas Pharmaceuticals is developing Cp40, a third-generation non-PEGylated compstatin analog, as the clinical candidate drug AMY-101. AMY-101 is developed for therapeutic interventions in C3 glomerulopathy (C3G), complications of ABO-incompatible kidney transplantation, paroxysmal nocturnal hemoglobinuria (PNH), and periodontal disease (124, 125). The PEGylated version of an earlier compstatin analog, POT-4/4(1MeW) (APL-2, Apellis Pharmaceuticals) is clinically developed for use in complement-mediated disorders including age-related macular degeneration and PNH. In a Phase II trial, prolonged APL-2 treatment was shown to be safe and reduced the growth rate of geographic atrophy associated with age-related macular degeneration. AMY-101 has obtained orphan drug designation for C3G and PNH from the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2016 and more recently, in 2017, successfully completed a phase I safety trial (125, 126). Targeted modifications of the *N*- and *C*-terminus of Cp40/AMY-101 have led to a series of fourth-generation compstatins with higher solubility, improved PK profiles thus broadening the spectrum of administration routes and likely reducing the dosing frequency of these peptidic drugs in chronic regimens (127). Overall, more than 20 candidate complement-targeted drugs that inhibit distinct points of the cascade are currently being tested in clinical trials for a variety of inflammatory and degenerative diseases (125). The documented safety of Cp40/AMY-101 and its protective effects in highly relevant preclinical models of periodontitis merits investigation in future clinical trials for the treatment of human periodontitis.

AUTHOR CONTRIBUTIONS

GH and JL: conceptualization; GH: original draft. All authors listed made a substantial intellectual contribution to the manuscript and edited and approved it for publication.

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Conflict of Interest Statement: JL is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation compstatin analogs, such as AMY-101). JL and GH are inventors of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. JL is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives].

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Targeting Complement Pathways in Polytrauma- and Sepsis-Induced Multiple-Organ Dysfunction

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Exposure to traumatic or infectious insults results in a rapid activation of the complement cascade as major fluid defense system of innate immunity. The complement system acts as a master alarm system during the molecular danger response after trauma and significantly contributes to the clearance of DAMPs and PAMPs. However, depending on the origin and extent of the damaged macro- and micro -milieu, the complement system can also be either excessively activated or inhibited. In both cases, this can lead to a maladaptive immune response and subsequent multiple cellular and organ dysfunction. The arsenal of complement-specific drugs offers promising strategies for various critical conditions after trauma, hemorrhagic shock, sepsis, and multiple organ failure. The imbalanced immune response needs to be detected in a rational and real-time manner before the translational therapeutic potential of these drugs can be fully utilized. Overall, the temporal-spatial complement response after tissue trauma and during sepsis remains somewhat enigmatic and demands a clinical triad: reliable tissue damage assessment, complement activation monitoring, and potent complement targeting to highly specific rebalance the fluid phase innate immune response.

Keywords: trauma, sepsis, hemorrhagic shock, MODS, complement activation, complement dysregulation, complement therapeutics, clinical trial

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INTRODUCTION

Complement activation as a major innate defense strategy occurs early after trauma, hemorrhagic shock and during sepsis in both the experimental and clinical settings (1–6). A recent comparison of severe trauma and septic patients in an intensive care unit (ICU) showed that during sepsis, excessive activation of the complement cascade is detectable as evidenced by significantly enhanced systemic C3a concentrations, whereas during trauma, complement activation is also existent but less pronounced (7). In trauma, rapid consumption of key complement components such as C3 or C5 seems to be the primary mechanisms (7) whereas in septic conditions, consumption of complement factors may occur later (if at all), secondary to the activation. Hyper-activated and consumed defense systems including the complement and coagulation cascade can result in imbalanced immune responses, impaired clearance of tissue debris and pathogens, dysregulated coagulation, perfusion disturbances, changes in tissue and cellular microenvironment, and barrier dysfunction. All these alterations culminate in multiple signaling-,

cellular-, and organ dysfunction (8, 9). Although highly specific complement inhibitors are available, translation of these observations into therapeutic strategies remains challenging and requires differential considerations (10). The immune response to damaged and infected tissue is not mono-dimensional; instead it comprises various compartmentalized responses and organ specific outcomes (11, 12). For example, in experimental sepsis there is a loss of C5aR expression on neutrophils whereas C5aR expression on various organs is significantly enhanced (13). Thus, the complement reaction to danger associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) needs to be reliably determined before any specific therapeutic intervention can be applied in the clinical setting of severe sterile or infectious insults. Furthermore, underlying triggers and mechanisms of the multiple organ dysfunction syndrome (MODS) need to be detected and further explored. Several hypotheses exist about the initiators and drivers of MODS. Main contributors seem to be barrier failure (9), electrophysiological alterations (14), microcirculatory disturbances, inflammation-induced cell dysfunction, protein alterations and microbiome shifts (15). Hibernation seems to be a contributing factor in the development of MODS, which allows the cell to shut down energy consuming functional efforts and therefore to preserve the cellular morphology (16). Although MODS can be induced by distinct injuries and infectious insults, once established, it seems to follow common pathways. For the individual and also the society, MODS remains a major burden with a high lethality rate and a high socio-economic impact and therefore requires an improved understanding, comprehensive mechanistic insights and basic as well as clinical research efforts (17).

POLYTRAUMA-INDUCED MODS—ROLE OF COMPLEMENT

Polytrauma comprises life-threatening multiple injuries that activate innate and adaptive immunity with multidimensional consequences for the host (9, 18). Although some reduction in the frequency of MODS after polytrauma has been noted in the last decades, it still remains a major cause of death after severe trauma (19). Within minutes after polytrauma there is a significant increase in circulating complement activation products such as C3a, C5a, and sC5b-9 and a drop in complement hemolytic activity (2–4, 6, 20) (**Figure 1**). Of note, an enhanced C3a/C3 ratio in plasma early after trauma was prognostic for lethal outcome (6). Another study showed enhanced C3a levels as an indicator for MODS (20). Activation via its amplification by the alternative pathway is observed early after trauma measured by Bb plasma levels, which was additionally correlated with injury severity and the development of organ failure such as acute lung injury and acute renal failure (3). The underlying mechanisms, however, are still elusive. For example, C3a may have a direct pathophysiological impact on the lungs as an "engine" of multiple organ failure. C3a alters not only the microvascular and airway tonus (21) but induces direct pro-inflammatory effects (22) which may contribute to perfusion disturbances and cellular dysfunction. In a murine model of blunt thorax trauma, we have found enhanced C3 and heme-oxygenase-1 (HMOX1) transcriptional expression levels in the lungs early after injury (23). A recent analyses of 81 polytrauma patients also revealed an enhanced expression of HMOX1, which was associated with septic complications (24). In this context, it is noteworthy that HMOX1 is downregulated in leukemic leukocytes by C3a and C5a resulting in an enhanced cellular mobility and infectious complications (25), indicating some interaction between HMOX1 and complement activation processes. Clinical trials, evaluating the effect of targeting at the C3or C3a/C3aR are lacking. Although in a different context, a recent study showed the potential of HMOX1 to protect endothelial cells against heme-mediated complement activation. Heme activates the alternative complement pathway and also up-regulates the cyto-protective and stress-response gene HMOX1 in an organ-specific manner. While it was highly upregulated in endothelial cells of large vessels, it was poorly upregulated in the renal endothelium, which makes the renal endothelium more vulnerably for complement over-activation and showed stronger C3 deposition (26).

On the C5 level, the generated anaphylatoxin C5a is a potent chemoattractant that enhances surface expression of intercellular adhesion molecules on the endothelium, and thereby effectively recruits inflammatory cells to the injured site (27). The migrated cells of the first line of defense can sense, phagocyte and clear damaged tissue and induced repair processes (9). In a recent polytrauma study, leukocytes with low C5-expression on day 1 after trauma correlated with an increased risk for the development of nosocomial infections during the later course (28). The corresponding C5a receptors, C5aR1 and C5aR2, are down-regulated on leukocytes early after trauma (29), something that has been proposed as a sign of enhanced risk for infectious complications (30). Of note, no clinical trial has been proposed or designed in regard to polytrauma using downstream modulation principals of the complement cascade, e.g., modulation of the membrane attack complex (MAC). This might represent an interesting pharmaceutical target, especially since significant amounts of sC5-9 are generated early after polytrauma (2).

HEMORRHAGIC-SHOCK-INDUCED MODS—ROLE OF COMPLEMENT

Hemorrhagic shock is a condition of disturbed tissue perfusion, resulting in the inadequate delivery of oxygen and nutrients and inadequate clearance of waste products, all of which are vital for regular cellular function. The involvement of complement is complex.

Complement activation products have been reported to directly or indirectly alter the vascular tonus. A C3a-analog

Abbreviations: CReg, complement regulator; DAMP, danger-associated molecular pattern; HMOX1, heme-oxygenase-1; HS, hemorrhagic shock; MAC, membrane attack complex; MODS, multiple organ dysfunction; MV, microvesicle; NHP, non-human primate; PAMP, pathogen-associated molecular patterns; PICS, persistent inflammation, immunosuppression and catabolism syndrome; PICS, persistent inflammation, immunosuppression and catabolism syndrome; PMN, polymorphonuclear leukocyte; sCR2, soluble complement receptor 2; TNF, tumor necrosis factor.

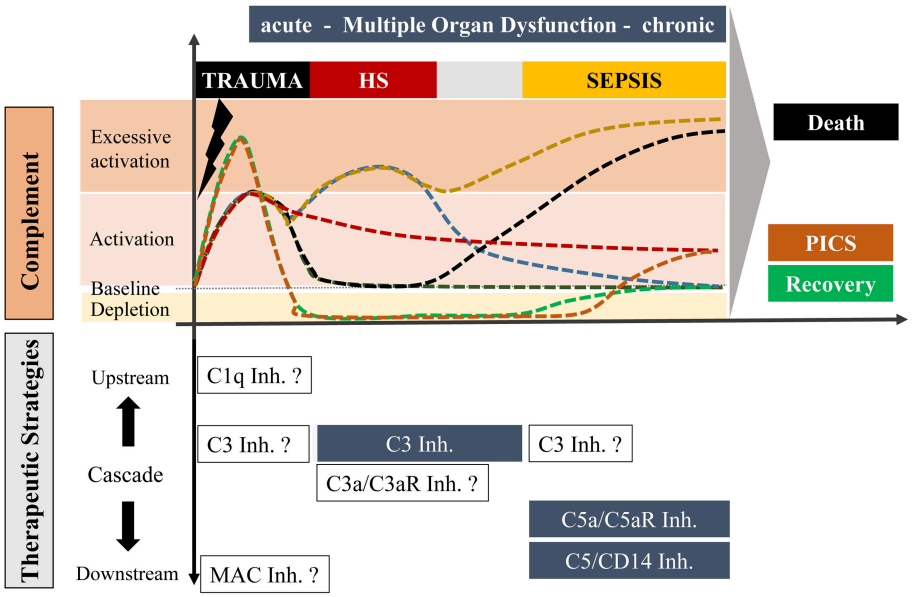

FIGURE 1 | Overview of potential complement responses after trauma with or without hemorrhagic shock and sepsis leading to either death or recovery or "Persistent Inflammation, Immunosuppression and Catabolism Syndrome" (PICS; with a constitutively active and chronic complement state) and potential complement-targeting strategies, respectively. The upper scaling represents the temporal course of complement activation with possible scenarios. Under optimal conditions, trauma induces an acute complement activation which follows a rapid decline to a physiological state with recovery (dark green). Alternatively, complement activity can also be maintained over a long period which may be associated with PICS (red). In an unfavorable state, the acute complement activation could be followed by secondary complication/s including sepsis leading to a complement hyperactivation and death (black). Trauma with an additional hemorrhagic shock can cause excessive complement activation, which can resolve in a physiological recovery. However, an additional hemorrhagic shock with hyperactivated complement can also result in a life-threatening sepsis phase (yellow). Trauma can further cause an acute complement hyperactivation with a rapid consumption of complement which may finally be resolved in a recovery phase (green) or to complement activation at the later stage leading to chronic inflammation (orange). The downwards orientated scaling includes possible therapeutic strategies of distinct stages of complement after trauma, hemorrhagic shock and sepsis. Blue highlighted boxes include therapeutic options which already revealed beneficial effects in pre-clinical studies. Non-highlighted boxes include possible strategies which need to be investigated in future experimental approaches.

peptide was able to cause pulmonary artery constriction in a thromboxan-dependent manner (31). Similarly, C5a caused transient vasoconstriction of an isolated pulmonary artery, which was dependent on the integrity of the endothelium (32). In other reports, C5a caused a leukocyte-dependent vasospasm and triggered thromboembolic events by C5a-induced release of thromboxane-like eicosanoids (33). In line with this observation, C5a is involved in the pathogenesis of acute kidney injury in hemorrhagic shock by reducing renal blood flow by ∼ 30% and the glomerular filtration rate by ∼ 45%, respectively (34). Of note, when either a leukotriene antagonist was applied or neutrophils were depleted, the C5a-induced reduction in renal perfusion was not observed indicating a leukotriene-dependent (34). In contrast, systemic application of C5a in rabbits induced a reversible drop in systemic arterial pressure (MAP) and a drop in central venous pressure (CVP), decreased cardiac output (CO), and neutropenia (35). In regard to coronary artery vasotonus, conflicting data have been reported for C5a both, vasoconstrictory (36) and vasodilatory effects (37). Other studies concluded that anaphylatoxins alter the vascular tonus with a high variability depending on the localization and pre-exposure to other mediators (38). Taken together, the available data suggest that complement activation changes tissue perfusion by macro- and micro-hemodynamic effects, especially when a shock is already established.

Another central pathomechanistic driver of hemorrhagic shock is the ischemia/reperfusion injury. In this regard, several studies have shown direct activation of complement with a significant increase in MBL and C3 on the endothelium by hypoxic and reperfusion conditions (39). Of note, clinically and experimentally, hemorrhagic shock is a major driver of further organ damage and development of coagulopathy, endotheliopathy, barrier failure, immune dysfunction and MODS after polytrauma (40–43). The close relation of hemorrhagic shock and complement activation reflected by C3 consumption has been previously modeled in baboons where cobra venom factor (CVF) was injected to activate and deplete C3 before a subsequent hemorrhagic shock was induced (20). In pigs, hemorrhagic shock resulted in a drop of CH50 during the hypovolemic phase and also during early resuscitation with enhanced plasma levels of C5a and early detection of endotoxin in the blood, which contributes to MODS (44). A proteomic approach using a rodent hemorrhagic shock model revealed a few alterations of the lymphatic fluid ("toxic lymph"), which included significantly enhanced C3 precursor protein concentrations (45). Since the "toxic lymph" is considered as an

important pathophysiological mechanism in the development of adult respiratory distress syndrome and MODS, these findings indicate C3 as a promising target for immune modulatory approaches (**Figure 1**).

SEPSIS-INDUCED MODS—ROLE OF COMPLEMENT

Sepsis was long considered as systemic inflammatory response syndrome with evidence of pathogenic microorganisms (46). In contrast, the new definitions describe sepsis rather as organ dysfunction induced by a dysregulated host response to infection (47). This paradigm shift (48) may also change the focus of therapeutic strategies toward support of organ functions, far beyond the established eradication of the pathogens. However, it remains somehow enigmatic what "dysregulation" of the host response means or if the "inadequacy" of the host response is central for development and progression of sepsis.

Multiple experimental sepsis studies have emphasized the detrimental effects of excessive complement activation for the host (**Figure 1**) (49, 50). This can be considered as a "complement paradox" since complement *per se* is central to the innate immune defense against invading microorganisms. However, in the context of MODS development caused by sepsis, complement activation seems to enhance rather than protecting against several organ dysfunctions, especially in the heart, lungs and kidneys, representing three central organs in MODS (51–53). The multi-organ gene expression profiles in experimental sepsis seems to be either organ-specific, or common to more than one organ, or distinctly opposite in some organs (54). Furthermore, a balanced pro- and anti-inflammatory genetic response was observed and a differential gene expression for mediators responsible for preventing tissue damage, e.g., protease inhibitors, oxidant neutralizing enzymes, decoy receptors, and proteins which can protect tissue barriers (54). Concerning complement, pre-pro-complement C3 was highly expressed in all organs except in the brain during the whole course of sepsis (54). However, genetic deficiency of C3 resulted in significantly enhanced lethality in comparison to C3-sufficient mice most likely due to a loss of C3b-dependent opsonization of invaded pathogens (55). In contrast, a blockade of C5a by various strategies in sepsis models, e.g., by anti-C5a antibodies, C5aR antibodies, small peptide C5aR1 antagonists, C5a-neutralizing mirror-image (l-)aptamer C5a aptamers, was coherently protective against biochemical and histological evidence of MODS and in general improved survival of sepsis (53, 55–61). All these experimental results demonstrate that C5a-C5aR interaction is clearly involved in the pathogenesis of MODS during sepsis and represents an important therapeutic sepsis target when the novel definitions of sepsis are applied (**Figure 1**) (47).

In translation to the clinical setting, several non-human primate experiments and studies in humans are in line with the findings in the rodent sepsis model. Evidence of systemic complement activation with reduction of complement hemolytic activity, C3 depletion and enhanced levels of C3a and C5a and corresponding loss of C5aR on neutrophils have been described in several human studies (62–64). Of note, the reduction of C5aR1 and C5aR2 on neutrophils has been correlated with the occurrence of infectious complications in ICU patients (30) and sepsis-induced MODS (62, 65, 66). Since loss of C5aR1 and C5aR2 has been concurrently correlated to the sequential organ failure assessment (SOFA) score (65), a flow-based rapid testing, might have a bedside monitoring potential to predict infectious problems and MODS development.

MODS—PATHOMECHANSISMS CAUSED BY MULTIPLE COMPLEMENT DYSFUNCTIONS

Several pathomechanisms contribute to the development of MODS, such as enhanced levels of DAMPs and PAMPs, reduced cytochrome P450 metabolism, macrophage activation syndrome, and cytokine-driven cellular dysfunction (67). Overall, it is clear that complement dysregulation contributes to MODS after trauma (**Figure 2**). Some of these aspects have been already mentioned and will be further discussed in this section.

Immune Paralysis

Though severely injured patients receive modern ICU management, many of them show signs of immunosuppression known as persistent inflammation-immunosuppressive catabolism syndrome (PICS) (**Figure 1**) (68). Clinically, PICS patients suffer from persistent inflammation, immune suppression and protein catabolism, which can lead to recurrent nosocomial infections with sepsis, MODS and death (68). Severe immune suppression of the fluid-phase and cellular immune response has been proposed as "immune paralysis" of the host response to sterile and infectious insults. In a clinical case report, an inadequate response to infection with signs of systemic depletion of complement (dropping C3 and C4 levels) has been associated with the development of acute kidney injury and multiple organ failure in a 17-day old newborn (69). C5b-9 and C5a have been described as contributors to cell death, immune paralysis, cardiac dysfunction, and multiple organ failure (**Figure 2**) (49, 70). In support, a baboon model of *Escherichia coli* sepsis showed that blockade of C5 protected organs from "immune paralysis" and improved the sepsis survival rate (**Table 1**) (75).

Dysregulated Complement Regulators

Additional connection between MODS and signs of complementopathy are supported by the fact that soluble and membrane-bound regulators of complement activity show alterations after trauma, sepsis and hemorrhagic shock. It is well-established that severe tissue injury causes an excessive systemic intravascular activation of the complement system resulting in a loss over the control mechanisms (8). In this context, the soluble form of the complement receptor 2 (sCR2) was shown to be present after nerve injury in rodents (80). After polytrauma in humans, leukocyte expression profiles of the complement regulators (CRegs) CD55 (decay accelerating factor), CD59 (membrane attack complex inhibitor), CD46

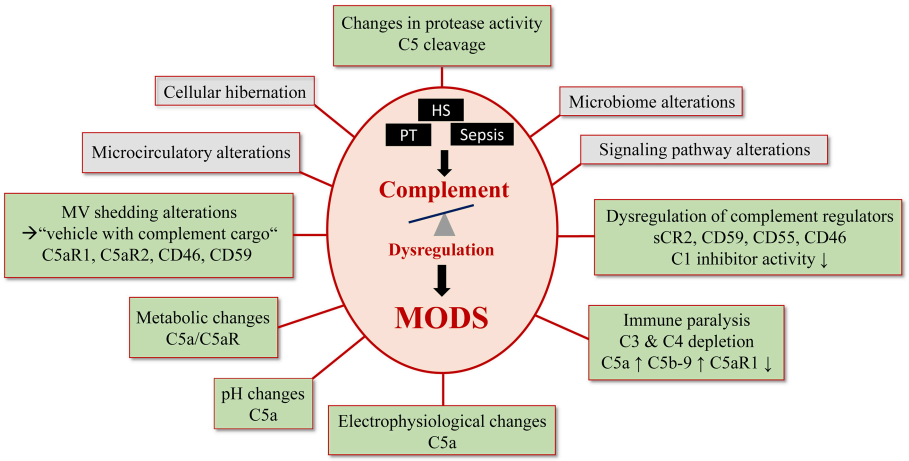

FIGURE 2 | Complement- mediated pathomechanisms in MODS development. Polytrauma, sepsis and hemorrhagic shock cause a critical complement dysregulation, which causes dysfunction in multiple organs. This figure summarizes potential and known pathomechansims for MODS development caused by complement dysregulation. Established complement involvement is highlighted in light green and proposed complement involvement is highlighted in gray.

(membrane cofactor protein), and CD35 (complement receptor 1) were cell-type- and time-dependently altered, reflecting manifestation of posttraumatic complementopathy (4, 29). Furthermore, an observational study including critically ill patients with multiple injuries and sepsis revealed that trauma patients with MODS have significantly lower C1-inhibitor activities (**Figure 2**) (7). A rare mutation in the complement regulatory gene factor H has been also implicated with a severe form of complement-mediated hemolytic uremic syndrome with multiple organ involvement, showing the general importance of complement regulation for organ (dys)functions (81).

Alteration of Signaling Pathway Activity

Experimental studies showed an interplay of complement with specific signaling pathways including the activation of PKC, MAPKs, and ERK (82). In a non-traumatic renal damage model, NF-κB contributed to enhanced complement activation (83), while in a similar mouse model the C5a/C5aR axis activated MAPK signaling (84). During sepsis, the activation of MAPKs and Akt signaling was complement-induced (51). These mechanistic insights require further elaboration to identify other participating mediators downstream to complement activation in trauma and hemorrhagic shock.

Changes in Protease Activity

Excessive systemic activation of proteases feed in the pathomechanisms of MODS and is known to create a vicious cycle of complement activation. Such proteases include immunoreactive trypsins and neutrophil elastase, which may directly interact with complement and have been extensively described in the case of polytrauma (9). A specific subtype of MODS has been introduced as thrombocytopenia-associated MODS, with low ADAMTS13 activity and defects in inhibitory complement regulators which may result in hyperactivation of coagulation and complement with resultant thrombosis (85). This form of MODS has been successfully treated by C5 blockade (eculizumab) and ADMTS13 reduction by plasma exchange (67). Furthermore, an unspecific and unregulated protease hyperactivity may directly cleave various complement components in a non-canonical manner, which represents a promising future research field for drug development. In this case, the intestine plays an important role in trauma and during shock, which can proceed to a proposed autodigestion phenomenon, since digestive enzymes from the pancreas are activated by enterokinases (86). Under physiological conditions, the auto-digestion is prevented by epithelial and mucosal barriers. However, during shock digestive peptides can pass the mucus and reach the epithelial cell membranes, where they disrupt junctional complexes, further activate complement and contribute barrier and organ dysfunction (87).

Microcirculatory Alteration

Patients who succumbed to traumatic-hemorrhagic shock showed an impaired microcirculation for at least 72 h, which also was a reliable predictor for a high Sequential Organ Failure Assessment (SOFA) score (88). In an ischemia rat model, application of soluble complement receptor type 1 significantly improved microvascular perfusion in the liver assessed by *in vivo* microscopy, suggesting an essential role of complement in microcirculatory disorders (89). Activated complement with microvascular alterations can also cause a disruption of cellular barriers leading to edema formation in lung, brain, and liver (84, 90, 91). However, hypothetical complement-caused damage of specific tight junction molecules in specific organs needs still scientific verification. Such mechanisms of MODS development may also be supported by simultaneous activation of complement and coagulation e.g., by complement-dependent generation of thrombin, which is known to efficiently break down various endothelial barriers. Furthermore, complement hyperactivation has been associated with thrombotic microangiopathies causing

TABLE 1 | Most representative preclinical studies addressing trauma, HS and sepsis, respectively.

	Model	Intervention	Outcome	References
Trauma	Mouse TBI	CR2-fH: C3 deposition Inhibition	Less C3 deposition in brain; decreased microglia activation; less neuronal cell death	(71)
	Mouse TBI	C6 antisense oligonucleotide Inhibition of MAC formation Coversin (OMCI) C5 inhibition	Inhibition of C6: less cerebral MAC (up to 96%); inhibition of C6 synthesis (> 80%)	(72)
	Mouse TBI	CD59-2a-CRIg Terminal pathway (MAC) inhibition	Less axonal damage; enhanced neurological recovery; inhibition of C5: decreased MAC formation; improved neurological outcome	(73)
	Mouse TBI	CR2-CD59: Terminal pathway (MAC) inhibition CR2-Crry: all complement pathways inhibition CR2-fH: alternative complement pathway inhibition	Significantly improved chronic outcomes	(74)
Sepsis	Baboon septic shock	RA101295: C5 inhibition	Significantly improved survival; reduced inflammation and coagulopathy; significantly improved organ function	(75)
	Mouse meningococcal sepsis	PMX 205: C5aR antagonist	Protection from invasive meningococcal infection; enhanced mouse survival; ameliorated inflammatory cytokine response	(76)
	Piglets poly-microbial sepsis	Coversin (OMCI) C5 inhibition And Anti-CD14	Combined C5 and CD14 inhibition: significantly improved survival; significantly lower plasma sC5b-9 levels, which correlated with mortality	(77)
	Baboon septic shock	Compstatin: C3 inhibition	Significantly decreased procoagulant response; organ protection by significantly improved vascular barrier function; less leukocyte infiltration and cell death	(5)
HS	NHP HS	Comstatin C3 blockade	Protection of organ function; reduced intestinal edema; improved kidney function	(78)
	Swine trauma with HS	C1 inhibition	less TNF; less complement deposition[C3, C5 and C5b-9 (MAC)] in the small intestine and lungs; improvement of metabolic acidosis; less renal, intestinal, and lung tissue damage	(79)

HS, hemorrhagic shock; MAC, membrane attack complex; NHP, non-human primate; TBI, traumatic brain injury; TNF, tumor necrosis factor.

endothelial cell activation and thrombus formation leading to hemolytic anemia, thrombocytopenia, and organ failure (91).

Metabolic Changes

Besides microcirculatory problems, massive blood loss due to hemorrhage can cause acute circulatory failure resulting in lactic acidosis. Acute liver or renal dysfunctions are most often associated with decreased lactate clearance and a pronounced increase in blood lactate levels compared with shock patients without signs of liver or renal dysfunction (92). It is also known that shock states initiate a pronounced compensatory vasoconstriction, consequently leading to hypoxia and accumulation of metabolites associated with lactate acidosis and a low pH milieu (92, 93). In this context, complement dysfunction has a deleterious influence and correlates with a worse outcome after shock. Especially the anaphylatoxin C5a and its following signaling via the C5aR1 have been described to further enhance acidosis after septic shock. More precisely, *in vitro* stimulation of neutrophils from healthy donors with C5a causes C5aR1 signaling-mediated immunometabolic changes in neutrophils with an enhanced glucose uptake and enhanced glycolytic flux (**Figure 2**) (94). In turn, it can initiate an increased proton secretion and further lower the extracellular pH. In response to C5a exposure, intracellular pH in neutrophils significantly increases via activation of the Na+/H+ exchanger type 1 (NHE-1) leading to an impairment in neutrophil function *in vitro*. Supporting this evidence, neutrophils isolated from septic patients has been shown to exhibit an increased intracellular pH compared to healthy donors (94). These recent findings indicate that inflammatory processes with produced C5a is solely capable to significantly change the micro-milieu with lactate acidotic features even in the absence of an oxygen deficit. It only can be speculated on, that in the case of an additional oxygen deficit, caused by shock conditions and vascular dysfunction, the anaphylatoxins may even function as a metabolic switch toward lactate acidosis and MODS.

Microbiome Alterations

The intestinal complement is suggested to cooperate in a close relationship with the gut microbiome (95). Therefore, another

contributor to MODS seems to be the alteration of the gut microbiome after trauma/hemorrhagic shock and/or sepsis. The microbiome, which is described by the phylogenetic composition and taxon relative abundance of the bacteria, is significantly altered in the first 72 h after injury. This rapid change in intestinal microbiota represents a critical phenomenon that may influence outcomes after severe trauma (15). The composition of the microbiome may influence the activation/dysregulation of complement pathways or dysregulated complement may change the microbiome composition. In the skin, complement activation modulates the inflammatory milieu by changing the cutaneous microbiota (96). Considering MODS, a mechanistic explanation for complement-microbiome interaction still remains elusive and needs further research.

Alterations in Microvesicle (MV) Shedding

Communication is essential for cellular homeostasis and vesicle shedding has been described to play a crucial role for maintaining proper immune cell function. Extracellular vesicle shedding is altered after inflammation and is considered as a crucial contributor to MODS after multiple injury and sepsis (97). Especially shedding of microvesicles (MV) have been implicated in several inflammatory conditions including sepsis and trauma. Increased amounts of CD41+ and CD31+/CD41–/AnnexinV– MV after sepsis, released by activated platelets and leukocytes have been shown to correlate with unfavorable outcomes (98).

Furthermore, MVs from patients with multiple organ failure support the coagulation system in triggering inflammation. In respect to complement, phosphatidylserine containing MVs also serve as platform for complement activation (99). Besides activation of complement on their surfaces, MVs represent transport vehicles sending complement as cargo to neighboring as well as cells in distance (99). Hence, MV from different cellular origins may contain complement receptors including C5aR but also CRegs such as CD46 and CD59, suggesting a putative role for complement activity (99). In accordance with this, loss of C5aR1, C5aR2, and C3aR on neutrophils after multiple injury was clinically present and was correlated to infectious complications and multiple organ dysfunction (**Figure 2**) (18).

Electrophysiological Changes

Another concurrent theory of MODS addresses electrophysiological changes of the cellular membrane which have recently been found in neutrophils from septic pigs (14). The anaphylatoxin C5a was able to alter the membrane potential of neutrophils but not in the case of neutrophils during septic MODS where the electrophysiological response to C5a was somehow frozen (**Figure 2**) (14). Whether complement inhibitory strategies will stabilize the cellular membrane electrophysiology is currently under investigation.

Cellular Hibernation

Trauma causes an alarming stress situation for the whole body with an extensive inflammatory response (9). Some studies indicate that trauma especially followed by additional sepsis causes hibernation in the cellular as well as fluid phase of innate immunity including the complement system. Reflecting the evolved habit of conserving physiological resources in the event of environmental stress, with inflammation ensues a similar mechanism where energy-consuming processes are shut down in the organism. Supporting this evidence, hibernation has been observed in the septic heart with ongoing metabolic changes including the upregulation of specific glucose transporters in cardiomyocytes (100). Besides its effects on metabolism, hibernation is known to affect various immune function including leukocyte migration, as well as adaptive immune responses and interestingly complement function, by lowering complement levels and reduced expression of C3 mRNA in the liver, which depicts a suggestive link post-shock. (101). However, another study demonstrated that hibernators are protected from shock-induced injury, inflammation, and organ function (102). Strikingly, arctic ground squirrels challenged with cardiac arrest or hemorrhagic shock showed no markers of organ damage, systemic inflammation, or loss of acid/base balance as indicated by a negative base excess. Neither reduced body temperature nor hibernation season are components of this protection, indicating still unknown mechanisms involved (102).

Unfortunately, no supporting data is available indicating that future research on the complement function during hibernation, especially following trauma and shock is needed. Further, the ability to induce a fully reversible state of immune suppression in humans by artificial hibernation might aid the treatment of several inflammatory and immune-mediated diseases.

TARGETING COMPLEMENT PATHWAYS IN MODS

Despite improvements in trauma care, the morbidity and mortality of MODS remains very high.

Therefore, new therapeutic strategies are urgently needed. Since complement is critically involved in initiation and progression of MODS, targeting complement as well as molecules contributing to complement activation represent promising future clinical approaches. Other complement-associated inflammatory conditions already addressed such a targeting strategy. Above 20 complement-interfering drugs have been evaluated in clinical settings so far. A few of them received FDA approval for inflammatory indications including eculizumab targeting the terminal complement pathway starting from C5, which is currently used for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) (103). As existing complement therapeutics cannot target every complement-driven disease status, it is a requisite to evaluate the different complement stages, their relevance, and target them in a disease-specific manner (10).

In the context of trauma, hemorrhagic shock and sepsis a few preclinical experimental studies focus on complement-targeting studies (**Table 1**). Besides complement activation products, other molecules including CRP, HMGB1, or mitochondrial DNA play crucial roles in contributing to complement dysregulation after sepsis and trauma, thus profoundly influencing secondary outcomes. Therefore, a rational approach seems to address molecules, which may further boost complement activity. Likewise, HMGB1 is a relevant danger molecule and complement

has been described to regulate HMGB1 release from human neutrophils (104) such that, complement inhibition has proved to be protective in blast-induced acute lung injury in rats by ameliorating HMGB1-mediated inflammation (105).

Targeting Complement After (poly)Trauma

In polytrauma patients, the only study of complement intervention early after trauma with RCT quality used C1 esterase inhibitor. However, this study has been terminated based on the heterogeneity of the patients (106). Experimentally, the majority of the complement targeting studies were performed in mouse traumatic brain injury (TBI) models. In this case, various interventions to inhibit complement activity, such as inhibitors for MAC formation and C3 deposition inhibitors have been applied, analyzed and reviewed elsewhere (9, 107, 108). To further clarify the impact of distinct complement activation pathways on neuroinflammation, a recent study compared the effect of complement inhibitors targeting upstream as well as downstream complement activity, respectively. This study brought into light that instead of downstream complement activation complexes, upstream complement activation products of the alternative pathway predominantly modulate and propagate chronic inflammation (74) (**Table 1**).

Targeting Complement in Hemorrhagic Shock

Based on the deduced potential of complement inhibitors to improve micro-perfusion disturbances, ischemia/reperfusion injury and the inflammatory response during hemorrhagic shock, various shock models have been tested for possible benefits of complement interventions. In a rat model of pressure controlled hemorrhagic shock, complement depletion by CVF improved the recovery of the mean arterial pressure (MAP) post shock (109). In a pig model of pressure controlled hemorrhagic shock, application of a C1-inhibitor improved the functional performance and reduced C3 deposition on a multi-organ level including liver, small intestine and lungs (**Table 1**) (79). Limitation of organ injury and sustained survival by C1 inhibitor therapy has also been reported for a porcine injury model mimicking battlefield injury (110). Further downstream, C3 deficient mice with bilateral femur fracture and hemorrhagic shock resulted in protective effects with reduced circulating DAMPs (e.g., dsDNA), decreased systemic inflammatory response and improved organ performance (e.g., liver enzymes) (111). In a rat model of hemorrhagic shock, C3 depletion by CVF, and the soluble form of CR1 to inhibit C3 action restored vascular reactivity to norepinephrine in the superior mesenteric artery (112). In the clinical setting, C3 inhibition has not been tested in hemorrhagic shock so far except in a recent study from our group, where non-human primates were modeled with severe hemorrhagic shock of 30 mmHg MAP for 1 h, and a delayed application of CP40 was tested (78). C3 blockade by the compstatin compound CP40 seems most promising since there was evidence of improved renal function, attenuated intestinal edema and reduced signs of systemic inflammation and coagulopathy (78). However, the transfer into clinical reality is pending although C3 inhibition seems rather safe in the case of hemorrhagic shock. Most likely, an early inhibition to avoid excessive C3 activation might be enough to improve the outcome (113).

On a C5 level, intestinal injury caused by hypovolemic shock in mice was ameliorated by application of a small peptide C5aR1 antagonist and C5 deficient littermates (114). Similarly, in a rodent model of ruptured aortic abdominal aneurysma with shock, C5 blockade by an anti-C5 antibody revealed some protective effects on remote lung injury with improvement of the bronchoalveolar permeability and myeloperoxidase (MPO) concentrations reflecting reduced neutrophil recruitment and activation (115). Combined inhibition of the complement component C5 and the Toll-like receptor co-factor CD14 in a porcine sepsis model, showed beneficial effects in regard to survival, hemodynamic parameters and systemic inflammation including complement activation (**Figure 1**, **Table 1**). First preclinical studies in non-human primates indicate appearance of sC5b-9 in the serum of traumatic-hemorrhagic shock and *ex vivo* effectiveness of C5 inhibitors in prevention of hemolysis, e.g., the small, inhibitory peptide RA101348, commercially-available C5 inhibitory antibodies and Quidel's A217 antibody (106). However, a RCT-clinical study with C5 inhibitory strategies in hemorrhagic shock has not been performed so far most likely based on the rareness and uncertain evidence of a great impact on MODS development in the so far performed preclinical non-human primate studies. Therefore, research providing information about the histological and biochemical data on the multi-organ level in non-human primate studies is necessary for final assessment.

Targeting Complement in Sepsis

In the case of septic shock, targeting the C5a/C5aR axis seems to be of practical importance since it correlated with the disease severity and mortality and showed promising improvements in different sepsis models (**Table 1**). Concerning complement-targeted therapies in sepsis, application of RA101295, a 2-kDa macrocyclic peptide inhibitor of C5 cleavage, improved organ performance and survival in an *E. coli*-sepsis model of baboons (75) with lessened evidence of coagulopathy and preserved endothelial and barrier functions. Furthermore, the inhibition of C5 cleavage lead to improved histomorphology of lungs, liver, kidneys, spleen, and adrenal glands suggesting improvement of sepsis-induced MODS (75).

In the same sepsis model, systemic blockade of C3 by compstatin also revealed organ protection on multiple levels. Compstatin showed evidence of sepsis-induced coagulopathy and preserved anti-coagulatory features of the endothelium. Furthermore, C3 blockade improved hemodynamics and heart function and biochemical damage markers of the kidney and liver, indicating protective effects in sepsis-induced MODS (5). For blocking the central complement component C3 during development of sepsis-caused MODS, clinical trials might be safe (113) but these trials are pending and require a special focus on the benefits or maladies for mental alterations during sepsis.

In terms of applicability in the clinical setting, it has been recently shown that blocking solely C5 activation by canonical C5-convertase (e.g., by eculizumab) might not be specific enough

TABLE 2 | Overview of observational clinical trials evaluating complement activation after polytrauma and sepsis.

Indication	Endpoint	Participants	Outcome	References
Polytrauma	Inflammatory pattern of complement activation and CRegs on leukocytes	60	Significantly increased serum C3a, C5a, and C5b-9 levels; decreased C5aR expression on neutrophils, which inversely correlated with the clinical outcome; significantly enhanced cC5aR levels, correlating with lethality	NCT00710411
Severe abdominal sepsis	Complement C3 depletion and its association with the down-regulated adaptive immunity	75	C3 depletion was connected to poor prognosis; depletion was associated with coagulopathy and aggravated infection during sepsis	NCT01568853
Polytrauma	Danger response to polytrauma	1000	still recruiting participants	NCT02682550

cC5aR, circulating form of C5aR; CRegs, complement-regulating proteins; CRP, C—reactive protein.

TABLE 3 | Overview of complement therapeutics for clinical trials.

Target	Indication	Primary endpoint	Participants	References
C1 esterase	Trauma or sepsis	Measurement of C1-inhibitor levels, complement concentration and activity, and cytokines; Analysis of neutrophil phenotype and hemodynamic response	Terminated: Study showed limited feasibility	NCT01275976
C1 esterase	Endotoxemia Inflammation MODS	Measurement of C1-inhibitor levels, complement concentration and activity, Cytokines, and markers of inflammation; Analysis of neutrophil phenotype, and hemodynamic response; Assessment of renal injury	20	NCT00785018
C5a	Severe sepsis Septic shock	Evaluation of pharmacodynamic (PD) effects of the C5a antibody	72	NCT02246595

since other serine proteases such as trypsin or thrombin can still cleave and activate C5. Therefore, a C5a blocking approach e.g., by IFX-1 anti-C5a antibody has been proposed as a targeted approach in local or systemic infection (116, 117). Overall, the specific complement targets for sepsis-induced MODS might be different than for polytrauma- or hemorrhagic shock-caused MODS especially when given early (**Figure 1**). The complement target might also change along the course of the disease. It is for example feasible, that early after polytrauma or during hemorrhagic shock a specific C3 inhibition is required, whereas later during development of septic complications, C5a inhibition might help against MODS development (**Figure 1**). And if complete depletion of a specific complement factor occurs, it might be even wise to replace it, which bolsters the importance of monitoring complement for any clinical trial.

In conclusion, MODS is a culmination of highly heterogeneous events which can involve different and complex stages of complement activation, making it nearly impossible to generate a drug addressing MODS generally. More precisely, the most prominent complement pathways and their down-stream signaling pathways involving multiple trauma with or without hemorrhagic shock and septic shock may be addressed in further preclinical studies.

Clinical Trials

Considering clinical trials of MODS caused by polytrauma, hemorrhagic shock and sepsis, three observational studies addressed complement activation after polytrauma and severe abdominal sepsis (**Table 2**). One study focused on the complement activation and expression on leukocytes in polytrauma patients revealing increased complement activity early after trauma and an increased shedding of complement receptors from neutrophil surfaces (NTC00710411; **Table 2**) (2, 62). Another observational study focuses on the molecular danger response after polytrauma. It is still recruiting patients, and the study aims to include up to 1,000 patients in a collective national data bank in collaboration with the Trauma Research Network (NTF) of the German Society for Orthopedics and Trauma (DGOU) and thus to obtain novel insight into the molecular pattern of complement activity following severe multiple injury (NTF-PT; NCT02682550; **Table 2**).

A further observational study has set its focus on the central complement C3 by investigation of C3 alterations in patients with severe abdominal sepsis. The aim was to evaluate the impact of C3 on patients' prognosis (NCT01568853; **Table 2**). This study revealed that C3 depletion is associated with a poor prognosis due to dysregulated coagulation and increased susceptibility for infections (118).

In regard to human studies, three interventional trials have been initiated so far (**Table 3**). One study addressed the inhibition of upstream complement component (C1) by targeting C1 esterase. This mono-centered double-blind randomized placebo-controlled trial (CAESAR; NCT01275976, **Table 3**) has been designed using a C1-esterase inhibitor in severely injured patients

with femur fracture. Although the rationale of a complement blockade on this level is very well founded because it may also reveal beneficial effects by synchronically inhibition of excessive activation of the coagulation pathway (119), the study has been terminated based on the heterogeneity of patients and challenges in recruitment. A further study has addressed the effect of the C1-esterase inhibitor on human endotoxemia by evaluating its effect on inflammation and marker of organ dysfunction (VECTOR; NCT00785018, **Table 3**). Detailed results of the study are still pending.

Another clinical trial has been designed and performed to study complement inhibition in early, newly developing septic organ dysfunction (SCIENS; NCT02246595, **Table 3**) applying a monoclonal antibody against C5a, though the detailed results of this trial have not been published so far. Regarding one cardinal clinical sign of sepsis, the occult or evident alterations of the mental status, inhibitory strategies against C5a might reveal "Janus faced" effects (120). On one hand, C5a inhibition could improve sepsis-impaired blood-brain-barrier, on the other hand, neuroprotective effects by C5a might be compromised (120, 121). Therefore, alterations of the mental status need to be carefully addressed and monitored in any clinical trial using C5a inhibitory strategies.

It is important to note, that it is in general rather difficult to perform interventional studies on polytrauma patients since an informed written consent cannot be provided and the legal representatives are usually difficult to determine within the first hours after severe injury. Therefore, innovative studies early after polytrauma addressing the complement cascade are rather rare and have not been performed on the C3 or C5 level yet.

CLINICAL PERSPECTIVES

Although various clinically relevant models of trauma, hemorrhagic shock and sepsis have been tested already in non-human primates for the benefit of complement interventions (75, 78, 106), clinical trials in these multidimensional pathophysiologic conditions remain rare (119, 122). When complement intervention strategies are designed for the clinics, the targeted complement factor or activation product needs to be measured before the therapy can be applied. Especially when complex intensive care is necessary which can alter complement levels within a short time period, e.g., by infusion of blood products which contain highly variable concentrations of complement (activation) factors (123) or by extra-corporal circulation devices with large artificial surfaces which may deplete key complement components (124, 125), the exact status of complement activation needs to be determined. This would allow precise and timely intervention either by inhibiting or supporting the complement response after trauma or during sepsis in order to rebalance the immune response. Whereas, various highly effective and specific complement intervention strategies have been developed within the last two decades and are available now (10), in the context of the complex immune response after trauma, hemorrhagic shock and sepsis (9, 126), specific organ damage and function assessment including the immune function at bedside seems far beyond. Therefore, functional monitoring of the organ and immune systems can be considered as a prerequisite before complement interventions move into clinical routine in diseases with a complex pathophysiology. In conclusion, further scientific knowledge and translational efforts are demanded for targeting complement pathways in the setting of trauma, hemorrhagic shock and sepsis with the aim to offer causal therapy and improved outcome.

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Conflict of Interest Statement: JL is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation compstatin analogs, such as AMY-101) and is the inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. JL is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives].

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Corrigendum: Targeting Complement Pathways in Polytrauma- and Sepsis-Induced Multiple-Organ Dysfunction

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A Corrigendum on

Targeting Complement Pathways in Polytrauma- and Sepsis-Induced Multiple-Organ Dysfunction

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In the original article, we neglected to include a conflict of interest statement of Prof. John D. Lambris.

JL is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation compstatin analogs, such as AMY-101) and is the inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. JL is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives].

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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Complement Therapeutics in Autoimmune Disease

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Many autoimmune diseases are characterized by generation of autoantibodies that bind to host proteins or deposit within tissues as a component of immune complexes. The autoantibodies can activate the complement system, which can mediate tissue damage and trigger systemic inflammation. Complement inhibitory drugs may, therefore, be beneficial across a large number of different autoimmune diseases. Many new anti-complement drugs that target specific activation mechanisms or downstream activation fragments are in development. Based on the shared pathophysiology of autoimmune diseases, some of these complement inhibitory drugs may provide benefit across multiple different diseases. In some antibody-mediated autoimmune diseases, however, unique features of the autoantibodies, the target antigens, or the affected tissues may make it advantageous to block individual components or pathways of the complement system. This paper reviews the evidence that complement is involved in various autoimmune diseases, as well as the studies that have examined whether or not complement inhibitors are effective for treating these diseases.

Keywords: complement, autoimmunity, antibody, immune complex, therapeutic

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INTRODUCTION

Autoimmune diseases are conditions in which immunologic tolerance for specific self-proteins is lost, and the adaptive immune system orchestrates injury of organs expressing those proteins. The skin, joints, and kidneys are commonly affected, but any organ in the body can be the target of autoimmunity. Most autoimmune disorders are associated with autoantibodies that are reactive against self-proteins. Systemic lupus erythematosus (SLE), for example, is associated with autoantibodies to nuclear antigens as well as many other intracellular and extracellular antigens (1, 2). Myasthenia gravis is associated with autoantibodies specific for the acetylcholine receptor (AChR). Although not all of the identified autoantibodies have been established as causative of disease, in some cases experiments have convincingly shown that the autoantibodies replicate the disease when transferred into animals that express the target antigen. In membranous nephropathy, for example, there are reported cases in which passage of autoantibodies from mother to fetus have caused antenatal disease (3).

Autoimmunity can be regarded as having two components: (1) the loss of tolerance to self-antigens, and (2) downstream immune-mediated effector mechanisms of injury. Autoantibodies may contribute to disease pathogenesis via several effector mechanisms. They can crosslink target antigens or activate receptors directly on cell surfaces. In Grave's disease, for example, autoantibodies to the thyrotropin receptor trigger release of thyroid hormone, and in myasthenia gravis antibodies to the AChR can cross-link the receptor (4, 5). The inflammatory effects of autoantibodies can also be mediated through ligation of Fc receptors on leukocytes. In

some models of autoimmunity this accounts for nearly all of the downstream tissue injury (6). Autoantibodies can also directly damage target tissues, and complement activation is a downstream mediator of this injury in some diseases.

Antibodies bound to cell surface antigens activate complement on the target cells, usually through activation of the classical pathway. Immune-complexes (ICs) can also deposit in small capillaries where they activate complement on bystander cells. Once activated, the complement cascade generates multiple different biologically active fragments. C3a and C5a contribute to chemoattraction and activation of leukocytes (7). C3 fragments (C3b, iC3b, C3dg) fixed to host tissues can activate leukocytes through ligation of complement receptors (CRs) 1–4 (8, 9). C5b-9 causes direct cell activation and cytotoxicity. Although most C5b-9 forms directly on cell membranes, it can also insert into nearby cells causing "bystander" injury (10). Thus, once the complement cascade is activated within a tissue it can trigger multiple local and systemic effects.

Given the prevalence of autoantibodies in the various autoimmune and rheumatic diseases, complement inhibition holds promise as an effective strategy for blocking multiple pathways of injury common to these diseases. In many of the diseases there is also pre-clinical or biomarker evidence to support the use of anti-complement therapeutics. Therapeutic complement inhibitors may be effective at blocking direct tissue injury by the complement cascade. Consequently, they may have a role in rapidly reducing tissue inflammation while other immunosuppressive drugs to block the adaptive immune response. Complement inhibitors may also reduce the adaptive immune response by decreasing stimulation of dendritic cells, T cells, and B cells via the complement receptors (11). This class of drugs could therefore be effective in patients with acute disease flares, but also for chronic treatment of these diseases.

A large number of anti-complement drugs are in development, providing tools for blocking all complement activity, specific activation pathways, or isolated complement fragments (12, 13). Intuitively, drugs that block the classical pathway should be beneficial in antibody-mediated diseases. The classical pathway also helps to prevent autoimmunity and to solubilize ICs, however, so drugs that block downstream targets within the complement cascade while leaving the early classical pathway intact may be preferable in some diseases (discussed below). Drugs that block specific complement fragments, such as C5a receptor (C5aR) antagonists, may also have fewer side effects than drugs that more completely block the complement system. It is also noteworthy that complement activation is not always an important component of disease pathogenesis, even in models in which ICs and complement are deposited within tissues. In some models this is due to the effects of other pathways, such as signaling through Fc receptors (6). In other instances this may be due to intrinsic differences in susceptibility or resistance of target tissues to injury (14). Given these considerations, a benefit to complement inhibition cannot simply be inferred from detection of autoantibodies or complement activation. The efficacy of complement inhibition in autoimmune diseases needs to be studied on a case-by-case basis.

SYSTEMIC AUTOIMMUNE DISEASES

Systemic Lupus Erythematosus

SLE is the paradigmatic autoimmune disease. It is a complex disorder characterized by formation of autoantibodies to multiple different nuclear antigens, including DNA, histones, and ribonucleoproteins. There is also evidence that autoantibodies react with antigens expressed in specific tissues, including alpha-enolase, annexin A1, and annexin A2 (15, 16). SLE frequently affects the joints, skin, and kidneys, but essentially any organ can be affected. Organ involvement is usually associated with the deposition of ICs and complement activation. Other downstream mediators of immunologic injury have been identified, however, including cytokines and chemokines, Fc receptor ligation, cellular infiltrates, and toll-like receptor activation (6, 17).

The Role of Complement in SLE

The complement system plays a paradoxical role in the pathogenesis of SLE—it seems to lower the risk of developing SLE while also mediating end organ injury in the disease (**Figure 1**). Congenital deficiency of classical pathway proteins are strong risk factors for developing SLE, presumably because they help protect against autoimmunity. Individuals with deficiencies of C1q, C1s, C1r, C2, and C4, for example, are all at increased risk of the disease (18).

Classical pathway proteins may be protective against SLE though several mechanisms. C1q and C4 opsonize apoptotic cell debris and ICs, and facilitate their clearance [**Figure 2**, and (19)]. C1q binds directly to molecules displayed on the surface of apoptotic bodies, including DNA and phosphatidylserine (20, 21). Mice with targeted deletion of the *C1qa* gene have a greater abundance of apoptotic bodies in the kidney than control mice, and they develop auto-antibodies to nuclear antigens (22). C1q deficient mice on a pure C57BL/6 background do not develop disease, however, demonstrating that other background factors are also important (23). A recent study also demonstrated that C1q can directly modulate CD8 T cell metabolism via receptors on the cell surface, reducing the response of the cells to self-antigens (24). In this study, C1q deficiency increased renal pathology in a CD8-dependent fashion. Work in animals demonstrates that C4 helps to maintain immune tolerance to self-antigens (25). An intact classical pathway, therefore, may reduce the abundance of nuclear proteins in the extracellular space and also suppress the immune response to these proteins.

Although the classical pathway helps to prevent development of SLE, the complement system is also an important mediator of tissue injury. Complement proteins are seen in kidney biopsies from patients with lupus nephritis and co-localize with deposited ICs (26). Glomerular complement deposits are associated with a worse prognosis (27), suggesting that complement activation contributes to tissue injury. Work in mouse models of lupus-like disease demonstrate that deficiency of classical pathway proteins is not protective [consistent with a protective role for these proteins (28)], whereas deficiency of alternative pathway proteins is protective (29, 30). Several therapies have been shown effective in preclinical studies using models of lupus-like disease, including agents that block the alternative pathway (31, 32),

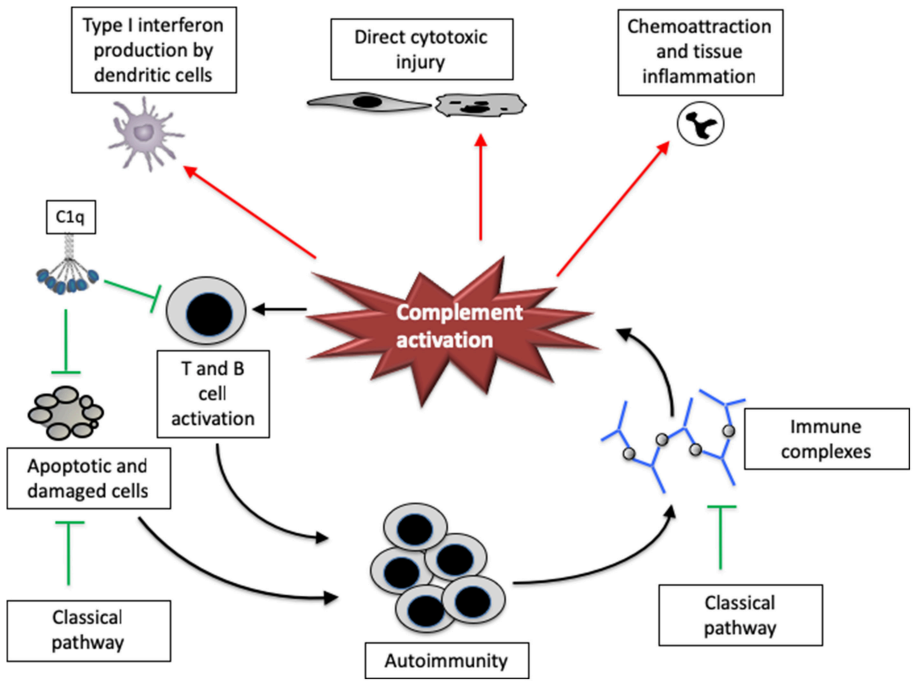

FIGURE 1 | The complement system has both pathogenic and protective roles in systemic lupus erythematosus. Complement proteins have multiple functions that affect lupus, both positively and negatively. The classical pathway may help facilitate the removal of apoptotic and damaged cells as well as immune complexes, reducing the risk of developing autoimmunity to nuclear components. C1q may also directly reduce CD8 T cell activation, thereby attenuating downstream immunity and autoantibody generation. Once activated, however, the complement system can promote tissue injury and disease severity. The complement system can directly cause cytotoxic injury in tissues, and also increases inflammation by attracting leukocytes and inducing interferon production by dendritic cells. Complement fragments may also increase T cell and B cell responsiveness, increasing the response to autoantigens.

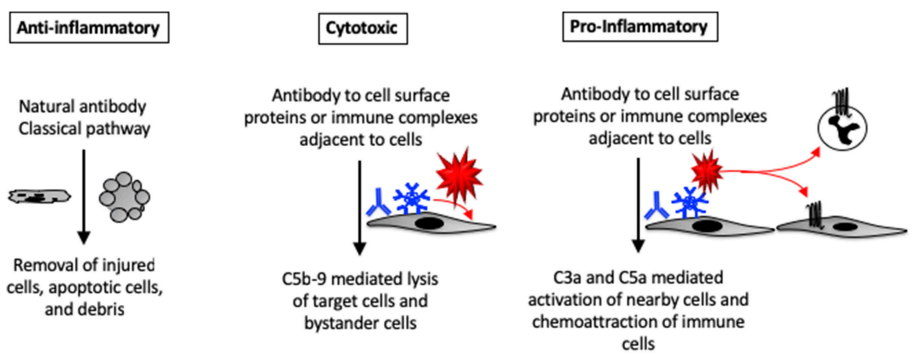

FIGURE 2 | Pro- and anti-inflammatory effects of complement activation in tissues. Complement proteins can directly bind to injured and apoptotic cells. The complement system can also be activated by natural antibodies bound to injured cells. Autoantibodies that are bound to self-antigens can activate complement directly on the cell surface or on immune complexes deposited near the cells. C5b-9 formed on the cell surface can lead to cell activation or lysis. C3a and C5a generated within tissues can promote tissue inflammation by activating nearby cells (which may then secrete cytokines and chemokines) or by causing infiltration of the tissue by circulating leukocytes.

activation at the level of C3 (33), C3a activity (34), and C5a activity (35). Furthermore, treatment with an inhibitory antibody to mouse C5 for 6 months improved renal function and survival in a mouse model of disease (36).

Based on the complex role of complement in SLE, a clinical trial of a complement inhibitory drugs in SLE patients must be designed with equipoise. The treatment should probably not block the classical pathway upstream of C4, even though this is an IC-mediated disease characterized by classical pathway activation.

Therapeutic Complement Inhibitors in SLE

A clinical trial of eculizumab in patients with lupus nephritis was planned, but was canceled after enrolling only one patient (37). Eculizumab has been used off-label in patients with proliferative lupus nephritis, and it may be effective in patients with severe

disease (38, 39). Several papers have also reported use of eculizumab in SLE patients with thrombotic microangiopathy (40, 41). Apellis is a company that is developing an agent (APL-2) that blocks complement activation at the level of C3. The company is currently conducting a phase 2 trial of the drug that will include patients with lupus nephritis.

Anti-neutrophil Cytoplasmic Antibody Vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA) vasculitis comprises a group of severe diseases that can affect many organs, including the lungs and upper airways, kidneys, skin, and joints. ANCA associated vasculitis (AAV) is often referred to as "pauci-immune" because immunoglobulin and complement proteins are not prominently seen in tissue biopsies. These diseases are antibody-mediated, however, and the ANCA can cause disease when injected into rodents (42). Antibodies and complement fragments probably function on the surface of neutrophils, and thus are not abundantly deposited within affected tissues. That said, complement fragments are frequently present in tissues biopsies, even if they are less abundant than in IC deposition diseases such as SLE (43).

The Role of Complement in ANCA Vasculitis

The important role of the complement system in AAV was revealed by an elegant series of experiments using a murine model of the disease. Using mice with various complement deficiencies, investigators found that factor B deficiency was protective but that C4 deficiency was not (44). Therefore, even though this an antibody-mediated disease, the alternative pathway appears to be more important than the classical pathway for disease pathogenesis. C5 deficiency is also protective in this model but C6 deficiency was not, suggesting that C5a is more important than C5b-9 formation in disease pathogenesis. Although referred to as "pauci-immune," there is clinical evidence of complement activation in human patients with AAV. Glomerular C3 deposits can be seen in more than 40% of patients (45). Complement activation fragments are also elevated in plasma and urine of patients with AAV, including the fragment Bb [an alternative pathway activation fragment (46)].

Therapeutic Complement Inhibitors in ANCA Vasculitis

In the murine model of AAV, a monoclonal antibody to C5 (BB5.1) and an orally delivered small molecule C5a receptor antagonist (CCX168) were both nearly completely protective (47, 48). These results demonstrated that that C5a blockade is sufficient for blocking the complement effects in AAV. A subsequent phase 2 study of CCX168 (conducted by the company ChemoCentryx) tested whether the drug could be used in lieu of corticosteroids (49). This study included a group of patients that received cyclophosphamide and CCX168 without corticosteroids. That treatment approach was non-inferior when compared to standard therapy (corticosteroids + cyclophosphamide). A phase 3 study is currently underway evaluating the efficacy of the drug in combination with rituximab or cyclophosphamide. The study will compare CCX168 to prednisone in combination with the same other immunosuppressive agents.

Antiphospholipid Antibody Syndrome

Anti-phospholipid antibodies (APLA) are autoantibodies that bind to endothelial cells, triggering thrombosis. The antiphospholipid syndrome (APS) is diagnosed by detection of these antibodies in patients who have had a thromboembolic event (50). The antibodies associated with APS can be reactive to cardiolipin, β2-glycoprotein 1, or they can be detected as "lupus anticoagulants." The lupus anticoagulant assay detects the antibodies indirectly, through the interference of clotting on phospholipid surfaces *in vitro*.

The Role of Complement in APS

Clinically, APS is associated with thrombosis or fetal loss. In a passive transfer model in rats, anti-β2 glycoprotein I antibodies caused thrombosis in rats only if they had previously been treated with lipopolysaccharide (51). Complement deficiency or inhibition prevented thrombosis in this model. A murine model of fetal loss caused by passive transfer of antiphospholipid antibodies also demonstrated a role for complement activation in the disease (52). The disease requires an intact alternative pathway, and treatment of mice with an inhibitory antibody to factor B protected them from fetal loss (53). Use of a C5a receptor antagonist was also protective in this model (54). There is evidence of complement consumption in patients with APS. Plasma C3 and C4 levels are lower than in healthy and disease-control subjects, and complement activation fragments are elevated (55). Detailed examination of a patient with APS has also demonstrated elevated levels of circulating complement fragments and also complement fragments deposited within an area of arterial thrombosis (56).

Therapeutic Complement Inhibitors in APS

The standard therapy for APS is anticoagulation. Catastrophic APS (CAPS) is a syndrome in which patients have thrombosis of multiple organs, and eculizumab has been used in patients with disease refractory to anticoagulation or conventional immunosuppression (57). There are also published case series of CAPS patients who were treated with eculizumab to prevent disease recurrence in renal transplants (58). An open-label phase 3 trial of eculizumab for preventing recurrence of CAPS in renal transplant patients was started, although it is apparently not recruiting patients currently.

ORGAN SPECIFIC AUTOIMMUNE DISEASES

Membranous Nephropathy

Membranous nephropathy (MN) is a form of kidney disease in which ICs deposit in the glomerular capillary wall between the podocyte and the basement membrane ("subepithelial ICs"). The podocyte is a specialized cell that sits on the urine side of the glomerular capillary, and it provides a barrier to leakage of cells and protein into the urine. The subepithelial ICs cause podocyte injury, thereby disrupting the filtration barrier. This leads to the

leakage of protein into the urine, and over time it can cause irreversible glomerular damage and a loss of kidney function.

In most cases of MN, autoantibodies bind to proteins expressed on the podocyte surface (referred to as primary MN). In some settings, however, antigens from elsewhere in the body become trapped under the podocyte. Antibodies can then bind the planted antigens *in situ* (referred to as secondary MN). Primary MN is an autoimmune disease in which the antibodies bind to self-proteins expressed by the podocyte, and several different target podocyte antigens have been identified. The first podocyte antigen identified in MN was neutral endopeptidase (NEP) (3). Mothers who are genetically deficient in NEP can be exposed to the protein during pregnancy, eliciting an immune response. During subsequent pregnancies, the maternal anti-NEP antibodies are transferred to the infant, leading to transient MN in the baby.

The most common target antigen in primary MN is the M-type phospholipase A2 receptor (PLA2R), a transmembrane protein expressed by the podocyte (59). Antibodies to PLA2R are seen in 70–80% of cases of MN. Approximately 5% of patients with primary MN have autoantibodies against thrombospondin type-1 domain-containing 7A (THSD7A) (60). It is not known why immune tolerance to these podocyte proteins is lost in patients with MN, but the disease is associated with certain HLA alleles (61), and the autoantibodies bind to specific epitopes on the target proteins (62, 63).

Autoantibodies to several intracellular proteins have also been found, including antibodies reactive with alpha enolase, aldose reductase, and manganese superoxide dismutase (SOD2) (64, 65). Initial injury to the podocyte may be required to cause release of these proteins into the extracellular space. Secondary MN is associated with numerous different systemic diseases, including SLE, several types of cancer, and various infections. Cancer-associated antigens and infection-associated antigens have been eluted from the glomerular deposits in cases of secondary MN, suggesting that these proteins reach the kidney via the circulation and become trapped under the podocytes. Antibodies then bind to the target antigens, and presumably injure the podocyte through the same inflammatory mechanisms as in primary disease.

The Role of Complement in MN

The subepithelial ICs in MN activate complement either directly on the surface of podocytes or immediately adjacent to the cells, and C3 is detected in most biopsies. The C3a and C5a generated by complement activation at this location likely pass directly into the urine, explaining why neutrophils are not usually seen in kidney biopsies (**Figure 3**). Similarly, histologic hallmarks of inflammation are not usually seen in biopsies. C5b-9 that is formed on the podocyte, on the other hand, can directly injure the cell. Early studies using animal models of MN indicated that it is a complement dependent disease (66). Furthermore, animals deficient in C6 are protected from injury, further implicating C5b-9 in podocyte injury and the pathogenesis of the disease (67, 68).

Interestingly, in primary MN the autoantibodies are predominantly of the IgG4 subclass, which does not activate the classical pathway of complement (69). Furthermore, C1q is not detected in most biopsies, whereas C4 is usually detected. These observations suggest that the autoantibodies may activate complement through the mannose binding lectin (MBL) pathway. Investigators have proposed that the carbohydrate side chains of IgG4 may bind to MBL and activate the lectin pathway (70). There are, however, reports of patients with congenital MBL deficiency who have developed MN (71). These patients had C3 deposits in the absence of C1q or C4 deposits, indicating that activation can occur solely through the alternative pathway and that the MBL pathway is not always required. Further complicating things, some IgG1 and IgG3 autoantibodies are detected in the circulation and in the glomerular deposits in primary MN (59, 72). IgG1, IgG2, and IgG3 are also usually seen in biopsies from patients with secondary MN (73, 74). Therefore, although complement is activated in both primary and secondary MN, the mechanisms may vary from patient to patient.

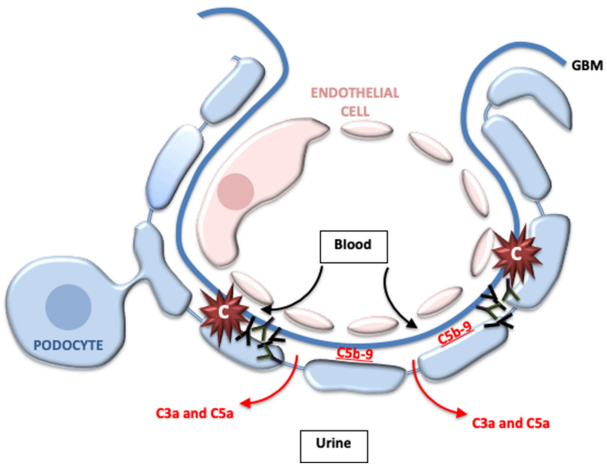

FIGURE 3 | Complement activation may directly injure the podocytes in membranous nephropathy. In membranous nephropathy, immune complexes are located between the glomerular basement membrane (GBM) and the podocyte foot processes. Activation at this location generates C5b-9, which may directly damage podocytes. Because of the location of the immune complexes, however, most of the C3a and C5a that is generated probably passes into the urine and does not enter the bloodstream. This may explain why leukocytes do not typically infiltrate the glomeruli in this disease.

Therapeutic Complement Inhibitors in MN

Based on the pre-clinical evidence that C5b-9 formation is important to the pathogenesis of MN, a clinical trial was conducted in which 122 MN patients were treated with eculizumab or placebo for 16 weeks (75). Treatment with eculizumab was not associated with a significant reduction in proteinuria, indicating that complement inhibition may not be of benefit in the disease. It is possible, though, that the lack of benefit was due to the short duration of treatment. It is also noteworthy that the doses of eculizumab used in the study may not have completely blocked terminal complement activation (76). Many new complement inhibitors are currently in development (13, 77), and several ongoing clinical trials are including patients with MN. Omeros has developed an inhibitory antibody to MASP2 (OMS721). OMS721 and APL-2 (a C3 activation inhibitor) are

currently being tested in Phase 2 studies that include patients with MN (13).

Myasthenia Gravis

Myasthenia gravis (MG) is an autoimmune disease caused by antibodies to proteins expressed at the neuromuscular junction, most commonly the AChR (78). The antibodies can block the function of the receptor. Complement activation on the muscle membrane surface also decreases expression of AChRs on the muscle cell. This results in weakness of skeletal muscles, and almost always includes eye muscles (79).

The Role of Complement in MG

Immunohistologic examination of the post-synaptic membranes from patients with MG demonstrates deposition of immunoglobulin and C3 in nearly all cases (80). C5b-9 has also been detected at this location (81). C5 deficiency was protective in a passive model of the disease in rats. In a mouse model of MG, C4 and C5 deficiency were both similarly protective, indicating that disease is caused through activation of the classical pathway by pathologic antibodies and is mediated by C5a or C5b-9 (82, 83). Conversely, deficiency of CD55 or CD59 exacerbates disease, indicating a role for these complement regulators in attenuating injury (84).

Several therapeutic complement inhibitors have shown efficacy in pre-clinical models. A soluble CR1 construct was protective in the rat models (85), and complement blockade with monoclonal antibodies to C5 or C6 has also proven effective in rodent models (86, 87).

Therapeutic Complement Inhibitors in MG

Based on the promising pre-clinical data, eculizumab has studied in several clinical trials of patients with relapsing or refractory MG. A phase 2 placebo controlled study showed that treatment with the drug was associated with improved muscle strength (88). This was followed by a randomized, double-blind, phase 3 study (REGAIN) (89). In this study, 126 patients were randomized to eculizumab or placebo for 26 weeks. The primary endpoint for the study was the Myasthenia Gravis-Activities of Daily Living (MG-ADL) score. This endpoint was not significantly different in the eculizumab group when evaluated by worst-rank ANCOVA. There was a significant improvement when pre-specified secondary endpoints were evaluated, however. Based on these clinical studies, eculizumab has been approved in the United States, Europe, and in Japan for treatment of MG (90). As more MG patients are treated with complement inhibitors the benefits and limitations of this approach will become clearer.

Neuromyelitis Optica

Neuromyelitis optica (NMO) is an autoimmune disease of the nervous system caused by autoantibodies, usually to aquaporin 4 expressed on astrocytes (91). Antibody deposition on the cells leads to demyelination. NMO primarily affects the optic nerve and spinal cord, but it can also affect the cerebral cortex. Immunosuppressive drugs are beneficial in some cases, and therapeutic plasma exchange ameliorates disease by removing the pathogenic autoantibodies.

The Role of Complement in NMO

Complement proteins, including C1q, C3, C4, and C5b-9, are deposited in nearly all cases of active NMO (92, 93). In animal models, the injection of AQP4-IgG alone is insufficient to initiate demyelination, but intracerebral co-injection of the antibodies with human complement sufficient serum induces lesions with the seminal histopathologic features of NMO (94). In the absence of complement or in the presence of complement inhibition, on the other hand, there is no evidence astrocyte or oligodendrocyte loss. In a rat model, peripherally administered AQP4-IgG caused NMO-like lesions in rats that received an intracerebral needle injury, to permit passage of the antibodies into the central nervous system (95). Injury was prevented, however, by depletion of complement with cobra venom factor (95). Similarly, in mice, it has been shown that lesions induced by injection of anti AQP4 autoantibodies require complement activation by the immunoglobulin (96).

Therapeutic Complement Inhibitors in NMO

Eculizumab successfully reduced the number of disease flares in an open label study that included 14 patients with NMO who had previously had relapsing disease (97). Patients were treated with the drug for 1 year. During that period 12 of the patients did not have any disease flares, and the other two patients had only single, minor flares. A phase 3 randomized controlled double-blind trial to test the efficacy of eculizumab in patients with relapsing disease (PREVENT study) recently completed its recruitment phase. This study enrolled 143 seropositive patients, who had had active disease within the previous 2 years.

CONCLUSIONS

The complement system is activated in almost all antibody-mediated autoimmune diseases. Therefore, drugs that block complement activation may block downstream mediators of injury that are common to most, if not all, of these diseases. The complement cascade plays a central role in the immune response. Complement inhibition may rapidly reduce tissue inflammation and damage, and it may also attenuate T cell and B cell activation, thereby reducing autoimmunity (11). Most ongoing trials are using complement inhibitors to block acute tissue inflammation. As these studies are analyzed, however, it will be interesting to see whether an effect on autoantibody titers can also be detected.

The complement system is activated by antibodies in the autoimmune diseases discussed above, and activation usually occurs through the classical pathway. In some of these diseases, such as MN, activation may occur through the alternative or lectin pathways. Furthermore, the classical pathway may help to eliminate nuclear antigens in diseases such as lupus. Thus, there is a rationale to develop and test inhibitors that can specifically block these other pathways or specific downstream complement activation fragments, such as C5a. Many new complement inhibitory drugs are currently in development (12, 13). As these drugs enter the clinic, it will be fascinating to test whether the benefits of complement inhibition are similar among the various autoimmune diseases, or whether disease specific approaches will be needed.

AUTHOR CONTRIBUTIONS

JT contributed to the conception, writing, and editing of this manuscript. RY contributed to the conception and writing of this manuscript.

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REVIEW
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Complementing the Cancer-Immunity Cycle

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Reactivation of cytotoxic $CD8^+$ T-cell responses has set a new direction for cancer immunotherapy. Neutralizing antibodies targeting immune checkpoint programmed cell death protein 1 (PD-1) or its ligand (PD-L1) have been particularly successful for tumor types with limited therapeutic options such as melanoma and lung cancer. However, reactivation of T cells is only one step toward tumor elimination, and a substantial fraction of patients fails to respond to these therapies. In this context, combination therapies targeting more than one of the steps of the cancer-immune cycle may provide significant benefits. To find the best combinations, it is of upmost importance to understand the interplay between cancer cells and all the components of the immune response. This review focuses on the elements of the complement system that come into play in the cancer-immunity cycle. The complement system, an essential part of innate immunity, has emerged as a major regulator of cancer immunity. Complement effectors such as C1q, anaphylatoxins C3a and C5a, and their receptors C3aR and C5aR1, have been associated with tolerogenic cell death and inhibition of antitumor T-cell responses through the recruitment and/or activation of immunosuppressive cell subpopulations such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), or M2 tumor-associated macrophages (TAMs). Evidence is provided to support the idea that complement blocks many of the effector routes associated with the cancer-immunity cycle, providing the rationale for new therapeutic combinations aimed to enhance the antitumor efficacy of anti-PD-1/PD-L1 checkpoint inhibitors.

Keywords: cancer immunity, immunotherapy, complement system, C3a, C5a, C1q, PD-1, PD-L1

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INTRODUCTION

Profound advances in our understanding of the interactions between tumors and the immune system have allowed the development of therapeutic approaches that boost the body's natural defenses against cancer. These therapies are aimed to mount effective antitumor immune responses and include immunomodulators, vaccines, and adoptive transfer of immune cells (1). Some of the most clinically effective immunotherapies to date target the programmed cell death protein 1 (PD-1) immune checkpoint. PD-1 is expressed by T cells during priming or expansion and binds to one of its two ligands PD-L1 or PD-L2 (2). Tumor cells upregulate PD-L1 in response to cytokines

such as interferon (IFN)-γ (3). Interaction of PD-L1 with PD-1 on T cells causes T-cell apoptosis, anergy, and exhaustion, protecting tumor cells from $CD8^+$ T cell-mediated cytolysis (3). PD-L1 can also deliver intrinsic intracellular signals that enhance cancer cell survival, regulate stress responses, and confer resistance toward apoptotic stimuli (4, 5). PD-1 and other checkpoints are also expressed by NK cells which may contribute to the antitumor activity of some therapeutic strategies under development (6, 7).

Neutralizing monoclonal antibodies against PD-1 or PD-L1 have transformed the therapeutic landscape of a wide range of cancers (**Table 1**), being particularly successful for tumors with limited therapeutic options such as melanoma or lung cancer (3). Notably, these antibodies generate durable responses without causing serious side effects. However, a significant fraction of patients manifests innate or acquired resistance to these therapies. Immune escape mechanisms stem from different cell interactions within the tumor microenvironment, and emphasize the need of developing rational combination strategies to obtain more potent anticancer responses (8).

In 2013, Daniel Chen and Ira Mellman described a series of self-sustaining stepwise events, referred as the cancer-immunity cycle, by which the anti-cancer immune responses lead to an effective elimination of cancer cells (9). The existence of negative feedback mechanisms developed by tumors hinders this cycle of cancer immunity and may pose a barrier to the development of effective clinical responses. Anticancer immunotherapies should be aimed to reactivate all the steps of the cycle, which include immunogenic cell death, maturation of antigen-presenting cells, T-cell priming and activation, promotion of immune infiltration, blockade of immunosuppression, and enhancement of effector T-cell activity. In this context, combination therapies would provide synergistic effects for the maintenance of the cancer-immunity cycle.

In the last years, the complement system, an essential part of innate immunity, has surged as a master regulator of cancer immunity (10). We, and others, have actively contributed to this field, leading to the proposal that modulation of complement activation can improve the antitumor efficacy of inhibitors targeting the PD-1/PD-L1 pathway. In 2008, making a paradigm shift in tumor immunology, we demonstrated that complement activation, followed by C5a signaling, has a tumor-promoting role in cancer (11). In 2012, using a lung cancer model, we first demonstrated an association between the inhibition of C5a receptor 1 (C5aR1) and the expression of PD-L1 within the tumor microenvironment (12). These results suggested the possibility of blocking complement factors to increase the efficacy of other immune therapeutic strategies (12). Following this line, we demonstrated that inhibition of PD-1/PD-L1 synergizes with the inhibition of C5a/C5aR1 in various preclinical models of lung cancer (13). This rationale provided the basis for a clinical trial in which the anti-C5aR1 antibody IPH5401 is being evaluated in combination with the anti-PD-L1 antibody durvalumab in patients with solid tumors (STELLAR-001).

In this review we describe the participation of complement elements in the steps of the cancer-immunity cycle. We propose that a combinational therapy using anti-PD-1/PD-L1 antibodies together with modulators of the complement system may open new therapeutic opportunities for tumors resistant to PD-1/PD-L1 blockade.

TABLE 1 | FDA-approved immune-checkpoint inhibitors (monoclonal antibodies) for cancer treatment.

Drug	Brand name	Target	Antibody subclass	Cancer type
Nivolumab	Opdivo	PD-1	Human IgG4	Melanoma Non-small cell lung cancer Small cell lung cancer Renal cell cancer Hodgkin lymphoma Head and neck cancer Urothelial cancer Colorectal cancer* Hepatocellular cancer
Pembrolizumab	Keytruda	PD-1	Humanized IgG4	Melanoma Non-small cell lung cancer Head and neck cancer Hodgkin lymphoma Primary mediastinal B-cell lymphoma Urothelial cancer Solid tumors* Gastric cancer Cervical cancer
Atezolizumab	Tecentriq	PD-L1	Humanized IgG1	Urothelial cancer Non-small cell lung cancer
Durvalumab	Imfinzi	PD-L1	Human IgG1	Urothelial cancer Non-small cell lung cancer
Avelumab	Bavencio	PD-L1	Human IgG1	Merkel cell cancer Urothelial cancer

**For patients with mismatch repair deficiency (dMMR) or microsatellite instability high (MSI-H).*

THE CANCER-IMMUNITY CYCLE

The cancer-immunity cycle is defined as a series of functional stepwise events needed to obtain an efficient control of cancer growth by the immune system (9). The process is initiated by the release of neo-antigens generated as a result of genomic instability. Cancer-associated antigens are captured by dendritic cells which, upon migration to lymph nodes, prime and activate tumor-specific cytolytic $CD8^+$ T cells. These effector cells migrate and infiltrate the tumor stroma, where potentially are able to recognize and eliminate cancer cells. T cell-mediated cytotoxic responses release new tumor antigens, fueling the cancer-immunity cycle. Interestingly, this model provides the rationale for targeting different steps of the cycle in order to maintain its functionality. An effective cancer immunotherapy should be designed based on the specific resistance mechanisms underlining the rate-limiting steps in each particular patient (14, 15). In the case of anti-PD-1/PD-L1 therapies, a variety of biological factors contribute to treatment resistance, including lack of cancer antigens recognizable by T

cells, impaired cancer-antigen presentation, impaired activation of cancer-specific T cells, poor infiltration of T cells into tumors, and accumulation of immunosuppressive factors and cells in the tumor microenvironment (15). The evading strategies present in a given tumor would determine whether this tumor shows an inflamed or a noninflamed phenotype (16). Clinical evidence suggests that anti-PD-1/PD-L1 inhibitors are most effective in inflamed tumors characterized by high tumor PD-L1 expression, $CD8^+$ T-cell infiltration or mutational burden (17–20). Jerby-Arnon et al. recently analyzed the relationship between malignant cell states and $CD8^+$ T-cell infiltration and identified a T-cell exclusion program that predicts responses to PD-1/PD-L1 blockade (21). This program was enriched for genes involved in predictable processes, such as antigen processing and presentation, IFN-γ signaling, and immune modulation; but also identified genes associated with activation and modulation of the complement system (21). Elements of the complement cascade are also present in a signature of serum proteins that predicts survival in patients receiving PD-1 blocking antibodies, suggesting that complement activation may inhibit the efficacy of adaptive antitumor immunity (22).

THE COMPLEMENT SYSTEM

The complement system, a central element of innate immunity, represents a first line of defense against unwanted non-self and host elements, and orchestrates many immunological and inflammatory processes that substantially contribute to body homeostasis (23, 24). Complement activities are mediated by more than 50 circulating, cell surface-bound and intracellular proteins. There are three main mechanisms of complement activation, known as classical, lectin, and alternative pathways. The classical pathway is commonly initiated by the binding of C1q to complement-fixing antibodies (mostly IgM and IgG types); although C1q can also recognize non-immunoglobulin ligands such as C-reactive protein (CRP), pentraxin 3 (PTX3), or apoptotic cells (25). The lectin pathway is activated by homologous proteins to C1q (mannose-binding lectin, collectins, and ficolins) that recognize repetitive carbohydrate patterns (26). Lastly, the alternative pathway is initiated by spontaneous cleavage of C3 on activating surfaces (27). Although the three complement pathways differ in their mechanisms of target recognition, in all cases, initiation of the complement cascade leads to the formation of C3 convertases and the activation of the central component C3. After this activation, C5 convertases are formed, C5 is cleaved, and the assembly of the pore-like membrane attack complex (MAC) is initiated. The enzymatic cleavage of complement elements leads to the release of proteolytic fragments such as C3a and C5a, and the deposition of other fragments such as C3b and iC3b. These molecules modulate a diverse set of processes (23), including the initiation and regulation of effector T-cell responses (28). Prevention of inappropriate activation by complement regulators takes place at three main levels: inhibition of protease activities in the activation cascade, decay and destruction of convertases, and control of MAC formation (29). Finally, recent experimental and clinical evidences suggest that intracellular complement components have important roles in cell physiology (30).

Complement has been traditionally regarded as playing a role in the elimination of tumor cells. Accordingly, an effective control of tumor growth may be achieved by complement-fixing antibodies (31). However, growing evidence, starting for the initial observation in a model of cervical cancer (11), strongly supports a tumor-promoting role of complement in several tumor types. This topic has been extensively reviewed elsewhere (10, 32–35). Briefly, complement establishes an immunosuppressive microenvironment, promotes angiogenesis, sustains cellular proliferation, and participates in tumor cell invasion and migration. In light of the various contributions of complement to cancer progression, it is not surprising that expression of complement effectors and receptors is associated with disease progression and poor prognosis (36–42). Among all the complement elements with potential pro-cancer activities, C1q, C3-derived fragments, and C5a are recognized as major modulators of tumor progression (43–45).

COMPLEMENT IN THE REGULATION OF THE CANCER-IMMUNITY CYCLE

In this section we will discuss the potential implication of effectors and regulators of the complement system in the steps of the cancer-immunity cycle. In light of the breadth and complexity of the immune response, we will focus our review on the specific aspects of the regulation of $CD8^+$ cytotoxic T cells, which are in large part the mediators of anti-PD-1/PD-L1 therapies. We will also examine evidence supporting the participation of complement in the regulation of the type 1 T helper (Th1) response, as it has a profound influence on the quality and extension of cytotoxic T-cell responses (46). Recent reviews have extensively addressed other complement-mediated immune functions not covered in the present review (10, 28, 47–50).

Modulation of the Initiation of T-Cell Immunity by the Complement System

The cancer-immunity cycle is initiated by tumor-specific neo-antigens generated by somatic mutations (51). Dying cancer cells release these antigens to the tumor microenvironment, where are captured and processed by dendritic cells, the principal cell type responsible for instructing naïve T cells to undergo antigen-specific effector functions. Depending on the stimuli provided by dying cancer cells, their interaction with dendritic cells can have immunogenic or tolerogenic consequences (52). The generation of an immunogenic or a tolerogenic cell death is mainly regulated by damage-associated molecular patterns (DAMPs). DAMPs are endogenous co-stimulatory signals secreted or presented on the cell surface of dying cells that interact with pattern-recognition receptors (PRRs) alerting the host of danger. Complement is required for efficient sensing of DAMPs (53–55). The specific interactions of danger sensors with complement elements allow to differentiate between physiological and pathological danger, shaping the maturation of dendritic cells

(23). This activity depends mainly on the classical complement pathway. Direct binding of complement C1q to apoptotic cells promotes a phagocytic-mediated uptake of dying cells, which sustains an anti-inflammatory innate immune response through the expression of cytokines such as transforming growth factor (TGF)-β (56). In fact, genetic deficiencies in C1q, as well as other elements of the classical complement pathway, can compromise the induction of self-tolerance and result in systemic autoimmune diseases (57, 58). Another complement element involved in the recognition of danger signals is factor H, a soluble complement inhibitor produced and secreted by cancer cells (59, 60). Upon opsonization of apoptotic cells, factor H induces an anti-inflammatory cytokine profile (61, 62) and a tolerogenic stage (63). CD46, a membrane-bound complement regulatory protein able to interact with C3 activation fragments and found at high levels in some cancer types (64, 65), has also been proposed as a negative regulator of immune recognition (66). Complement proteins are easily detectable in various types of cancer, consistent with complement activation by these tumors (32). Therefore, upregulation of complement components in the surface of dying cancer cells may be associated with a tolerogenic cell death, in contrast to the immunogenic cell death required for an effective anticancer immune response (67, 68).

Modulation of Priming and Activation of T Cells by the Complement System

Progress of the cancer-immunity cycle requires the presence of activation signals that allow dendritic cells to mature, migrate to the lymph nodes, and present the neo-antigens to naïve T cells. Efficient priming also relies on the contextual information provided by the microenvironment. Mature dendritic cells in the presence of suitable signals are able to induce T-cell effector functions; whereas in the absence of appropriate conditions, antigen presentation leads to T-cell anergy or generation of regulatory T cells (Tregs) that suppress effector responses.

Locally-produced complement elements determine the state of dendritic cell activation (69), and are critical in the regulation of T-cell responses (28). Production of C1q and C3 by dendritic cells induces their maturation and their capacity to stimulate Th1-cell responses (70, 71). C3 may also facilitate intracellular antigen processing and presentation (72). In agreement with these observations, optimal priming and expansion of $CD4^+$ and $CD8^+$ T cells in infection models is dampened by C3 deficiency (73, 74), and the complement fragment C3d amplifies antitumor T-cell responses (75). In the case of C3a and C5a, through activation of their respective receptors C3aR and C5aR1, these anaphylatoxins enhance the capacity of human monocyte-derived dendritic cells to stimulate T cells (76). In accordance, C3aR pathway inhibition in dendritic cells results in defective T-cell priming, associated with a reduced surface expression of major histocompatibility complex (MHC) and costimulatory molecules (77). Finally, downregulation of the expression of the complement regulator CD55 in antigen-presenting cells during T-cell activation increases the local production of C3a and C5a, providing costimulatory signals to induce T-cell proliferation and differentiation (78, 79).

Complement elements can also exert a direct influence on T cells. Activation of human $CD4^+$ T cells by CD46 stimulates the effector potential of Th1 cells (80–82). As on dendritic cells, paracrine and autocrine interactions of C3a and C5a with their respective receptors C3aR and C5aR1 mediate Th1 cytokine production and T-cell induction (78, 83). It has also been suggested that Tregs express C3aR and C5aR1, and that signaling through these receptors inhibits Treg function (84, 85).

A central role in T-cell homeostasis has been recently assigned to intracellular elements of the complement system (30). Activation of lysosomal C3aR by intracellularly generated C3a contributes to the survival of resting $CD4^+$ T cells. Upon activation, the intracellular stores of the C3 system translocate to the cell surface triggering the upregulation of IFN-γ and Th1-cell responses in conjunction with the extracellular engagement of C3b to CD46 (86). Intracellular C5a can also be generated from endogenous C5. Upon T-cell activation, C5a binds to intracellular C5aR1, inducing the activation of the NLRP3 inflammasome and, consequently, the initiation of a Th1 response (87).

All these activities underline the importance of complement effectors and regulators in the initiation of T-cell responses. In contrast, the complement system has also been associated with the prevention of T-cell priming and the induction of tolerance; probably as a regulatory mechanism to facilitate the timely resolution of the immune response. Thus, C1q can suppress macrophage-mediated inflammation and dendritic cell-mediated Th1-cell proliferation (88, 89). Binding of the C3 fragment iC3b to complement receptor type 3 (CR3) on antigen-presenting cells results in the production of TGF-β2 and interleukin (IL)-10, and the induction of antigen-specific tolerance (90). C3a and C3b also participate in the contraction phase of human Th1 responses (80, 82). CD46 promotes the switching of $CD4^+$ T cells toward IL-10 producing cells with a regulatory phenotype (80, 91), and negatively regulates Th1 activity through the binding of endogenous C5a to surface-expressed C5aR2 (87). C5aR2 was first proposed as a negative regulator of C5aR1, but some specific functions have been ascribed to this C5a receptor (92). CD55 may also have a role in the suppression of adaptive immune responses. Mice lacking CD55 experience enhanced T-cell responses to active immunization, characterized by an increased production of INF-γ and IL-2, as well as downregulation of IL-10 (93).

All these studies point to the dual role played by complement in the activation of effector T-cell responses. On the one hand, complement elements are central in the primary phase of effector expansion. On the other hand, complement can mediate a suboptimal T-cell activation associated with the contraction phase or the establishment of tolerance. With this duality in mind, it is interesting to analyze the relative role played by complement in the context of well-established tumors. It has been suggested that C5a affects T-cell responses in a concentration-dependent manner (94). Tumor-bearing mice with low C5a-producing tumor cells exhibit a reduced tumor burden with increased IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the spleen and tumor-draining lymph nodes. In contrast, tumor-bearing mice with high C5a-producing cancer cells have an

accelerated tumor progression with less $CD4^+$ and $CD8^+$ T cells in the tumor, tumor-draining lymph nodes, and the spleen (94). This effect was associated with the presence of more myeloid-derived suppressor cells (MDSCs) in the spleen. Interestingly, other studies have found elevated levels of C5a in cancer patients (12), which have been implicated in the recruitment of MDSCs to tumors (11). MDSCs are immunosuppressive immature myeloid cells able to disrupt major mechanisms of antitumor immune responses (95–97). In models of breast cancer, C5aR1 signaling in MDSCs induces the production of immunosuppressive cytokines, such as TGF-β, and reduces Th1 immune responses (98, 99). Treatment of mouse squamous cell carcinomas with paclitaxel and PMX-53, a C5aR1 inhibitor, results in peripheral priming and expansion of antigen-specific clones (100). Therefore, we can conclude that complement-mediated effects may have evolved at established tumors to interfere with the generation of antitumor T-cell responses.

Modulation of T-Cell Trafficking by the Complement System

A range of tumors escape from antitumor immune responses even after activation and expansion of T cells. In some cases, this can be attributed to the ability of the tumor endothelium to prevent T-cell trafficking. A complex network of endothelial adhesion molecules, Th1 cytokines, and surface receptors regulates T-cell homing and infiltration (101). Tumor cells, in concert with the endothelium, interfere with T-cell infiltration through a variety of molecular mechanisms, including the downregulation of endothelial adhesion molecules and the expression of T-cell inhibitory ligands (102).

Although complement elements can directly act on the endothelium (103–105), little is known about its contribution to the biology of tumor-associated endothelial cells. In the context of adoptive T-cell transfer, the capacity of tumor-reactive $CD4^+$ and $CD8^+$ T cells to infiltrate tumors requires the local production of C3, complement activation and release of C5a (106). Accordingly, the abrogation of CD55 expression enhances tumor T-cell infiltration (106). Radiotherapy has been found to upregulate the release of C3a and C5a within the irradiated tumors, leading to pronounced infiltration of $CD8^+$ effector T cells (107). The presence of C3d on melanoma cells yields greater infiltration by $CD4^+$ and $CD8^+$ lymphocytes (75). However, in apparent contradiction to these findings, blocking of C3aR or C5aR1 in most cancer models has been associated with an increased infiltration of T cells both in the primary tumor (11, 13, 42, 99, 100, 108, 109) and the metastatic niche (98, 109). The mechanisms underlying this outcome are not fully understood but may be related to the downregulation of immunosuppressive cell populations, such as MDSCs, able to impair T-cell trafficking (11, 12). Complement inhibition also reduces the levels of VEGF (110), which may normalize the tumor vasculature, increasing the infiltration of lymphocytes into tumors (111).

Modulation of Cytotoxic T-Cell Activity by Complement

An immunosuppressive microenvironment hampers the killing capacity of cytotoxic $CD8^+$ T-cells. The pro-tumorigenic effect of complement activity is mediated, in an important way, by promoting immunosuppressing responses within the tumor microenvironment. The critical contribution of complement to regulating immunosuppressive cell populations, such as TAMs,

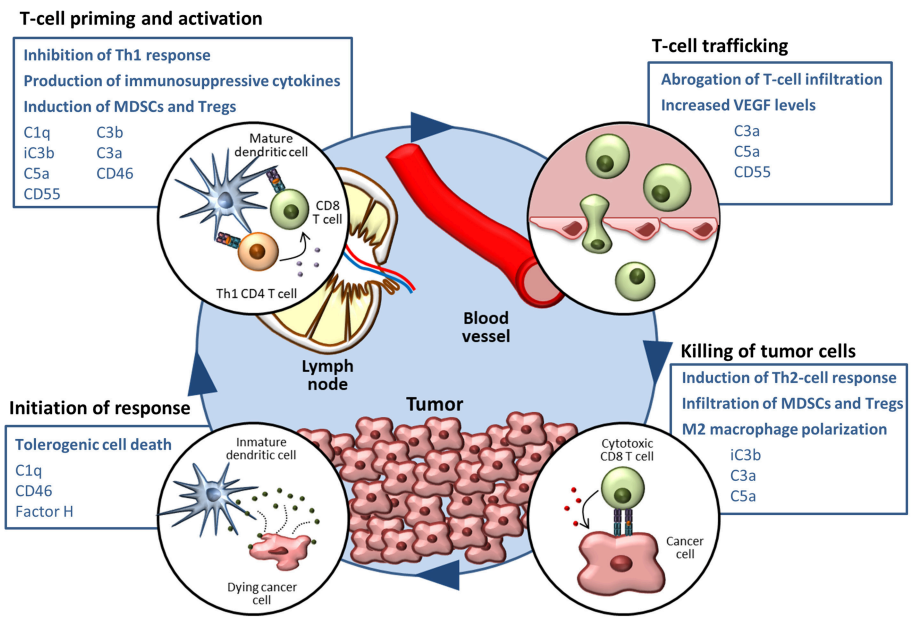

FIGURE 1 | The complement system in the cancer-immunity cycle. The cancer-immunity cycle is summarized in four steps. Complement-mediated mechanisms associated with the inhibition of the cancer-immunity cycle, together with complement components that participate in these processes, are shown in blue boxes.

MDSCs, or Tregs, has been recently reviewed (47). C5a acts as a potent chemoattractant for polymorphonuclear MDSCs and stimulates the production of immunosuppressive reactive oxygen and nitrogen species by tumor infiltrating monocytic MDSCs (11). Accordingly, pharmacological blockade of C5aR1 decreases the frequency of MDSCs and impairs tumor growth (11–13, 112). C5aR1 inhibition also downregulates the expression of immunosuppression-related genes within the tumor milieu (12). In addition, C5a contributes to conditioning the premetastatic niche through TGF-β and IL-10-mediated accumulation of Tregs, proliferation of resident alveolar macrophages, and decrease in number and maturation of dendritic cells (98). As a consequence, effector $CD4^+$ T-cell responses skew toward a Th2 phenotype, limiting Th1 responses (98, 113).

C5a also affects the biology of macrophages. C5a skews macrophage polarization toward an M2 phenotype via C5aR1 signaling upon *Leishmania* infection (114). After *ex vivo* challenge of human whole blood with heat-killed *Pseudomonas aeruginosa*, C5a induces PD-L1 expression on monocytes, and the production of IL-10 and TGF-β (115). Elevation of PD-L1 expression has also been reported after C1q-mediated polarization of macrophages (89). M2 tumor-associated macrophages (TAMs) are an essential component of the tumor microenvironment that contribute to tumor progression by blocking $CD8^+$ T-cell responses (116). Recruitment of tumor-promoting TAMs with a M2-like phenotype is also observed in mouse sarcomas induced in a PTX3-deficient context and characterized by an increase in C5a and CCL2 (44). C5a also promotes hepatic metastases of colon cancer associated with an increase of monocyte chemoattractant protein-1 (MCP1), anti-inflammatory modulators such as arginase-1, IL-10, or TGF-β, and M2-like macrophages (117, 118). Similarly, in a model of squamous carcinogenesis, C5a regulates the protumorogenic properties of C5aR1-expressing mast cells and macrophages, leading to hampered antitumor $CD8^+$ T-cell responses (100). A combined treatment with cytotoxic chemotherapy and the blockade of C5aR1 synergistically inhibits the recruitment of effector memory $CD8^+$ T cells by both the modification of macrophage- and IFNγ-dependent mechanisms (100). Interestingly, this study suggests that C5a is not generated in the tumors through C3 activation, although further studies are needed to rule out this possibility (119).

Complement C3 activation fragments can also precondition the tumor microenvironment toward immunosuppression. The C3 degradation product iC3b promotes the development of MDSCs *in vitro* (120). Inhibition of complement C3 abrogates the suppressor phenotype of polymorphonuclear MDSCs in the ovarian tumor microenvironment (121). Deletion of C3 in tumor cells also inhibits M2 polarization (122). Signaling mediated by C3a contributes to melanoma tumorigenesis by inhibiting neutrophil and $CD4^+$ T-cell responses (108). Interestingly, some studies have suggested a direct effect of complement effectors in the functionality of T cells. C3 inhibits IL-10-mediated cytotoxic properties of tumor-infiltrating $CD8^+$ T lymphocytes in an autocrine manner, enhancing melanoma and breast cancer growth (123). Alterations in $CD4^+$ T cells by C3/C5-dependent pathways may also have a major role in lung cancer progression (109).

Finally, complement can also slow down the feeding of the cancer-immunity cycle by dying cancer cells. Ribosomal protein S19 (RPS19), upon release from dying tumor cells, interacts with C5aR1 expressed on MDSCs, promoting its recruitment to tumors, the generation of Tregs, the production of immunosuppressive cytokines (including TGF-β), and the reduction of $CD8^+$ T-cell tumor infiltration (99).

Overall, tumor-associated complement activation deeply influences the tumor microenvironment, leading to an immunosuppressive state and the attenuation of tumor-specific cytotoxic T-cell responses.

TABLE 2 | Contribution of some elements of the complement system to the inhibition of the cancer-immunity cycle.

Entity	Role	Affected cancer-immunity step
C1q	Tolerogenic clearance of dying tumor cells	Initiation of anti-tumor immunity
	Inhibition of antitumor Th1 response	T-cell priming and activation
C3 fragments (C3b, iC3b, C3a)	Tolerogenic clearance of dying tumor cells	Initiation of anti-tumor immunity
	Inhibition of antitumor Th1 response	T-cell priming and activation
	Abrogation of T-cell infiltration	T-cell trafficking
	Differentiation of MDSCs	Killing of cancer cells
	Impaired T-cell cytotoxicity	Killing of cancer cells
C5a	Inhibition of antitumor Th1 response	T-cell priming and activation
	Abrogation of T-cell infiltration	T-cell trafficking
	Angiogenesis	T-cell trafficking
	Tumor infiltration of MDSC and Tregs	Killing of cancer cells
	Polarization toward an M2 phenotype	Killing of cancer cells
	Impaired T-cell cytotoxicity	Killing of cancer cells

COMPLEMENTING THE CANCER-IMMUNITY CYCLE

As reviewed in the previous section, a growing body of evidence supports the notion that complement activities support cancer growth and metastasis in the context of established tumors (124). Many mechanisms related to immune escape and resistance to checkpoint inhibitors can be modulated by elements of the complement system (summarized in **Figure 1**). The non-immunology-related effects of complement on cancer cell biology, including cancer cell proliferation, survival and invasion capacity (42, 43, 117, 125–137), further reinforces the impact of complement activation in cancer progression.

Based on the regulatory functions of complement in the cancer-immunity cycle, we sought to evaluate whether complement inhibition may represent an effective target for combined immunotherapies in preclinical syngeneic models of

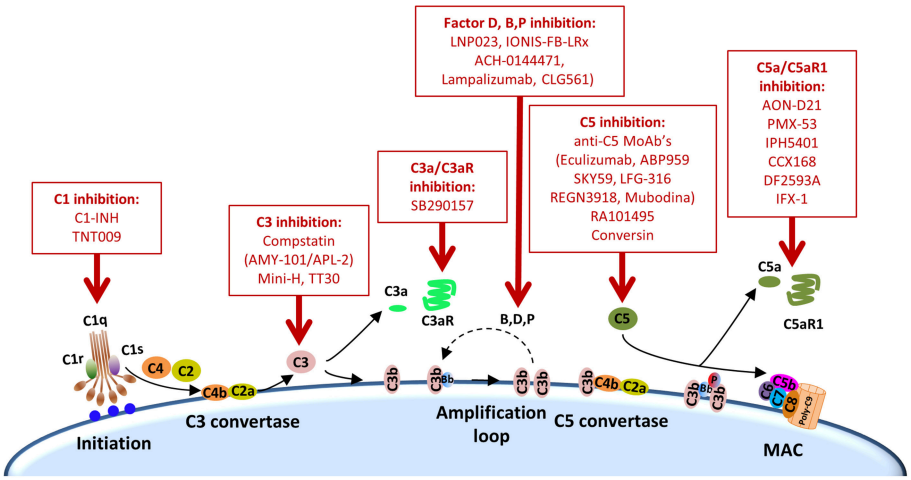

FIGURE 2 | Points of complement inhibition. Steps of the classical complement activation pathway and some inhibitors available for targeting these steps are shown. These points of therapeutic intervention may render synergistic antitumor activities in combination with anti-PD-1/PD-L1 therapies.

cancer. Clinical successes and limitations of anti-PD-1/PD-L1 monotherapy prompted us to use this target as the primary building block for the combination. The C5a/C5aR1 axis was selected as the complement-related target based on the abundant evidence supporting the role of this pathway in the establishment of an immunosuppressive microenvironment (**Table 2**) (45). Using different lung cancer models, we observed a remarkable synergistic control of lung tumor burden and metastatic progression in animals simultaneously treated with an aptamer against C5a (AON-D21) and an anti-PD-1 monoclonal antibody (13). This effect is accompanied by a negative association between the frequency of $CD8^+$ T cells and the presence of MDSCs within tumors, and by a reduction of $CD8^+$ T-cell exhaustion markers (13). The synergistic benefit of this combination was later confirmed in models of melanoma and colon cancer (138). Interestingly, PD-1/PD-L1 antibodies induce the production of C5a (138), establishing a regulatory loop between both pathways.

Other complement elements, such as C1q, C3, or C3a, may be also targeted to re-educate the tumor microenvironment and sensitize it to the subsequent administration of immune checkpoint blockers (**Table 2**). A multifaceted repertoire of therapeutic inhibitors targeting these complement elements has been developed, and are currently in preclinical or clinical development (139, 140). **Figure 2** shows examples of compounds that may be used to target complement in the context of cancer immunotherapy. Complement C3, the centerpiece of complement activation, represents a particularly attractive target for therapeutic complement inhibition (141). Nonresponsive patients to PD-1/PD-L1 blockade frequently have noninflamed tumors with a defect in the early stages of the cancer-immunity cycle (15). Opsonization of dying cells with C3 fragments induces the production of anti-inflammatory cytokines and reduces the costimulatory molecules needed for the maturation of dendritic cells, resulting in T-cell tolerance (90). Therefore, C3 blockade may have a beneficial impact in the early stages of the cancer-immunity cycle, converting a noninflamed tumor into an inflamed tumor susceptible to PD-1/PD-L1 blockade. Moreover, both C3a and C5a production would be impaired (at least in the case of inhibitors, such as compstatin, that blocks C3 activation by all pathways). Interestingly, a simultaneous blockade of C3aR and C5aR1 has been reported to enhance the efficacy of anti-PD-1 therapy against melanoma cells (123). Deletion of C3 in tumor cells that had high C3 expression enhanced efficacy of anti–PD-L1 treatment (122). Additionally, complement C3 inhibition may have antitumor potential in the context of other immune combinations. For example, C3 inhibition by complement depletion or the use of the inhibitor compstatin enhances the antitumor efficacy of oncolytic virus (142, 143) and induces natural killer (NK)-mediated antitumoral responses (144). Inhibition of complement activation upstream of all complement effectors also appears to be a rational approach. In this sense, combinatorial therapies involving inhibitors of C1q (e.g., C1-INH), which presents both complement-dependent and -independent tumor promoting activities, merit further investigation. Finally, it has to be noted that most complement inhibitors target the extracellular complement system, preserving its intracellular activity. This may be of upmost importance, since intracellular C3aR and C5aR1 signaling pathways seem to be required for T-cell survival (28).

Finally, it is interesting to point that the preclinical findings showing the feasibility and value of blocking C5a/C5aR1 to increase tumor-killing efficacy of checkpoint inhibitors have been the basis for the design of a phase I/II study (STELLAR-001). In this trial, the safety and efficacy of durvalumab (an anti-PD-L1 monoclonal antibody) is being tested in combination with IPH5401 (an anti-C5aR1 monoclonal antibody) in patients with selected solid tumors, including non-small cell lung cancer and hepatocellular carcinoma (NCT03665129). We are looking forward to the outcome of this trial, as well as

to the clinical evaluation of novel combinations involving complement inhibitors.

CONCLUDING REMARKS

This is an exciting time for the complement field, in which new biological concepts have brought new therapeutic opportunities. Based on the extensive literature associating complement activation and cancer progression, we propose here that substantial clinical benefits can be achieved by multi-modal anticancer immunotherapies targeting both complement-mediated mechanisms (to reverse immunosuppression), and PD-1/PD-L1 immune checkpoints (to re-activate T-cell functionality). Our preclinical studies supporting the idea that C5a/C5aR1 inhibition creates a "window of opportunity" for the administration of anti-PD-1/PD-L1 checkpoint inhibitors pave the way for the evaluation of other complement-based combinations. The challenge being that many potential combinations can be evaluated. Insights into how complement switches from tumor suppressing to tumor promoting activities at the onset of disease, as well as how to manage this dichotomy should be important research areas in order to establish the best therapeutic strategies. The differences between mice and humans in complement-mediated T-cell responses should also be considered (28, 87). To overcome this limitation, faithful mouse models that recapitulate the complexity of the human immune context in the tumor microenvironment are urgently needed (145).

AUTHOR CONTRIBUTIONS

RP and JL designed the concept. All authors wrote the manuscript. SO-E prepared the figures. All authors read and approved the final version of the manuscript.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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The Challenges and Promise of Complement Therapeutics for Ocular Diseases

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Ocular inflammation is a defining feature of sight threating diseases and its dysregulation can catalyze and or propagate ocular neurodegenerative maladies such as age-related macular degeneration (AMD). The complement system, an intrinsic component of the innate immunity, has an integral role in maintaining immune-surveillance and homeostasis in the ocular microenvironment; however, overstimulation can drive ocular inflammatory diseases. The mechanism for complement disease propagation in AMD is not fully understood, although there is accumulating evidence showing that targeted modulation of complement-specific proteins has the potential to become a viable therapeutic approach. To date, a major focus of complement therapeutics has been on targeting the alternative complement system in AMD. Recent studies have outlined potential complement cascade inhibitors that might mitigate AMD disease progression. First-in-class complement inhibitors target the modulation of complement proteins C3, C5, factor B, factor D, and properdin. Herein, we will summarize ocular inflammation in the context of AMD disease progression, current clinical outcomes and complications of complement-mediated therapeutics. Given the need for additional therapeutic approaches for ocular inflammatory diseases, targeted complement modulation has emerged as a leading candidate for eliminating inflammation-driven ocular maladies.

Keywords: age-related macular degeneration, complement system, immune modulation, ocular inflammation, therapeutics

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Age-related macular degeneration (AMD) is the leading cause of blindness of the elderly in the Western world (1). Its prevalence is expected to rise as aging populations rise and ∼288 million people will be affected by AMD by 2040 (2). The buildup of debris within retinal pigment epithelial (RPE) cells and under the RPE layer is considered a hallmark of AMD development and progression (3). Photoreceptors require a large membrane surface area for phototransduction, and have a large outer segment that must be continuously replaced. The RPE are specialized cells that phagocytize the shed outer segments of photoreceptors, and are the most active phagocytizing cells of the body (4). As aging progresses, it is thought that the machinery of these cells deteriorates, along with their capacity to degrade and recycle photoreceptor metabolic waste. During the lifetime of a person, the choroidal capillary area becomes thinner, and Bruch's membrane may also accumulate lipoprotein material from the choroidal capillaries, which adds to the incompletely digested material deposited by RPE cells. Collectively, this accumulating material is referred to as drusen, which is a major pathological feature of AMD.

Drusen size can predict the development of AMD. Accordingly, a new clinical classification scheme for AMD using drusen size and presence of pigmentary abnormalities has been introduced by the Beckman Initiative for Macular Research Classification Committee (5). In this scheme, individuals over 55 years of age with small (<63 μm) drusen were considered to have normal aging. Early AMD is characterized by the presence of medium-sized drusen (≥63 and <125 μm) with no pigmentary abnormalities, and is accompanied by a mild loss of vision. Intermediate AMD is characterized by larger drusen deposits (≥125 μm) and/or presence of pigmentary abnormalities, and is associated with a moderate loss of vision. Late/Advanced AMD is characterized by the presence of any choroidal neovascularization (CNV), known as neovascular AMD, or gross pigment abnormalities and cellular degeneration such as geographic atrophy (GA). Collectively, advanced AMD causes severe loss of vision and can lead to blindness. The incidence of late-stage AMD increases exponentially with age, with the two forms of late AMD, neovascular AMD and GA, occurring with roughly equal prevalence (2, 6). An estimated 1.22 million and 973,000 people in the United States have neovascular AMD and GA in at least one eye, respectively (7). As the population ages, the prevalence of late AMD is expected to rise from 9.6 million to 11.3 million in 2020 and 18.6 million in 2040 (2).

Currently, there is no effective prevention, individual risk estimation, or reliable prognostic evaluation available for the clinical management of AMD. Further, there are no therapeutics approved for the treatment of GA. The development of AMD depends on a complex interplay of risk factors such as age, genetics (8), and behavior including; smoking (9), diet (10), and sunlight exposure (11). Genetic variations in genes involved in the complement system, as well as others, are associated with risk for developing AMD, or risk of progression from early to late AMD (12). Overall, these findings suggest that AMD is a progressive neurodegenerative disease involving inflammation (13), and in particular an inflammatory immune response (14).

THE COMPLEMENT SYSTEM IS A VITAL COMPONENT OF INNATE IMMUNITY

The immune system is divided into two distinct types, the innate and adaptive systems. The complement system, as part of the innate immune system, plays an integral role in maintaining immune-surveillance and homeostasis in the ocular microenvironment (15, 16). The complement system consists of three systems classical, lectin, and alternative (17). The classical system is mediated by the binding of the complement component C1q of C1 protein to antigen–antibody complexes. The lectin system is activated by mannose-binding lectin recognition of the polysaccharide or glycoprotein motifs on the cell surface of damaged host and non-host cells (17, 18). Lastly, the alternative complement system is constitutively active through the spontaneous cleavage of an internal C3 thioester bond (17, 19) (**Figure 1**). The spontaneous hydrolysis of this internal thioester bond within the complement protein C3 forms C3(H_2O), and while not cleaved, C3(H_2O) can function in the same manner as C3b in a C3 convertase. This "tick-over" of C3 to C3 (H_2O) enables it to complex with complement factor B, following by its cleavage by complement factor D, a serine protease, into Ba and Bb fragments. The resulting C3(H_2O)Bb complex is a C3 convertase, which can efficiently cleave C3 into C3a and C3b. Factor B enables further downstream activation of the alternative system through its binding to C3b (17–21). These components form the C3 convertase enzyme, C3bBb, promoting the cleavage of C3 and creating a positive feedback loop (19, 21). Subsequently, C5 convertase is created by the combination of the C3 convertase with an additional C3b molecule. The C5 convertase then cleaves C5 into C5a and C5b (17–19). C5b recruits C6, − 7, −8, and−9, forming the membrane attack complex (MAC), which forms a pore in the cell membrane causing cell lysis and death, which can be down-regulated by complement inhibitors such as CD59 (22, 23). However, according to a previous immunohistochemical study from human donor eyes with drusen, the antibody against C5b-9 indicating MAC showed a restricted pattern, while the C3 or C5 antibody showed a diffuse pattern throughout the drusen and choroid (24). Thus, it is unclear if MAC formation is linked to AMD disease progression. It may be complement's upstream opsonization functions that are perturbed in this pathology.

ALTERNATIVE COMPLEMENT CASCADE DYSFUNCTION IN AGE-RELATED MACULAR DEGENERATION

Complement Factor H and Related Proteins

Over a lifetime, the photoreceptors and RPE cells of the retina encounter a number of innate immune activators, such that tight regulation of immunity is necessary to prevent deleterious inflammatory events. Dysregulation of the complement system may potentially drive ocular inflammation, which contributes to vision loss in AMD. Complement byproducts such as complement factor H (FH) in the drusen were the first indication that the complement system is involved in AMD progression (25). In 2005, genome-wide association studies (GWAS) suggested that the complement cascade was involved in AMD disease progression. **Table 1** summarizes the genes for the alternative complement pathway that are known to be involved in AMD. Early GWAS and targeted sequencing studies identified a common single nucleotide polymorphism (SNP) in the *CFH* gene (rs1061170) that is associated with an increased risk of developing AMD (26–29). This SNP replaces a tyrosine residue with a histidine residue at position 402 (Y402H) (49). Heterozygous individuals and homozygotes for the Y402H polymorphism have a 2.3 and 5.2-fold increased risk of developing AMD, respectively (30). Approximately 30% of individuals of European descents are carriers of at least one of the Y402H risk alleles (30).

FH is an important regulator of the complement system (50). Increasingly, it has been found that complement activation is linked with AMD associated with genetic variants on chromosome 1 locus (1q32) that contains the *CFH* and

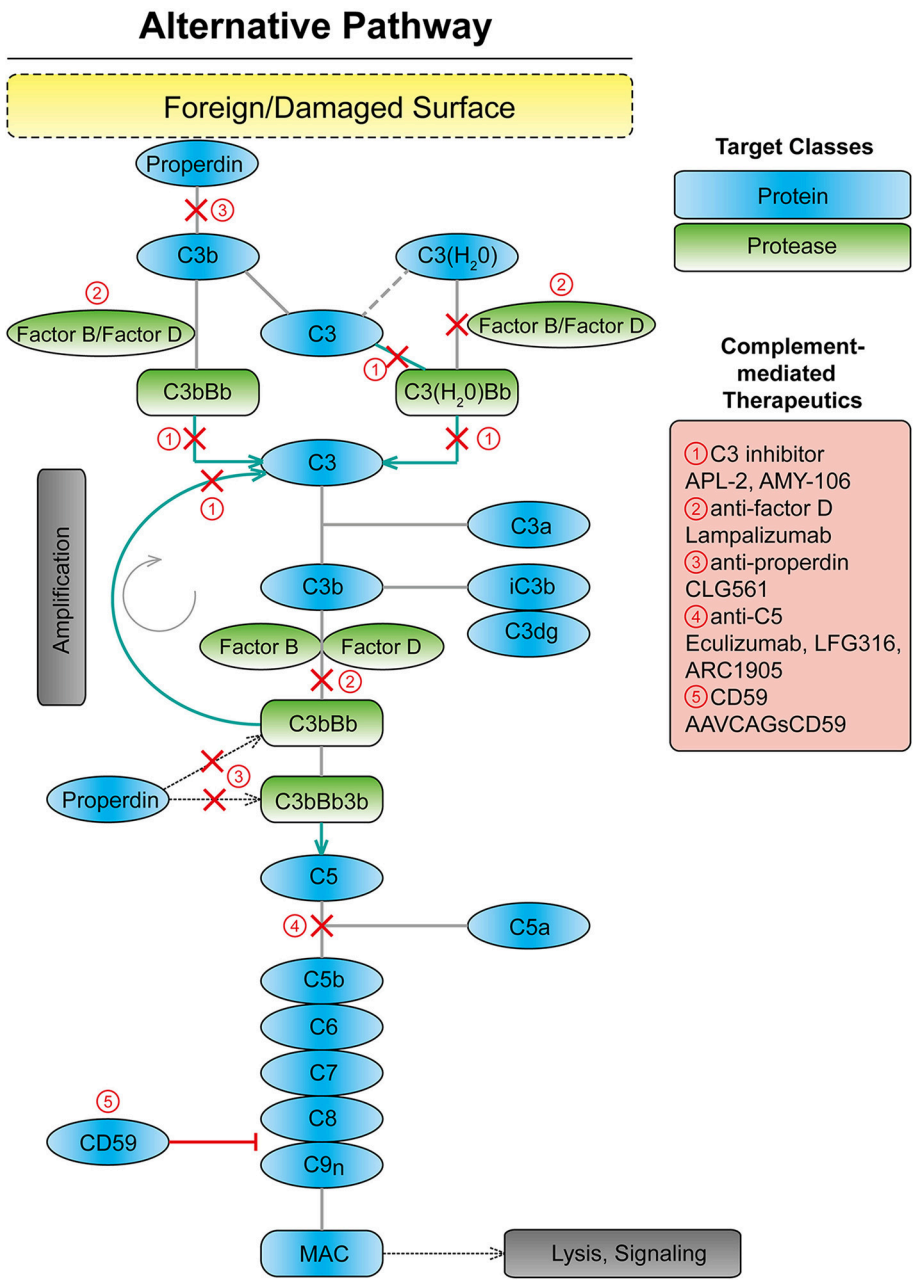

FIGURE 1 | Scheme showing the alternative complement system with targets for complement mediated therapeutics. MAC, membrane attack complex.

CHFR genes, rather than chromosome 10 (10q31) around the *ARMS2/HTRA1* genes (51). AMD patients with chromosome 1 mutations have significant complement over-activation in the extracellular matrix of the choriocapillaris, underlying Bruch's membrane. Complement activation and turnover have been identified in the choriocapillaris layer as well, and increased levels are found in individuals with AMD (52, 53). This is also found in donor eyes from individuals genetically at risk for AMD, but who do not yet have clinical manifestations of the disease (54). The activation of the alternative complement pathway is initiated by the formation of the C3bBb complex, also known as C3 convertase. Formation of this complex leads to the amplification of complement signaling and immune response. FH accelerates dissociation of this C3bBb complex, inhibiting alternative complement system activation. Furthermore, FH, as a cofactor, facilitates factor I-mediated C3b inactivation. FH is anchored to the extracellular matrix and the cell surface through interactions with glycosaminoglycans (GAGs) (55). The Y402H polymorphism does not alter the overall protein structure (56). However, the Y402H polymorphism disrupts binding of

TABLE 1 | Genes in the alternative complement pathway that are involved in age-related macular degeneration.

Complement	Gene location	Variants	Role in complement system	References
Complement factor H (FH)	1q31.3	Y402H (common variant) R1210C, R53C, and D90G (rare variants)	FH is an important regulator of the complement system. Dissociation of the C3Bb complex, known as C3 convertase, is accelerated by FH, thus alterations of FH can lead to abnormally increased complement activity. Furthermore, it acts as a cofactor for factor I.	Common variants (26–30) Rare variants (31–33)
Complement factor H-related (FHR)	1q31.3		Although the exact function is unclear, some of the FHR proteins may act as competitors for FH binding to various ligands and can help to form a novel C3 convertase by binding C3b.	(34–36)
C3	19p13.3	R80G, R102G (common variants) K155Q (rare variants)	C3 is the central component of all three complement systems	Common variants (37–39) Rare variants (40, 41)
Factor B	6p21.33	L9H, R32Q (common variants)	Factor B is cleaved by factor D in the presence of C3b, and its Bb fragment forms the C3bBb, C3 convertase, in the alternative system	Common variants (42–44)
Factor D	19p13.3	rs3826945 (common variants)	Factor D cleaves factor B within this complex into Ba and Bb fragments. This is a rate-limiting step in the formation of the C3bBb complex, which is crucial for the activation of the alternative complement system	Common variants (45)
Factor I	4q25	rs10033900 (common variant) G119R and G188A (rare variants)	This serine protease domain regulates the complement system by cleaving and inactivating C4b and C3b which is regulated by C4bp and FH, respectively	Common variants (41, 46) Rare variants (47)
C9	5p13.1	P167S, R95S (rare variants)	Encodes complement component 9, the final component of the complement cascade and component of the membrane attack complex (C5b-9).	Rare variants (41, 48)

the complement control region to GAG chains in the Bruch's membrane (55). Because the Y402H variant has decreased binding affinity to numerous components of the damaged retina (57–59), the inhibitory effect of FH on the complement system is thought to be decreased. This could result in poorly controlled complement turnover and excessive chronic local inflammation. The FH and factor H-like protein (FHL-1), components of the alternative system, are capable of suppressing complement activation on the extracellular matrix (51). The shortened splice variant of FH and FHL-1 appears to prevail in the ECM in/around Bruch's membrane (60–63). Because FHL-1 only has the one GAG-mediated anchoring site in its CCP7 domain which anchors FHL-1 to Bruch's membrane and the intercapillary septa, this GAG-binding site is affected by the Y402H polymorphism. In contrast, FH has two anchoring sites and is not particularly affected by the Y402H polymorphism (51). This may explain why the Y402H polymorphism has a disproportionate effect on protein anchoring via GAGs.

Other rare variants in *CFH* SNPs have also been reported to affect AMD, including the R1210C, R53C and D90G polymorphisms (31, 32). R1210C is extremely rare, with a minor allele frequency of 0.0173 %, but has an even stronger association with AMD than Y402H, potentially by acting as a functionally null allele (31). The highly penetrant R1210C variant is associated with a 6-year earlier onset of AMD with drusen phenotype (33). Advanced age and decreased FH induced sub-RPE deposit formation leading to complement activation, which contributed to RPE damage and visual function impairment (64).

A number of other SNPs further downstream on chromosome 1 are associated with AMD, suggesting involvement of the five factor H-related (FHR) proteins in disease pathogenesis (34). Although the exact function of FHR proteins is unclear at present, some of them may bind competitively to ligands involved in FH binding and may facilitate formation of a novel C3 convertase by binding C3b (65). Furthermore, a haplotype of complete deletion of the genes for *FHR-1* and *FHR-3* was associated with a decreased risk of AMD in human studies (34–36).

C3

C3 is the central component of all three complement systems, and functional changes in C3 directly affect the downstream

cascade (66). A common SNP, rs2230199 (R80G), is associated with increased risk of AMD (odds ratio 2.6) (37, 38). The R102G polymorphism of *C3* results in reduced FH binding to the 102G variant. As FH acts as a cofactor for factor I-mediated cleavage of C3b, R102G polymorphism of *C3* decreased the factor I-mediated cofactor activity. By extending the convertase lifetime, activation of the alternative complement system is enhanced (39). A single rare variant in *C3*, K155Q, is positively associated with AMD risk (odds ratio 3.8) (41). K155 in *C3* is in close proximity to the FH binding site (40). In surface plasmon resonance experiments, binding of this *C3* mutant to FH was reduced compared to wild type *C3*. The K155Q allele in *C3* results in resistance to proteolytic inactivation by FH and CFI. Taken together, these findings implicate that loss of C3 functionality contributes to AMD pathogenesis (41). Increased plasma complement protein is also associated with advanced stages of AMD, further supporting the hypothesis that complement activation may be correlated with disease progression as well (67, 68). C3a was significantly increased in AMD patients, ranging from 4.6 to 87.2 % (69). Furthermore, C3a and C5a are markers of acute activation of the alternative complement system. Thus, the plasma concentrations of activation peptides including C3a and C5a are positively correlated biomarkers of AMD (69).

Factor B

Both haplotypes, L9H variant of *factor B* and the E318D variant in *C2*, as well as a variant in intron 10 of *C2* and the R32Q variant of *factor B*, are considered highly protective against AMD development (odds ratio 0.45 and 0.36, respectively), although the protection is likely mediated by *factor B* mutations (42, 43). Factor B fragments are found in the drusen at similar levels to that of factor H, and convertase formation, which strengthens complement activation, is decreased by the R32Q mutation (44). Furthermore, studies in mouse models of complement dysregulation support a causative role for the complement cascade in multiple retinal pathologies. Factor B is up-regulated in the retinal detachment mouse model, and in the vitreous from patients with retinal detachment (70). In addition, *factor B* knockout mice are protected from retinal detachment associated photoreceptor death which further emphasizes a role for this system in photoreceptor degeneration.

Factor D

Factor D is one of the serine proteases that regulates the activation of the alternative complement system (71). Factor D becomes transiently active and is able to cleave factor B within this complex into Ba and Bb fragments. This is a rate limiting step in the formation of C3bBb which strengthens the signal and consequently activates the complement system (21, 71). A small case-control series showed that *factor D* gene SNP rs3826945 is positively associated with AMD risk (odds ratio 1.44) (45).

Factor I

The *factor I* gene spans 63 kb and contains 13 exons, the first 8 of which encode the heavy chain and the last 5 the light chain, which contains a serine protease domain (72). The serine protease domain is responsible for cleaving and inactivating C4b and C3b (73). Factor I-mediated C3b inactivation is assisted by FH. The FH acts as a cofactor for factor I-mediated cleavage of C3b (74), and thus accelerating breakdown of the alternative system C3 convertase, C3bBb. A common polymorphism near the *factor I* gene, rs10033900, has been described (46). A cohort study of 2,493 advanced AMD cases and controls showed that 7.8% of AMD cases compared to 2.3% of controls are carriers of rare missense *factor I* variants (odds ratio 3.6) (41). Another cohort study reported that two variants of *factor I*, G119R and G188A remained significant (47). The rare *factor I* G119R variant conferred a particularly high risk of AMD (odds ratio 22.2). Functional studies on the G119R variant showed that the secretion of the mutant protein was lower than that of the wild type protein, which led to a decrease in factor I-mediated cleavage of C3b.

Complement 9

Complement component 9 (C9) is the most downstream component of the terminal complement complex cascade, acting as the terminal effector molecule of all three complement activation systems. Because the ultrastructure terminal complement complex is partially dependent on the amount of C9 molecules relative to C5b-8 molecules, a significant reduction in C9 can alter the terminal complement complex stoichiometry, reducing cytolytic activity (75, 76). Genotyping in 5,115 independent samples confirmed associations to AMD with a P167S allele in *C9* (41). When compared to the controls, a two-fold increase was observed for this variant in the AMD cases. Another R95X variant was negatively associated with AMD risk in a small study (48), but has not been confirmed. Together, these findings suggest that the terminal complement pathway has a potential role in AMD pathogenesis.

THERAPEUTIC APPROACHES AND CLINICAL TRIALS USING COMPLEMENT-MEDIATED DRUGS

In addition to strong genetic and basic research studies supporting a pathological role for the complement system in AMD, many clinical trials for therapeutics targeting alternative complement cascade are at various stages or completed (**Table 2**). Clinical trials for eight drugs that target the alternative complement cascade include two targeting C3, one targeting factor D, one targeting properdin, three targeting C5, and one targeting CD59.

C3-Mediated Therapeutics

Inhibiting the C3 protein, including the C3 convertase, may be a desirable therapeutic approach. C3 modulates amplification of all initiation pathways, the generation of anaphylatoxins (C3a, C5a), and the membrane attack complex (MAC), while C3 inhibition attenuates these events. C3 functions as a central hub that mediates and controls the upstream activation and downstream effector functions of the complement cascade. Initial attempts in developing small molecules that inhibit conversion of C3 failed due to lack of potency and specificity (85).

TABLE 2 | Complement-mediated therapeutics in clinical trials for the treatment of age-related macular degeneration.

Target	Drug (Company)	Class	Phase (Name) trial number	Route of administration	Indication	Design	Trial status and primary outcomes
C3	POT-4/AL78898A (Potentia/Alcon)	Peptide	1 (AsAP) NCT00473928	IVT	nAMD	SAD, $n = 27$	Completed, no safety concerns (77)
			2 (RACE) NCT01157065	IVT	nAMD	Single dose POT-4 (50 μL) vs. ranibizumab (50 μL), $n = 49$	Completed, POT-4 group did not show a reduction in the central subfield retinal thickness from baseline.
			2 NCT01603043	IVT	GA	Monthly POT4 vs. Sham, $n = 10$, 1 year	Terminated
C3	APL-2 (Apellis)	Peptide-PEGylated	1 NCT02461771	IVT	nAMD	SAD	Completed, unpublished
			2 (FILLY) NCT02503332	IVT	GA	Monthly vs. every other month vs. Sham, $n = 246$, 18 months	29% significant reduction in GA growth at 12 months in the monthly injection group
			3 NCT03525613	IVT	GA	Monthly vs. every other month vs. Sham, $n = 600$, 30 months	Recruiting
			1b/2 NCT03465709	IVT	nAMD	Target $n = 17$, 18 months	Recruiting
Factor D	Lampalizumab (Roche)	Fab	1a NCT00973011	IVT	GA	SAD	No safety concerns (78)
			2 (MAHALO) NCT01229215	IVT	GA	Monthly vs. every other month vs. Sham, $n = 123$	There was a trend for the reduction of GA progression by 20% in the monthly group (79)
			3 (CHROMA, SPECTRI) NCT02247479 NCT02247531	IVT	GA	Duplicate trials, $n = 906$ (CHROMA) and $n = 975$ (SPECTRI), every 4 weeks vs. every 6 weeks vs. Sham for 96 weeks	No treatment benefits compared to the sham group in both trials (80)
			2 NCT02288559	IVT	GA	Every 2 weeks vs. every 4 weeks vs. Sham, target $n = 99$, 6 months	Completed, unpublished
Properdin	CLG561 (Novartis)	Monoclonal Ab	2 NCT02515942	IVT	GA	CLG561 vs. CLG561+LFG316 vs. Sham, 12 monthly injections, $n = 114$	Completed, unpublished
C5	Eculizumab (Alexion)	Monoclonal Ab	2 (COMPLETE) NCT00935883	IV	GA, drusen	Low dose (600 mg) weekly for 4 weeks followed by 900 mg every 2 weeks vs. high dose (900 mg) weekly for 4 weeks followed by 1,200 mg every 2 weeks for 24 weeks, then FU 6 months, $n = 30$ GA (COMPLETE) $n = 30$ drusen	Eculizumab was well-tolerated through 6 months but did not decrease the growth rate of GA significantly at 6 or 12 months (81). No reduction of the drusen volume at 6 months (82)
C5	LFG316 (Novartis)	Monoclonal Ab	1 NCT01255462	IVT	GA or nAMD	SAD, $n = 24$	Completed, unpublished
			2 NCT01527500	IVT	GA	Low dose (5 mg/50 μL) vs. Sham, monthly for 1 year, $n = 150$	No reduction of the GA lesion in the treatment group compared to the sham group
			2 NCT01535950	IVT	nAMD	Active vs. Sham, 113 days $n = 43$	Completed, unpublished
			2 NCT01624636	IV	nAMD	Placebo vs. 2 doses of LFG316, 113 days	Terminated, unpublished
C5	ARC1905 (Ophthotech)	Aptamer	1 NCT00950638	IVT	GA	Dose 1 (0.3 mg) vs. Dose 2 (1 mg), $n = 47$, 1 year	Completed, unpublished

(Continued)

TABLE 2 | Continued

Target	Drug (Company)	Class	Phase (Name) trial number	Route of administration	Indication	Design	Trial status and primary outcomes
			1 NCT00709527	IVT	nAMD	6 monthly ARC1905 (0.3, 1, or 2 mg) in combination with ranibizumab (0.5 mg), $n = 43$, 2 years	Completed, well-tolerated without evidence of acute toxicity (83)
			2b NCT02686658	IVT	GA	Dose 1 vs. Dose 2 vs. Sham, 12 months, $n = 200$	Recruiting
			2a NCT03362190	IVT	nAMD	ARC1905 Dose 1 vs. Dose 2 vs. Dose 3 vs. Dose 4 in combination with ranibizumab 0.5 mg, $n = 64$	Generally well tolerated for 6 months (84)
			2a NCT03374670	IVT	PCV	ARC1905 Dose 1 vs. Dose 2 in combination with aflibercept 2 mg, $n = 20$	Recruiting
			2b NCT03364153	IVT	STGD1	ARC1905 vs. Sham, $n = 120$	Recruiting
CD59	AAVCAGsCD59 (Hemera)	Virus	1 NCT03144999	IVT	GA	SAD (3 dose levels, expansion), $n = 17$	Recruiting

Ab, antibody; GA, geographic atrophy; IV, intravenous; IVT, intravitreal; nAMD, neovascular age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; SAD, single ascending dose; STGD1, autosomal recessive Stargardt disease 1.

In contrast, the peptidic inhibitor compstatin exerts its function via direct binding to native C3. Compstatin was discovered by screening phage-display libraries searching for C3b-binding peptides (86). Compstatin is a 13 amino acid cyclic peptide that inhibits complement activation by binding C3 and interfering with convertase formation and C3 cleavage (and the subsequent C3b opsonization, amplification, and generation of effectors) (85). Thus, compstatin could be a potent inhibitor, blocking all three complement systems (87).

POT-4 (Potentia Pharmaceuticals, Crestwood, KY), a compstatin derivative, is the first complement-specific drug that has entered clinical trials for the indication of AMD. A phase 1 clinical trial (AsAP, NCT00473928) using intravitreal POT-4 delivery was conducted in patients with neovascular AMD, and suggested that there were no safety concerns (77). Following that, a double-masked, randomized, multicenter phase 2 clinical trial for patients with neovascular AMD (RACE, NCT01157065) was performed. Forty-nine patients were randomized to receive either single POT-4 (50 μL) or single ranibizumab (Lucentis®, Roche AG, Basel, Switzerland) (50 μL) injections, and were followed up for 12 weeks. The primary outcome was a mean reduction from baseline in the central subfield retinal thickness at week 4. Compared to the ranibizumab group (199.9 μm), the POT-4 group showed no benefit (-12.1 μm) in the reduction of macular edema (88). For the indication of GA, a phase 2 clinical trial with 10 patients (NCT01603043) was terminated before completion, and product deposits was reported in four cases among seven patients (57.4%).

The observation of product precipitation, led to an attempt to increase its solubility by pegylation of POT-4 with a 40,000 Da PEG (termed APL-2, Apellis Pharmaceuticals Inc., Crestwood, KY); APL-2 is currently under clinical trials for patients with GA and neovascular AMD. A phase 2 clinical trial (FILLY, NCT02503332) evaluated the efficacy of APL-2 by comparing three groups: a monthly intravitreal APL-2 injection (15 mg/0.1 mL) group, every other month APL-2 injection (15 mg/0.1 mL) group, and the sham group. The monthly intravitreal APL-2 injection group ($n = 84$) showed a 29% significant reduction in GA growth at 12 months compared to the sham group ($n = 80$) (89). The every other month APL-2 injection group ($n = 78$) showed a 20% reduction in GA growth, though this decrease was not statistically significant. Best-corrected visual acuity (BCVA) did not differ among the three groups. Of concern was that individuals receiving APL-2 had an 8 and 18% conversion rate to neovascular AMD in the every other month and monthly injection groups, respectively (90). Conversion to neovascular AMD appeared to be dose dependent based on monthly and bi-monthly injections. A possible explanation is that PEG can induce CNV in a mouse model by upregulating complement proteins in the RPE-choroid tissue or that a complement inhibition-mediated shift of pro-inflammatory M1 to pro-angiogenic M2 macrophages as part of the repair process, may lead to small exudations (91, 92). Endophthalmitis occurred in two out of 86 patients (1.3%) and in one out of 79 patients (2.3%) in the every other month and monthly injection groups, respectively (93). At present, a new 30-month, Phase 3, multicenter clinical trial for GA is recruiting subjects (NCT03525613) (94). Patients will be randomized to monthly and every other month injections for both APL-2 (15 mg/0.1 mL) and sham groups. Additionally, a phase 2 clinical trial for patients with neovascular AMD has been initiated, and is currently recruiting subjects (NCT03465709) (95).

In the meantime, a novel non-pegylated C3 inhibitor is under development (termed AMY-106, Amyndas Pharmaceuticals) and has shown prolonged residence in ocular tissues at C3-saturating

levels, extending over 3 months after a single intravitreal injection in cynomolgus monkeys (96). The increased bioavailability of this C3 inhibitor, indicates its clinical potential for ocular indications associated with C3 convertase dysregulation (e.g., AMD).

Factor D-Mediated Therapeutics

FCFD4514S (Lampalizumab)

As a rate limiting enzyme, factor D cleaves factor B to generate C3 convertase and is a pivotal activator of the alternative complement system (21, 97). FCFD4514S (lampalizumab, Roche AG, Basel, Switzerland), a Fab fragment of humanized IgG murine anti-factor D antibody, was developed to block factor D-mediated formation of the C3 convertase (98). A phase 1 trial (NCT00973011) of intravitreal lampalizumab injection in patients with GA found that this modality is safe and well-tolerated, and there were no adverse events (78). This was followed by the phase 2 MAHALO study. "The phase 2 clinical trial (MAHALO, NCT01229215) was a multicenter, randomized, controlled study that evaluated intravitreal delivery of lampalizumab (10 mg/100 μL) administered monthly ($n = 42$) and every other month ($n = 41$) vs. sham control ($n = 40$) in patients with GA. The primary endpoint was the mean change in the lesion area from baseline to month 18 as measured by fundus autofluorescence (79)." The results showed a trend for a reduction in GA progression of 20% in the lampalizumab group, but there was no significant difference between the lampalizumab and sham groups (79). Interestingly, in a subgroup of *factor I* risk-allele carriers (57% of the patients), monthly treatment showed a 44% reduction in GA area progression compared to the sham control (95% CI, 15 to 73%). "The MAHALO study demonstrated an acceptable safety profile during the 18-month treatment period. The MAHALO study concluded that modulation of the complement system can affect the progression of GA, which is supported by human genetic studies for which various genetic variants in the alternative complement system increase the AMD risk (79)."

However, the subsequent following phase 3 trials showed conflicting results. Two similarly designed phase 3 double-masked, randomized, sham-controlled clinical trials (CHROMA, NCT02247479 and SPECTRI, NCT02247531) enrolled participants at 275 sites in 23 countries (99, 100). "Participants were aged 50 years or older, with bilateral GA and no prior or active CNV in either eye. Participants were randomized 2:1:2:1 to receive 10 mg of intravitreal lampalizumab every 4 weeks, a sham procedure every 4 weeks, 10 mg of lampalizumab every 6 weeks, or a sham procedure every 6 weeks, through 96 weeks. The primary endpoint was the mean change in the GA area at 48 weeks by fundus autofluorescence (80)." In contrast to the favorable results from the phase 2 study, the phase 3 study CHROMA ($n = 906$) showed both dose arms of the monthly and every 6-week injections did not show any significant efficacy compared to the sham group (80). The SPECTRI study ($n = 975$) also demonstrated no treatment benefit of lampalizumab. No benefit of lampalizumab was observed across prespecified subgroups, including by factor I, noted in the earlier phase 2 study. It was noted that development of new CNV in patients with bilateral GA occurred in <2% of the study or fellow eyes. This finding is consistent with observational studies, which reported conversion rates of 2% at 2 years and 11% at 4 years in patients with bilateral GA and no baseline CNV (101). CHROMA and SPECTRI, which are the largest GA complement studies conducted to date, concluded that lampalizumab did not reduce GA enlargement vs. sham over 48 weeks of treatment. The limitation of these studies comes from the selection criteria of enrolled patients which excluded patients with smaller or larger lesions, unilateral GA, autofluorescence patterns except for banded or diffuse, current or prior CNV, GA from causes other than AMD, or earlier stages. Recent diffusion studies with enriched Bruch's membrane from human donor eyes over a wide age-range demonstrated that there is distinct selectivity in the permeability of Bruch's membrane to complement proteins with size and glycosylation being the important determinants (102). Factor D can penetrate Bruch's membrane suggesting a possible reason as to why the clinical trials with lampalizumab may have failed. Furthermore, an *in vivo* study with mice reported that the absence of both factor H and factor D pushes the complement system identifying a factor D bypass mechanism that is likely always present but only clinically germane in association with factor H dysfunction (103).

Properdin-Mediated Therapeutics

C3 convertase (C3bBb) is unstable, and is stabilized by the binding of properdin. Stabilized C3bBb can cleave more C3, thus generating a feedback loop (17, 19). Therefore, it is hypothesized that use of an anti-properdin antibody should destabilize the C3 convertase and block the feedback loop. An anti-properdin monoclonal antibody (CLG561, Novartis International AG, Basel, Switzerland) was developed by Novartis, and a phase 2 clinical trial (NCT02515942) was completed. The purpose of that study was to evaluate the safety and efficacy of 12 (every 28 days) intravitreal injections of CLG561 as a monotherapy and as a combination therapy with LFG316 (anti-C5, discussed below) compared to the sham in subjects with GA. However, to date the results remain unpublished (104).

C5-Mediated Therapeutics

Cleavage of C5 generates C5a and the initiation of MAC, which are key mediators of the terminal complement pathway and complement activation (18). C5a is immunomodulatory, while the MAC initiates cell lysis. Histopathologic specimens of human dry AMD lesions strongly stain for C5 and MAC at the key sites of pathology (24). C5 inhibitors do not affect the formation of upstream complement components such as C3b, which are important in host defense mechanisms. It was suggested that by inhibiting C5-mediated inflammatory and MAC activities, a therapeutic benefit may be achieved in both dry and wet AMD while sparing the immunoprotective functions of the complement system, however, this has not yet been proven in the clinic (105). Three anti-C5 antibodies are in clinical trials: eculizumab (Soliris®; Alexion Pharmaceuticals, Cheshire, CT,

US), LFG316 (Novartis Pharma AG, Basel, Switzerland), and ARC1905 (Zimura®, Ophthotech Corp., Princeton, NJ, US).

Eculizumab

Eculizumab is a humanized monoclonal antibody derived from the murine anti-human C5 antibody m5G1. This antibody specifically binds the terminal complement protein C5, thereby inhibiting cleavage to C5a and C5b during complement activation and preventing MAC formation. The United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved eculizumab for the treatment of two rare genetic deficiencies of complement inhibition, atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria (106). Recently, the FDA/EMA approved eculizumab for the treatment of adult patients with generalized myasthenia gravis, who are anti-acetyl-choline receptor antibody positive (107).

A phase 2 clinical trial (COMPLETE, NCT00935883) was the first prospective, randomized, placebo-controlled investigation of complement inhibition for the treatment of AMD (108). This trial was conducted to evaluate the effect of intravenous injection of eculizumab, a systemic C5 inhibitor, on the expansion of GA in patients with AMD (81). "Patients were randomized 2:1 to receive intravenous eculizumab or placebo over 6 months. In the eculizumab treatment arm, the first 10 patients received a low-dose regimen of 600 mg weekly for 4 weeks followed by 900 mg every 2 weeks until week 24, and the next 10 patients received a high-dose regimen of 900 mg weekly for 4 weeks followed by 1200 mg every 2 weeks until week 24. At 26 weeks, the GA area increased by 0.19 ± 0.12 and 0.18 ± 0.15 mm in the eculizumab and placebo groups, respectively ($P = 0.96$). At 52 weeks, the GA area increased by 0.37 ± 0.22 mm in the eculizumab group and by 0.37 ± 0.21 mm in the placebo group ($P = 0.93$). None of the eyes converted to neovascular AMD (81)." The lack of any trend toward therapeutic efficacy with eculizumab led the study authors to conclude that eculizumab was well-tolerated through 6 months but did not decrease the growth rate of GA significantly. Furthermore, the effect of eculizumab treatment on the drusen volume was also evaluated (82). The mean drusen cube root volumes were 0.49 and 0.47 mm ($P = 0.64$) at baseline and 0.51 and 0.42 mm ($P = 0.17$) at 26 weeks in the eculizumab and placebo groups, respectively, which showed no beneficial effect of eculizumab in reducing the drusen volume compared to the placebo.

There are several limitations in the COMPLETE clinical trial, including the route of administration. The rationale for using a systemic drug in that clinical trial was based on the belief that complement activation in the choroid played an important role in the progression of GA. Furthermore, eculizumab already has been approved for systemic delivery for patients with paroxysmal nocturnal hemoglobinuria (109) and atypical hemolytic uremic syndrome (110); thus, systemic delivery of eculizumab was possible without additional safety studies. Although systemic complement inhibitors are successfully used for other systemic diseases including paroxysmal nocturnal hemoglobinuria, the amount of drug delivered systemically could be inadequate to penetrate the blood retinal barrier and affect the pathological progression of GA. Another possible explanation could be that the clinical trials focused on the wrong targets. Eculizumab for GA is no longer listed among Alexion's pipeline therapeutics (111).

LFG316

LFG316 is a human monoclonal C5 antibody. A phase 2 clinical trial (NCT01535950) to evaluate the efficacy of intravitreal LFG316 in patients with neovascular AMD was completed though the results remain unpublished (112). Another phase 2 clinical trial (NCT01527500) for 150 patients with GA was completed. However, the intravitreal LFG316 treatment group (5 mg/50 μL) did not have reduced GA lesions compared to the sham group over 1 year of follow-up (113). A phase 2 clinical trial (NCT01624636) evaluating the safety and tolerability of intravenous LFG316 injection was terminated before completion (114).

ARC1905

ARC1905 is an intravitreal anti-C5 aptamer. Results of the phase 1 study (NCT00709527) were presented at the Association for Research in Vision and Ophthalmology 2010 Annual Meeting (83). "Forty-three patients with subfoveal neovascular AMD received six monthly administrations of ARC1905 (0.3, 1, or 2 mg) in combination with ranibizumab (0.5 mg). The BCVA in the study eye was 20/63 to 20/200. It did not show any dose-limiting toxicity during 6 months. The mean change in BCVA at week 24 was an increase of +13.6, +11.7, and +15.3 letters at the doses of 0.3, 1, and 2 mg, respectively. Furthermore, 46%, 47%, and 60% of patients gained 3 or more lines of visual acuity at the doses of 0.3, 1, and 2 mg, respectively. The mean change in center point thickness by optical coherence tomography was−150 μm." The phase 1 study showed that the combined therapy of C5 and VEGF inhibition was well-tolerated without any toxicity issue. An additional phase 1 trial (NCT00950638), which was conducted to evaluate the safety and tolerability of ARC1905 in patients with GA, was completed though the results have not been published (115).

A phase 2 clinical trial for patients with GA started recruiting (NCT02686658) (116). Furthermore, 64 patients with neovascular AMD were enrolled in the randomized, dose-ranging, open-label, multicenter phase 2a safety trial (NCT03362190) of ARC1905 in combination with ranibizumab (117). Ophthotech announced that after 6 months of treatment, the ARC1905 combination therapy was generally well-tolerated in neovascular AMD. It is interesting that though this clinical trial, with a small sample size, was not designed to detect a significant difference in efficacy, 60% of patients who had received monthly ARC1905 (2 mg) in combination with ranibizumab (0.5 mg) gained greater than or equal to three lines of vision, or 15 Early Treatment of Diabetic Retinopathy Study letters, defined as a significant visual gain (84).

A phase 2a open-label trial to assess the safety of ARC1905 in combination with aflibercept (Eylea®, Bayer AG, Leverkusen, Germany) 2 mg, in patients with idiopathic polypoidal choroidal

vasculopathy has started recruiting (NCT03374670) (118). Furthermore, a phase 2b randomized, double-masked, controlled trial to evaluate the safety and efficacy of ARC1905 compared to sham in subjects with autosomal recessive Stargardt disease 1 is recruiting (NCT03364153) (119).

CD59-Mediated Therapeutics

Soluble and cell-bound regulators of complement including CD59 help to protect healthy host tissue from self-recognition and serve to prevent activation of a complement response (17). However, damaged or diseased host cells can down-regulate membrane-bound complement inhibitors which enables targeted clearance. An imbalance between complement recognition and initiation on healthy host cells can lead to unregulated complement activation, opsonization, and/or subsequent cellular damage. Moreover, accumulation of MAC on cell surfaces leads to cell damage and death, associated with several clinical findings observed in AMD. CD59 functions by binding the C5b678 terminal complement protein complex and preventing the incorporation of the multiple C9 molecules required to complete the formation of a pore in the cell membrane. Normal cells within the human body produce a surface protein, CD59, which blocks formation of the MAC (22, 23). In mouse models with retinal pathologies including retinal detachment (70) and oxygen-induced retinopathy (120, 121), *CD59* expression was suppressed compared to the control mice. The above animal studies showed that the presence of CD59 can have a dichotomous role. *Cd59* down-regulation can either be protective in pathologies involving dividing cells (e.g., vascular endothelial cells in neovascular disease), or can lead to neurodegeneration when *Cd59* down-regulation enables complement to target non-dividing cells of the central nervous system (e.g., photoreceptor cell). In AMD, the complement cascade is thought to be upregulated and it has been postulated that targeting MAC formation can protect against self-cell death. AAVCAGsCD59 (Hemera, QC, Canada), an ocular gene therapy product that is delivered intravitreally, causes normal retinal cells to increase the expression of a soluble form of CD59 (sCD59). This soluble recombinant CD59 is designed to protect retinal cells by inhibiting MAC formation. Adeno-associated virus (AAV) serotype 2 was used because it has been shown to be safe for use in humans and is generally considered less immunogenic than adenovirus vectors (122). A phase 1 trial (NCT03144999) was initiated to evaluate drug safety after a single injection of AAVCAGsCD59 administered in an office setting for patients with GA (123). Regarding the frequency of injections, the biggest advantage of this gene therapeutic approach is that it requires only one injection compared to other drugs with monthly or bimonthly intravitreal injections. This could be beneficial in minimizing potential endophthalmitis, which is a risk that can arise from multiple injections.

TREATMENT CHALLENGES

Vascular endothelial growth factor (VEGF) drives ocular neovascularization, and anti-VEGF therapies are highly effective in the treatment of neovascular AMD. Although these drugs are highly effective, they require frequent intraocular injections, and are costly, reducing patient compliance. Furthermore, anti-VEGF therapies are not effective in treating GA.

A significant body of work in animal models, genetic studies and clinical trials suggests an important but complex role for the complement system in AMD, including GA. However, therapies targeting the alternative complement cascade have thus far had only modest therapeutic effects in GA and neovascular AMD. The reasons for these unexpected outcomes have not been fully elucidated, but are likely due the disease stages treated, and in some cases, insufficient drug delivery. The complement system may be more relevant in the earlier stages of the disease, before clinical pathologies such as GA, CNV and decreased visual acuity develop. Drug delivery may also have been problematic in some studies such as intravenous eculizumab injection in the COMPLETE study. Intravitreal injections may be the most popular, but there are issues with continued dosing and getting to the right locations within the eye (i.e., monthly injections). Gene therapy has made great strides recently, and sub-retinal injections of AAV-delivered therapeutics are now FDA approved for other ocular indications such as inherited retinal dystrophy (i.e., Voretigene neparvovec, Luxterna, Spark Therapeutics Inc.) (124), which could show the beneficial mode of delivery of the future complement therapeutics. In addition, an emerging hypothesis points toward a dual role for complement in the progression of age-related and degenerative diseases, which are often driven by accumulating debris (125). However, a previous study using a primate model with early-onset macular degeneration, which develop drusen in <2 years after birth, showed that intravitreal C3 inhibitor compstatin injection for 6 months resulted in drusen disappearance (126). The "fitness" of the cascade, largely defined by the complotype of polymorphisms/mutations in complement genes (127), is likely of high importance in these chronic, slowly developing disorders.

CONCLUSION

Abundant evidence from pathological as well as genetic studies has contributed to a breakthrough in our understanding of the role of the complement system in the pathogenesis of AMD. Thus, local inhibition of complement activation has been considered a promising approach for treating both forms of late AMD. In light of the probable role of the complement system in development of AMD, many clinical trials investigating the effect of complement inhibitors have been conducted or are in progress. The results of clinical trials, in which often only a subgroup of patients responded favorably, has shown that careful stratification of indications and patient cohorts will be critical to identify patients that may benefit from complement-mediated therapies. In this aspect, genetic and/or clinical diagnostic tools will be important. Continued research, including studies on the initial

development of late AMD and the subsequent impairment of visual function, will be crucial to further understand the complement pathophysiology in AMD, and to identify additional potential therapeutic targets for complement modulation. Furthermore, the conceptual diversity of complement-mediated therapeutics could allow for accessible indications, treatment options, costs, and clinical availability. Thus, the results of ongoing clinical trials are eagerly awaited, with the hope of developing additional therapeutic modalities for this increasingly common malady.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: JL declares that he is the founder of Amyndas Pharmaceuticals, is named as an inventor on patents or patent applications describing the therapeutic use of complement inhibitors (some of which are being developed by Amyndas Pharmaceuticals) and is the inventor of the compstatin analog licensed to Apellis Pharmaceuticals termed 4(1MeW)7 W (also known as POT-4 and APL-1) and pegylated derivatives such as APL-2.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Anti-complement Treatment for Paroxysmal Nocturnal Hemoglobinuria: Time for Proximal Complement Inhibition? A Position Paper From the SAAWP of the EBMT

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The treatment of paroxysmal nocturnal hemoglobinuria has been revolutionized by the introduction of the anti-C5 agent eculizumab; however, eculizumab is not the cure for Paroxysmal nocturnal hemoglobinuria (PNH), and room for improvement remains. Indeed, the hematological benefit during eculizumab treatment for PNH is very heterogeneous among patients, and different response categories can be identified. Complete normalization of hemoglobin (complete and major hematological response), is seen in no more than one third of patients, while the remaining continue to experience some degree of anemia (good and partial hematological responses), in some cases requiring regular red blood cell transfusions (minor hematological response). Different factors contribute to residual anemia during eculizumab treatment: underlying bone marrow dysfunction, residual intravascular hemolysis and the emergence of C3-mediated extravascular hemolysis. These two latter pathogenic mechanisms are the target of novel strategies of anti-complement treatments, which can be split into terminal and proximal complement inhibitors. Many novel terminal complement inhibitors are now in clinical development: they all target C5 (as eculizumab), potentially paralleling the efficacy and safety profile of eculizumab. Possible advantages over eculizumab are long-lasting activity and subcutaneous self-administration. However, novel anti-C5 agents do not improve hematological response to eculizumab, even if some seem associated with a lower risk of breakthrough hemolysis caused by pharmacokinetic reasons (it remains unclear whether more effective inhibition of C5 is possible and clinically beneficial). Indeed, proximal inhibitors are designed to interfere with early phases of

complement activation, eventually preventing C3-mediated extravascular hemolysis in addition to intravascular hemolysis. At the moment there are three strategies of proximal complement inhibition: anti-C3 agents, anti-factor D agents and anti-factor B agents. These agents are available either subcutaneously or orally, and have been investigated in monotherapy or in association with eculizumab in PNH patients. Preliminary data clearly demonstrate that proximal complement inhibition is pharmacologically feasible and apparently safe, and may drastically improve the hematological response to complement inhibition in PNH. Indeed, we envision a new scenario of therapeutic complement inhibition, where proximal inhibitors (either anti-C3, anti-FD or anti-FB) may prove effective for the treatment of PNH, either in monotherapy or in combination with anti-C5 agents, eventually leading to drastic improvement of hematological response.

Keywords: paroxysmal nocturnal hemoglobinuria, intravascular hemolysis, extravascular hemolysis, complement inhibition, eculizumab, ravulizumab, compstatin

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematological disorder characterized by complement-mediated intravascular hemolysis, bone marrow failure, and severe thrombophilia (1). PNH is due to the expansion of hematopoietic stem cells (HSCs) bearing somatic loss-of-function mutations in the phosphatidylinositol N-acetylglucosaminyltransferase subunit A (*PIGA*) gene (2–4). The *PIGA* genetic lesion impairs the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor, and as a consequence all GPI-linked proteins are not expressed on affected HSC and their mature progeny blood cells (5–8). Among the missing GPI-linked proteins, the lack of the two complement inhibitors CD55 (9–11) and CD59 (12, 13) makes erythrocytes susceptible to complement lysis. However, the expansion of the mutated HSCs, which is essential to develop the disease, presumes a concomitant immune-mediated damage of normal hematopoiesis, from which *PIGA* mutated HSCs are spared (14–16). Treatment options for hemolytic PNH remained limited and often inadequate until eculizumab became available, a humanized monoclonal antibody (mAb) targeting the component 5 (C5) of the complement cascade (17). Indeed, by disabling the complement cascade at the level of the terminal complement step (i.e., membrane attack complex—MAC—formation) eculizumab prevents the lysis of PNH erythrocytes, which cannot properly curb complement activation on their surface (1). The efficacy of eculizumab in PNH patients was first demonstrated in a pilot study from the United Kingdom, which showed robust inhibition of complement-mediated intravascular hemolysis (18). Two subsequent large international phase III randomized studies demonstrated that eculizumab prevents intravascular hemolysis in PNH, eventually leading to hemoglobin stabilization, reduction/eradication of red blood cell transfusions, and resolution of most disease-related symptoms (19, 20). These data were confirmed in longer follow up analyses, which showed further hematological improvement on continuous maintenance treatment with eculizumab, with no safety concerns (21). Notably, eculizumab also reduced the thromboembolic risk (22), the most serious complication in PNH, thereby impacting on the disease course, morbidity and long-term survival. Indeed, with the caveat of the relatively short follow up, two independent studies have shown that PNH patients receiving continuous treatment with eculizumab have a 5 year survival >90% (23, 24). These survival rates appear superior to the rate reported on the natural history of PNH (25–27), elegantly shown in a retrospective comparison between eculizumab-treated patients and historical controls (24). Thus, after its approval in 2007, eculizumab is considered to this date the standard of care for PNH patients with hemolytic disease or thromboembolic complications. Despite the fact that eculizumab was a breakthrough therapy for PNH, recent efforts are aimed to further improve this current standard in PNH. In this manuscript, we review current gaps in anti-complement treatment for PNH, eventually setting the goals for future complement inhibitors in development for PNH.

HEMATOLOGICAL RESPONSE IN PNH DURING ECULIZUMAB

Anti-complement treatment with the anti-C5 monoclonal antibody eculizumab results in sustained inhibition of complement-mediated hemolysis in almost all PNH patients (19, 20); however, in the registration trials the endpoints were mostly set on transfusion independence and reduction of hemolysis, assessed by LDH. Although hemoglobin stabilization was achieved in most patients (including transfusion independent patients), many exhibited significant improvement in hemoglobin level but still remained variably anemic (19–21). However, well-defined response categories had not been established. In 2009, we empirically classified hematological response in PNH patients on eculizumab as follows: (i) optimal response (no transfusions, hemoglobin stable >11 g/dL); (ii) good response (no transfusion, hemoglobin ranging between 8 and 11 g/dL); (iii) partial response (still transfused, but with transfusion requirement reduced by at least 50%); (iv) minor response (transfusion requirement unchanged, or reduced by <50%) (28). In this study, we showed

TABLE 1 | Tentative classification of hematological response to anti-complement agents in PNH.

Response category	Red blood cell transfusions	Hemoglobin level	LDH level*‡	ARC*
Complete response	None	≥12 g/dL	≤1.5x ULN	***and*** ≤150,000/μL§
Major response	None	≥12 g/dL	>1.5x ULN	***or*** >150,000/μL§
Good response	None	≥10 and <12 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
Partial response	None or occasional (≤2 every 6 months)	≥8 and <10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
Minor response#	None or occasional (≤2 every 6 months) Regular (3–6 every 6 months) Reduction by ≥50%^	<8 g/dL <10 g/dL <10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°
No response#	Regular (>6 every 6 months)	<10 g/dL	A. ≤1.5x ULN B. >1.5x ULN	Rule out bone marrow failure°

*LDH, lactate dehydrogenase; ULN, upper limit of the normal; ARC: absolute reticulocyte count. *Response categories are mostly based on red blood cell transfusion and hemoglobin level, but LDH and ARC serve as ancillary indicators to discriminate between complete and major response, as well as within suboptimal response categories. ‡A. and B. indicate subcategories without or with residual significant intravascular hemolysis, respectively. §To rule out increased erythropoietic response to compensate ongoing hemolysis; the value of 150,000/μL is a tentative index based on 1.5x ULN (which in most laboratories is set at 100,000/μL). °To assess the relative contribution of the degree of bone marrow failure to any response less than complete: a value of ARC below 60,000/μl could be a tentative index to establish such a contribution; bone marrow investigation may be appropriate. ^For patients with previous transfusion history (with a pre-treatment follow up of at least 6 months). #For patients who do not accept red blood cell transfusions, minor response can be defined based on hemoglobin level ≥6 and <8 g/dL, and no response based on hemoglobin <6 g/dL. All hemoglobin, LDH and ARC values should be assessed based on the median value over a period of 6 months.*

that no more than one third of PNH patients on eculizumab achieved normal hemoglobin values, leading us to investigate possible explanations for this limited and less than anticipated hematological benefit. Ten years later, additional long-term data have confirmed that, despite of the overall sustained efficacy and improved survival under eculizumab treatment, hematological benefit from eculizumab can be variable (21, 23, 24); thus, in addition to transfusion independence, hemoglobin normalization appears to be a discrete endpoint which can be used to characterize hematological response. Thus, considering that nowadays most new PNH patients start anti-complement therapies before receiving many transfusions, the following response categories can be proposed (**Table 1**): (i) complete response (no transfusion with normal hemoglobin stable and no evidence of hemolysis); (ii) major response (no transfusion with normal hemoglobin, with evidence of intravascular or extravascular hemolysis); (iii) good response (no transfusion, with persistent chronic mild anemia or evidence of residual intravascular hemolysis); (iv) partial response (persistent chronic moderate anemia and/or occasional red blood cell transfusions); (v) minor response (regular red blood cell transfusions); (vi) no response (regular and frequent red blood cell transfusions). For PNH patients with documented history of regular blood cell transfusions before starting eculizumab, these two latter hematological response categories may also be defined based on the reduction of the transfusion burden: patients with reduction ≥50% may be classified as minor responders, whereas those with reduction <50% may be classified as non-responders. Patients with suboptimal hematological response can be further distinguished based on the evidence of persistent intravascular hemolysis (based on LDH ≤1.5 or >1.5 ULN). It is important to emphasize that no hematological response does not necessarily mean no clinical benefit from eculizumab (see the effect on thromboembolisms in PNH, eculizumab treatment section), or, in the future, to other anti-complement agents: it is a very useful tool to better understand the reasons underlying unsatisfactory hematological benefit, eventually driving therapeutic decisions (e.g., modified treatment schedules, or addition/switch to different inhibitors). Indeed, at the moment there is no clear evidence about the possible impact (if any) of a suboptimal hematological response to eculizumab on its prevention of thrombosis, and on its long-term survival benefit.

Intrinsic resistance to eculizumab has been reported, albeit very rare, and it is associated with inherited polymorphism of C5 which prevents eculizumab binding (29); but, in all other patients, eculizumab is biologically active and reduces intravascular hemolysis with unpredictable hematological benefits. There are several factors which contribute to such heterogeneity which are discussed herein (**Table 2**) (30). Bone marrow function is the most obvious contributor, since immune-mediated bone marrow failure is a key element of the pathophysiology of PNH (14). In this context, it is worth mentioning that impaired bone marrow function may become clinically meaningful even without overt aplastic anemia, given the lack of a compensatory increase in erythropoiesis with continuous hemolysis. Second is the efficacy of the inhibition of intravascular hemolysis; as discussed below, residual intravascular hemolysis is detectable in most PNH patients on eculizumab, and may become clinically relevant in specific conditions. And thirdly is the occurrence of C3-mediated extravascular hemolysis (28); this novel and unanticipated mechanism of hemolysis is mechanistically associated with anti-C5 therapies. Since many of these factors may contribute to the ultimate hematological response in PNH patients, it is essential that their contribution is adequately investigated during eculizumab therapy (31).

TABLE 2 | Reasons for inadequate hematological response to eculizumab and possible actions.

Reason	Cause	Prevalence	Mechanism	Clinical impact on hematological response	Corrective action
Intravascular hemolysis	Inherited C5 variants	Ultra-rare (<1%, usually in Japanese patients)	Intrinsic resistance due to impaired binding of eculizumab (and of ALXN1210)	Minimal (but very significant for the few patients for whom there is no available treatment)	Switch to other investigational agents (mostly alternative C5 inhibitors)
	Recurrent pharmacokinetic breakthrough	10–15% of patients	Inadequate plasma level of eculizumab	Significant	Decrease interval of dosing (10–12 days) or increase dose of eculizumab (1,200 mg), or consider novel investigational agents
	Sporadic pharmacodynamics breakthrough	May occur in any patients	Massive complement activation due to concomitant clinical events	Minimal	None (treat the underlying cause)
Extravascular hemolysis	C3-mediated extravascular hemolysis	25–50% of patients (even more considering subclinical events)	Persistent uncontrolled activation of proximal complement, leading to C3-fragment opsonization of PNH red blood cells and subsequent removal by professional hepato-splenic phagocytes	Very significant	Consider employing investigational proximal inhibitors of the complement
Bone marrow disorders	Bone marrow failure	10–35% (depending also on initial patient selection)	Inadequate production of red blood cells	Significant	Treat underlying aplastic anemia with either immunosuppression or bone marrow transplantation
	Clonal evolution to myeloid malignancies	1–5%	Additional stochastic somatic mutations	Relevant	Treat the myeloid malignancy

Thromboembolisms in PNH During Eculizumab Treatment

The clinical benefit of eculizumab in PNH goes beyond the inhibition of intravascular hemolysis and possible hemoglobin stabilization; indeed, another consequence of therapeutic complement blockade is the prevention of thromboembolism. In the registration trials, the rate of thromboembolism during eculizumab treatment was reduced by 85% as compared with the pretreatment rate in the same patients (from 7.37 to 1.07 events/100 patient-years) (22). This effect was demonstrated even in patients already on anti-thrombotic treatment (mostly patients with previous thromboembolic events, thus the population at the highest risk of new thromboembolisms), with rate of thromboembolism reduced from 10.61 to 0.62 events/100 patient-years with eculizumab treatment (22). Nevertheless, albeit rarely, thromboembolic events may appear even during eculizumab treatment (23, 24); in analogy with intravascular hemolysis, these events may be defined as *"breakthrough thromboembolisms."* It is not entirely clear how eculizumab mechanistically prevents thromboembolism in PNH (e.g., direct inhibition of complement-mediated activation on PNH platelets, or indirect effect due to reduced intravascular hemolysis). Thus, the possible relationship of breakthrough thromboembolisms with suboptimal complement blockade and/or residual intravascular hemolysis, as well as the contribution of non-PNH related factors, need to be assessed individually in each patient, and further mechanistic investigations would be welcome, including assessment of possible biomarkers of such a risk.

Bone Marrow Function in PNH

As stated earlier, PNH is not simply a hemolytic anemia; indeed, a bone marrow disorder is always assumed to allow for the expansion of *PIGA* mutated HSCs (14, 15), which may appear as immune-mediated aplastic anemia (AA) (16). About 40% of PNH patients develop meaningful AA during their disease course (27); but even in milder forms, immune-mediated bone marrow failure may contribute to cytopenias, including anemia. The treatment of AA in the context of PNH is out of the scope of this review and it will not be discussed in detail; however, two important points are worth highlighting. First, the presence of a PNH clone in the context of severe AA (SAA) does not change the management of SAA: patients younger than 40 years with a matched related donor should proceed to bone marrow transplantation (BMT) (32), whereas immunosuppression (horse anti-thymocyte globulin and cyclosporine) is the preferred first-line treatment for patients older than 40 years, or lacking a matched related donor (33, 34). The addition of the thrombopoietin-mimetic agent eltrombopag in combination with standard immunosuppression seems very promising and has been approved in the U.S. as first-line (35). Currently underway is a phase III randomized study conducted by the Severe Aplastic Anemia Working Party of the EBMT comparing horse anti-thymocyte globulin and cyclosporine ± eltrombopag (36). Second, more severe forms of AA in most cases represent a contraindication to anti-complement treatment, which does not reverse the marrow failure component and should be reserved for patients with more adequate bone marrow function (i.e., no severe neutropenia or thrombocytopenia, and compensatory

reticulocytosis adequate to hemoglobin levels). Nevertheless, sometimes clinically significant hemolytic anemia and bone marrow failure may appear concomitantly in the same patient, or, more commonly, may develop at different times during the disease course. In these circumstances, the indication for anti-complement treatment should be evaluated individually, since in selected cases complement inhibition can be clinically effective in AA-PNH and could be used concomitantly or sequentially to standard immunosuppression (eculizumab does not worsen other cytopenias) (24). Thus, continuous evaluation of bone marrow function is mandatory in all hemolytic PNH patients receiving anti-complement treatment. In addition, similarly to AA and other bone marrow failure syndromes, PNH harbors a risk, albeit a low one, of evolution into myeloid malignancies such as myelodysplastic syndromes and acute leukemia (27). If immune-mediated bone marrow failure develops in the context of a hemolytic PNH, concomitant or sequential treatment with immunosuppression and anti-complement agents may be considered (37–39). Other non-transplant therapies in AA that directly stimulate HSC (eltrombopag) are making a positive impact in some patients (35, 40, 41); however, the use of eltrombopag in AA/PNH will require specific investigations for the risk of expansion of the PNH clone. Since the complement cascade is not involved in the pathophysiology of immune-mediated bone marrow failure, the improvement of anti-complement therapeutic strategies is not expected to improve the treatment of marrow disorder underlying PNH.

UNMET CLINICAL NEEDS IN ANTI-COMPLEMENT TREATMENT FOR PNH

Intravascular Hemolysis in PNH During Eculizumab Treatment

As initially shown in the registration trials (19, 20), treatment with eculizumab results in sustained control of complement-mediated intravascular hemolysis in all PNH patients. Nevertheless, using lactate dehydrogenase (LDH) as the best biomarker of hemolysis, the vast majority of patients continue to show slightly increased LDH, usually ranging between 1- and 1.5 times the upper limit of normal (ULN), in addition to persistently undetectable haptoglobin (19–21). This observation has raised the notion that minimal, residual intravascular hemolysis is common during eculizumab treatment, even if it is clinically not relevant in the majority of patients. The reasons for this less than optimal complement inhibition *in vivo* have not been fully elucidated. Some of us have demonstrated that residual complement activity (assessed by a functional assay measuring the 50% of complement hemolytic activity—CH50) can be detected in several PNH patients on eculizumab, and it correlates with plasma LDH levels (42). This residual complement activity (CH50 >10%) also correlates with low plasma levels of free eculizumab, eventually suggesting that suboptimal (or even partial) C5 blockade may occur due to subtherapeutic plasma levels of eculizumab (42). Nevertheless, in this broad PNH population suboptimal C5 blockade does not seem to be associated with lower hemoglobin levels (in the sense that most patients remain anemic irrespective of full C5 blockade), eventually arguing against a predictive clinical value for LDH, CH50, or any other laboratory measurement in the context of eculizumab treatment. On the other hand, in a few patients meaningful laboratory findings (i.e., LDH >1.5x ULN) and clinical hemolysis can be detected during eculizumab treatment; in this condition CH50, or other more sophisticated functional complement assays, may confirm partial C5 blockade, eventually further justifying specific therapeutic intervention (43).

Breakthrough Hemolysis: Pharmacokinetics vs. Pharmacodynamics

The reappearance of hemolysis in a PNH patient on eculizumab has been described as "breakthrough hemolysis." There is no formal definition for this condition, but it seems very important to have it, since it will be eventually exploited as an endpoint in future trials investigating novel anti-complement agents, and its elimination may represent a clinical goal for any new therapy for PNH. Clinical breakthrough hemolysis is identified by the appearance of clinical symptoms such as painful hemolytic crises and dark urines (somehow subjective), associated with a rise in LDH and a drop in hemoglobin. Sometimes hemolysis may be evident just by laboratory data (i.e., LDH or hemoglobin) and hemoglobinuria: this may be referred to as subclinical breakthrough hemolysis. More robust definitions for clinical and subclinical breakthrough are needed, and we suggest the following classification (**Table 3**): breakthrough hemolysis should be individualized to each patient's steady-state LDH, and significant hemoglobin drop in a given period or clinically meaningful signs or symptoms of hemolysis should be acknowledged as a clinical event. For instance, ***clinical breakthrough hemolysis*** may be defined by a hemoglobin drop $\geq$ 2 g/dL within 2 weeks or the development of clinical signs or symptoms of hemolysis, in combination with laboratory demonstration of increased intravascular hemolysis (LDH >1.5x ULN, increased as compared to the latest available value). In contrast, the isolated laboratory evidence of increased intravascular hemolysis (>1.5x ULN, increased by at least 50% as compared to the latest available value, or a discrete hemoglobinuria), without meaningful drop in hemoglobin (<2 g/dL) and without other clinical signs or symptoms of hemolysis, may be defined ***subclinical breakthrough hemolysis***.

Irrespective of the reliability of the current definition, breakthrough hemolysis has been described since the very first experiences with eculizumab (19, 20). Mechanistically, breakthrough hemolysis obviously results from a temporary decrease in complement inhibition, leading to some perturbation of the previous steady-state. Indeed, semantically speaking, chronic, continuous residual intravascular hemolysis (or persistence of hemolysis seen in PNH patients carrying the R885H C5 polymorphism) (29) must not be defined as a breakthrough. PNH erythrocytes are exquisitely susceptible to complement-mediated lysis because they lack complement regulators, which are normally expressed on cell surface through a GPI anchor. Among all the GPI-linked proteins which are missing on PNH erythrocytes, there are the two main

TABLE 3 | Definition of clinical and subclinical *breakthrough* hemolysis during eculizumab treatment for PNH.

	Clinical criteria		Laboratory criteria
	Hemoglobin level	**Sign or symptoms**	**LDH level**
Clinical breakthrough*	Drop ≥2 g/dL (compared to the latest assessment, within 15 days)	Gross hemoglobinuria, painful crisis, dysphagia or any other significant clinical finding	>1.5x ULN (and increased as compared to the steady-state)
Subclinical breakthrough	Drop <2 g/dL (compared to previous assessment, within 15 days)	No clinical symptom or sign, except moderate hemoglobinuria	>1.5x ULN (and increased by at least 50% as compared to the steady-state)

*LDH, lactate dehydrogenase; ULN, upper limit of the normal. *The breakthrough is defined clinical if either one of the two clinical criteria is demonstrated, in presence of the laboratory evidence of intravascular hemolysis (LDH level).*

complement regulators CD55 (also named Decay Accelerating Factor, DAF) and CD59. As a result, both early complement activation (i.e., both assembly and decay of the C3 convertases C3bBb and C4b2a, regulated by CD55) (9–11) and terminal complement with its effector mechanism (i.e., assembly of the lytic C5b-C9 MAC, regulated by CD59) (12, 13) are uncontrolled, eventually leading to intravascular hemolysis (**Figure 1**) (1). Thus, the reappearance of intravascular hemolysis is mechanistically due to lack of effective complement inhibition; the event develops acutely because the PNH erythrocyte mass susceptible to complement activation is large (the proportion of affected erythrocytes increases during effective eculizumab treatment) (18–20). In the clinic, two distinct types of breakthrough hemolysis can be identified, which eventually imply different pathogenic mechanisms (**Table 4**) (44).

The first (and better defined) example of breakthrough hemolysis was described in about 10–15% of PNH patients on eculizumab, as the frequent (and somehow regular) reappearance of hemolysis in the few hours/days before the next administration of eculizumab without any obvious trigger or complement activating conditions (i.e., LDH increases by 2–3 folds as compared to values assessed at day 7 from previous eculizumab dosing). In this case, impaired C5 blockade has been associated with low trough plasma levels of eculizumab demonstrated at 12–14 days from the previous dosing (42); thus, the term "***pharmacokinetic (PK) breakthrough***" has been designated for this condition (44, 45). Notably, the final confirmation that this is a PK phenomenon comes from the observation that changes to the treatment schedule (i.e., decreasing the interval dosing to 10–12 days, or increasing the dose to 1,200 mg) eventually result in sustained C5 blockade, with evident clinical benefit (21, 42, 46).

The second type of breakthrough hemolysis during anti-complement treatment in PNH is rather more unpredictable, since it may occur at any time (with respect to last infusion of eculizumab) and it tends to be sporadic and not recurrent as the PK breakthrough. In most cases, it is associated with infectious episodes or other clinical conditions that trigger complement activation in addition to the basal, low-grade, steady-state activation deriving from C3 tick-over (47–50). For this condition, we have used the term "***pharmacodynamic (PD) breakthrough***" (31, 44, 45) since it appears to be caused by massive complement activation, exceeding the inhibitory ability of eculizumab that is independent of its plasma level. The most frequent causes triggering this massive complement activation are infectious events (both bacterial and viral, such as common seasonal viruses) or any event leading to inflammation (i.e., surgery, possible comorbidities). In the clinic, it is well-accepted that in this condition extra dosing of eculizumab is not appropriate, because these episodes tend to be self-limiting, and the temporary increase in plasma level of eculizumab is not necessarily effective, as the plasma drug levels are already high. Notably, these acute events mirror the well-known hemolytic paroxysms which gave the name to the disease in absence of treatment, explaining the concept that the degree of complement activation is fluctuating acutely during these "trigger" events and may became clinically significant even during anti-C5 treatment. Experimental observations that aid in the understanding of this phenomenon are available both in the recent and old scientific literature. Indeed, we have documented that eculizumab at the therapeutic dose does not result in complete inhibition of hemolysis in an *in vitro* model investigating hemolysis of PNH erythrocytes (51–53). Recently, we have further dissected this phenomenon, using different conditions of complement activation *in vitro*, in the presence of eculizumab: whereas with spontaneous complement activation (paralleling the spontaneous, low-grade complement activation in steady-state clinical conditions) residual hemolysis is minimal (i.e., corresponding to the clinical finding of LDH ranging between 0.75 and 1.5 fold ULN); after massive complement activation hemolysis remains >40%, even with excess concentrations of eculizumab (5–10 times the therapeutic plasma levels) (44, 51). Thus, in clinical circumstances triggering the complement cascade, excessive complement activation may override the C5 blockade delivered by eculizumab, eventually leading to hemolytic crises due to PD breakthrough. The actual mechanism by which massive complement activation overrides eculizumab requires a review of complement biology: C5 is the substrate of an enzymatic reaction catalyzed by the C5 convertase, which eventually enables MAC formation. Anti-C5 antibodies bind to C5 in its fluid phase, preventing its cleavage by the C5 convertase, either of the alternative or of the classical/mannose pathway (17). Thus, PK and PD of eculizumab depend not only on its target C5, but also on the degree of C5 convertase activity competing with eculizumab for free C5, which in turn varies based on the magnitude of complement activation (54). It is quite obvious that complement activation may increase the number of C5 convertases; but it is even more important that the affinity of C5 convertases for the substrate C5 may vary also,

TABLE 4 | Definition of pharmacokinetic and pharmacodynamic *breakthrough* hemolysis during eculizumab treatment for PNH.

	Timing	Frequency	Concomitant conditions	Free C5	Eculizumab plasma level	Mechanism	Intervention
Pharmacokinetic breakthrough	>7–10 days from previous dosing	Recurrent	Usually none*	Always >0.5–1 μg/mL	Inadequate	Residual free C5 available for steady-state (normal) C5 convertase activity	Decrease interval of dosing (10-12 days) or increase dose of eculizumab (1,200 mg)
Pharmacodynamic breakthrough	Any time	Sporadic	Infectious events (both bacterial and viral, such as common seasonal viruses) or any event leading to inflammation (i.e., surgery, possible comorbidities)	Usually ≤0.5–1 μg/mL (but it may occur with any free C5 plasma level)	Adequate	Massive complement activation leading to excess C5 convertase activity, which might displace C5 from eculizumab	None (treat the underlying cause triggering complement activation)

**Events leading to pharmacodynamic breakthrough (i.e., triggers of complement activation) may eventually contribute also to pharmacokinetic breakthrough.*

as membrane-bound convertases have a much higher affinity for C5 as compared with convertases in the fluid phase (55, 56). Moreover, this affinity is largely dependent on the density of surface-bound C3b: when this density increases (by 10–100 times, in presence of events triggering complement activation) the excess of C3 generates very high-affinity C5 convertases (57–59). These complexes with high C3b content, which are generated at high rate on PNH erythrocytes even in presence of C5 blockade (see below) (28, 44), may displace C5 from the complex eculizumab:C5 thus arming the MAC, irrespective of therapeutic plasma levels of eculizumab and of very low levels of free C5 (57–59). Indeed, this breakthrough may be referred also as "*breakthrough with minimal free C5 levels*"; however, while PK breakthrough is unequivocally marked by high free C5 levels, PD breakthrough may contribute to hemolysis even when free C5 levels are high (C5 cleavage by high-affinity C5 convertases is further increased in excess of substrate). This mechanism of endogenous regulation of C5 convertase activity has been demonstrated not only for the alternative pathway convertase C3bBbC3b, but also for the classical/lectin pathway one C4bC2aC3b (59–61). These mechanistic data have been recently reproduced in the context of therapeutic C5 inhibition (62); interestingly, at least *in vitro*, the association of two different C5 inhibitors (that are both only partially effective if used in monotherapy) appears to overcome this phenomenon (62). Taken together, all these data support the existence of a breakthrough hemolysis due to PD reasons (i.e., secondary to transient massive complement activation); however, the clinical relevance of this phenomenon as well as possible therapeutic strategies for its prevention remain to be delineated.

Therapeutic goals

Residual intravascular hemolysis may persist during eculizumab treatment, either as low-grade continuous hemolysis or as breakthrough hemolytic crisis due to PK or PD reasons, which may eventually impact hematological response (see **Table 1**). With the exception of recurrent PK severe breakthrough (namely requirement for transfusion), which obviously requires therapeutic intervention to improve hematological response (**Table 2**), the other conditions may be clinically mild, and the actual need to develop novel strategies specifically targeting these conditions is questionable. Thus, while the possible impact of all novel therapies on residual intravascular hemolysis has to be addressed, we have to acknowledge that residual intravascular hemolysis seems not to be the most pressing unmet clinical need during eculizumab treatment.

C3-Mediated Extravascular Hemolysis

Both residual intravascular hemolysis due to suboptimal C5 blockade and inadequate compensatory erythropoiesis due to underlying bone marrow failure may contribute to persistent anemia in PNH patients on eculizumab (31). However, most patients exhibit reasonable control of intravascular hemolysis (LDH stably <1.5 times the ULN) and adequate reticulocytosis (largely >100,000/μL). In contrast, all patients share a novel phenomenon which is the opsonization of surviving PNH erythrocytes with C3 fragments, which are detectable by flow cytometry (28, 63). Based on this finding, together with the demonstration of reduced *in vivo* half-life of ^{51}Cr-labeled erythrocytes (with increased hepatosplenic uptake of ^{51}Cr), we have described C3-mediated extravascular hemolysis as a novel disease mechanism which limits hematological benefit in most PNH patients on eculizumab (28, 64–66). C3-mediated extravascular hemolysis in PNH patients on eculizumab (or any anti-C5 agent) is not a complication, but rather a mechanistic phenomenon related to complement biology. We have discussed that PNH erythrocytes lack both CD55 and CD59 from their surface, and thus they are unable to control both early complement activation (i.e., assembly and decay of C3 and C5 convertases) and effector mechanisms of the terminal complement pathway (i.e., MAC assembly). Irrespective of the hierarchical contribution of CD55 and CD59 (the latter appears to be the most important surface endogenous complement modulator, at least for lysis prevention) (67), therapeutic C5 blockade prevents only MAC assembly, without interfering with early steps of the complement cascade. Thus, while PNH erythrocytes are kept alive by eculizumab because their lysis is precluded, surface complement activation on affected cells continues (mostly due to the constitutively active C3 tick-over of the alternative pathway), with covalent binding of C3b to erythrocyte surface (via glycophorin A, for example) and further generation of C3 convertase, which in turn amplifies C3

activation and C3 surface deposition. Then, PNH erythrocytes are progressively opsonized with different C3 split fragments (initially C3b, which then is quickly processed to C3d) (52), and they can be specifically recognized by C3 receptors (e.g., complement receptor 3) (68) leading to entrapment by professional phagocytes in the liver and spleen (28, 31, 64, 68). Different groups have confirmed opsonization by C3 split fragments as a common event in PNH patients on eculizumab (**Figure 1B**) (28, 66, 69); but its clinical relevance is not universally acknowledged by all experts (42, 70). The extent of this chronic extravascular hemolysis is very heterogeneous among patients, and the actual hemoglobin level reflects also residual intravascular hemolysis as well as compensatory erythropoiesis (even patients with normal hemoglobin levels exhibit massive reticulocytosis with increased bilirubin) (31, 64, 71). The clinical impact of this chronic anemia on quality of life (e.g., possible differences in distinct hematological response categories) and long-term organ damage has not been systematically investigated, even if some possible complications have emerged, such as iron overload, especially in patients still requiring transfusions (72–74). Inherited polymorphisms of different genes associated with complement regulation may shape the individual susceptibility of PNH patients to C3-mediated extravascular hemolysis; we have already shown that PNH patients carrying the hypomorphic variant of the complement receptor 1 gene have a much lower chance in achieving a good hematological response during eculizumab treatment (75). Given the number of proteins involved in complement activation and regulation (e.g., complement factor H, complement factor H related proteins, complement factor B, complement factor I, membrane cofactor protein, C3, etc.) (76, 77), it is likely that other gene variants associated with better or worse hematological benefit (as well as with residual intravascular hemolysis) may be identified in the near future.

Therapeutic goals

To date, there is no treatment option for C3-mediated extravascular hemolysis. The chronic use of steroids has been discouraged because of inefficacy and unacceptable side effects (65); splenectomy has been reported as possibly effective to ameliorate this condition (78, 79), but it is not considered a standard treatment (70). Thus, C3-mediated extravascular hemolysis represents an unmet clinical need in PNH, and it is the most reasonable therapeutic goal for any new strategy of complement inhibition.

THE ROLE OF BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) remains the only curative treatment for PNH (32, 80, 81), however its use is limited by treatment-related morbidity and mortality. As discussed above, BMT is a key treatment option in patients with AA/PNH syndrome; however, its role can be discussed even in patients with classic PNH. Indeed, the outcome of patients undergoing allogeneic BMT for classic, purely hemolytic, PNH is excellent, with a long-term survival of 80–90% (32). BMT remains the best treatment option for hemolytic PNH for patients who have no access to eculizumab treatment, which is the case for many developing countries. Indeed, for emerging markets the very high price of eculizumab (82) represents a major limitation to its use, even with approval from regulatory authorities (which does not necessarily imply allocation of financial resources and reimbursement). Since the cost of BMT can be equivalent to about 3–4 months of eculizumab treatment, BMT may be not only clinically appropriate, but even cost-effective. In addition, BMT might be considered even where eculizumab is fully available, in case of lack of hematological response to the treatment; however, no response (see **Table 1**) is rare, and even in case of minor hematological benefit eculizumab appears to retain obvious clinical benefits, with major impact on long-term survival (23, 24). Thus, BMT is not recommended for the majority of hemolytic PNH patients with unsatisfactory hematological response; and for this condition, novel strategies of complement inhibition represent an intriguing alternative to BMT.

THE SECOND GENERATION OF ANTI-COMPLEMENT AGENTS FOR PNH

The clinical development of eculizumab for PNH, and then also for other diseases, has been a unique experience in terms of both scientific and financial success. This growing interest in the field of complement therapeutics has generated several preclinical and clinical programs for the development of novel anti-complement agents (**Table 5** and **Figure 1**). We have already reviewed quite recently most of these programs (45, 126); here we focus on the possible therapies whose development appears more advanced, or more exciting for their possible breakthrough results. Indeed, our discussion is biased by our commitment to address the major unmet clinical needs in PNH, as described in the first part of this manuscript. Therapeutic agents interfering with complement activity may be grouped based on different factors; for this review, the most useful classification is based on their targets in the complement cascade. Two major classes of complement inhibitors may be identified: (i) inhibitors of the terminal complement pathway targeting C5 (even if agents targeting downstream complement components such as C6 have been announced); (ii) inhibitors of early phases of the complement cascade targeting either the key event of the cascade (C3 cleavage), or pathway-specific initiating events (for PNH, they include proteins of the alternative pathway such as complement factor D, factor B and properdin); all together, these agents can be classified as proximal complement inhibitors.

Novel Inhibitors of the Terminal Complement

There are at least seven novel anti-C5 agents (in addition to biosimilars of eculizumab, which have been announced as well), which have entered clinical development for PNH; most of them are monoclonal antibodies like eculizumab, but the list includes also small peptide inhibitors and small interfering RNA (siRNA). All these agents aim to reproduce the excellent data achieved with eculizumab, trying to address some other clinical needs mostly concerning patient (dis)comfort: indeed, current eculizumab

TABLE 5 | Complement inhibitors in clinical development for PNH.

Class	Agent	Target	Clinical trial ID	Design	Patient population	Study treatment	Results
Terminal inhibitors	ALXN1210	C5	N.A.	Phase I, randomized vs. placebo	Healthy volunteers	SAD, IV infusions	Yes
			NCT02598583 (83)	Phase I/II, open-label	Untreated PNH	Intra-patient DE by IV infusions	Yes (84)
			NCT02605993 (85)	Phase I/II, open-label	Untreated PNH	MAD; IV infusions	
			NCT02946463 (86)	Phase III, randomized vs. Ecu	Untreated PNH	IV infusions (every 8 weeks)	Yes (87)
			NCT03056040 (88)	Phase III, randomized vs. Ecu	Stable responders PNH	IV infusions (every 8 weeks)	Yes (89)
	SKY59	C5	NCT03157635 (90)	Phase I/II, multi-part study	Healthy volunteers	SAD, IV infusions	Yes (91)
					Untreated PNH	Intra-patient DE by IV infusions, followed by SC injections	Yes (92)
					Stable responders PNH		
	LFG316	C5	NCT02534909 (93)	Phase II, open-label	Untreated PNH	IV infusions	Pending
	REGN3918	C5	NCT03115996 (94)	Phase I	Healthy volunteers	IV and SC infusions	Yes (95)
	ABP959	C5	EudraCT 2017-001418-27 (96)	Phase III, randomized vs. Ecu	Stable responders PNH	IV infusions	Ongoing
	RA101495	C5	N.A.	Phase I, SAD and MD	Healthy volunteers	Daily, SC injections	Yes (97, 98)
			NCT03078582 (99)	Phase II, open label, fixed dose	Untreated PNH	Daily, SC injections	Yes (100)
					Poor responders PNH		
			NCT03030183 (101)	Phase II, open label, fixed dose	Poor responders PNH	Daily, SC injections	Ongoing
			NCT03225287 (102)	Phase II, open-label, extension	PNH exposed to RA101495	Daily, SC injections	Ongoing
	Coversin	C5	N.A.	Phase I, SAD and MD	Healthy volunteers	SC injections	Yes (103)
			NCT02591862 (104)	Phase II, open-label	Poor responder PNH	SC injections; intra-patient DE	Pending
			EudraCT 2016-002067-33 (105)	Phase II, open-label, fixed dose	Untreated PNH	SC injections	Yes (106)
			EudraCT 2016-004129-18 (107)	Phase II, open-label, extension	PNH exposed to coversin	SC injections	Ongoing
	ALNCC5	C5	NCT02352493 (108)	Phase I/II, randomized vs. Ecu, SAD and MAD	Healthy volunteers	SC injection (ALNCC5 or placebo)	Yes (109)
					Untreated PNH	SC injections (ALNCC5 only)	Yes (110)
			EudraCT 2016-002943-40 (111)	Phase II, open-label	Poor responder PNH	SC injections	Pending
Proximal inhibitors	TT30	CAP	NCT01335165 (112)	Phase I, SAD	Untreated PNH	SC injections and IV infusions	Yes (113)
	AMY-101	C3	NCT03316521 (114)	Phase I, SAD and MD	Healthy volunteers	SC and IV infusions	Pending
	APL-2	C3	N.A.	Phase I, SAD and MD	Healthy volunteers	SC and IV infusions	Yes (115)
			NCT02264639 (116)	Phase Ib, open label, MAD, POC	Poor responders PNH	Daily, SC infusions	Yes (117)

(Continued)

TABLE 5 | Continued

Class	Agent	Target	Clinical trial ID	Design	Patient population	Study treatment	Results
			NCT02588833 (118)	Phase Ib, open label, MAD, POC	Untreated PNH	Daily, SC infusions	
			NCT03531255 (119)	Phase III, open label, extension	PNH exposed to APL-2	Daily, SC infusions	
			NCT03500549 (120)	Phase III, randomized vs. ecu	Poor responders PNH	SC infusions, BIW	Ongoing
	ACH-4471	FD	N.A.	Phase I, SAD	Healthy volunteers	Orally, QD and BID	Yes (121)
			NCT03053102 (122)	Phase II, open label, MD, POC	Untreated PNH	Orally, TID	Pending
			NCT03181633 (123)	Phase II, open-label, extension	PNH exposed to ACH-4471	Orally, TID	Ongoing
			NCT03472885 (124)	Phase II, open label, MD, POC	Poor responders PNH	Orally, TID	Ongoing
	LNP023	FB	NCT03439839 (125)	Phase II, open label	Poor responders PNH	Orally, BID	Ongoing

N.A, not available; Ecu, eculizumab; SAD, single ascending dose; MAD, multiple ascending doses; MD, multiple doses; POC, proof-of-concept; DE, dose escalation; SC, subcutaneous; IV, intravenous; QOD, quaque die (once a day); BID, bis in die (twice a day); TID, ter in die (thrice a day); BIW, bis in week (twice a week); PK, pharmacokinetics; PD, pharmacodynamics; LDH, lactate dehydrogenase.

treatment requires intravenous (IV) infusions given every 14 days indefinitely. These agents have been designed trying to increase the interval between administrations, and/or switching from an IV dosing to a subcutaneous (SC) or even oral one.

ALXN1210 (Ravulizumab)

ALXN1210 (also known with the brand name of ravulizumab, Ultomiris®) is the first of the second-generation therapeutic complement inhibitors, as well as the one with the most advanced clinical program. ALXN1210 is another anti-C5 mAb which was generated through specific amino acid modifications of eculizumab aiming to improve its PK profile (127). The half-life of eculizumab is largely influenced by non-specific pinocytosis by endothelial cells and trafficking into the lysosome compartment, where it can be efficiently degraded (especially if bound to its target C5) (127). A potential strategy to reduce this degradation and to favor recycling to the vascular compartment may exploit a more efficient dissociation of the mAb:C5 immune complex in the low pH lysosome compartment, together with a higher affinity for the neonatal Fc receptor (FcRn), which is responsible for specific recycling to the extracellular, vascular compartment (127). ALXN1210 was designed to exploit targeted reengineering of eculizumab: two histidine switches were included in the complementary determining regions (CDRs) to promote more efficient pH-dependent dissociation of the mAb:C5 complex, and two additional amino acides changes were included in the Fc region to increase the affinity for the FcRn (127). Based on preclinical data, both these goals have been achieved. ALXN1210 exhibited a reduced target-dependent drug disposition (TDDM) and a longer half-life as compared to its parental molecule eculizumab, becoming an attractive long-acting anti-C5 mAb to be used in the clinic (127). Two phase Ib/II multicenter studies were conducted to investigate safety and preliminary efficacy of different IV doses of ALXN1210 in untreated PNH patients (84). In the first study (NCT02598583, study 103), 13 PNH patients received the drug every 4 weeks at the maintenance dose of either 900 (same as eculizumab) or 1800 mg (83); in the second study (NCT02605993, study 201), 26 PNH patients were treated with the maintenance dose of 1,000 mg every 4 weeks, 1,600 mg every 6 weeks, 2,400 mg every 8 weeks, or 5,400 every 12 weeks (85). Without focusing on details, rapid and sustained reduction in LDH (which was the primary endpoint) was achieved in all cohorts, without substantial difference in percentage change from the baseline (84). However, the percentage of patients achieving normal or near-normal LDH (<1.5 times of the ULN) was higher in those with the higher trough exposure to ALXN1210 (i.e., 1,800 mg every 4 weeks) (84). The safety profile of ALXN1210 was overlapping to that established for eculizumab, with no deaths, and no adverse events (either serious or non-serious) leading to drug discontinuation; however, two cases of sepsis by *N. Meningiditis* were observed (both patients completely recovered after ceftriaxone treatment, and continued ALXN1210 therapy) (84). These results led to the design of two large phase III, open-label, randomized trials where the maintenance dose of ALXN1210 was 3,300 mg every 8 weeks (with some dose adjustment based on patient weight: 3,000 mg for those <60 kg, 3,600 for those >100 kg); the data of these studies have become available recently (87, 89).

The study 301 (NCT02946463) tested for non-inferiority of ALXN1210 as compared with eculizumab in treatment-naïve hemolytic (LDH >1.5 times of the ULN) PNH patients (86). A total of 246 patients were randomized 1:1 to receive either ravulizumab (a single loading dose of 2,700 ± 300 mg for under- or overweight patients was followed by maintenance dose starting after 2 weeks) or eculizumab for 6 months; the co-primary endpoints were transfusion independence and LDH normalization (86). ALXN1210 was non-inferior to eculizumab for both the primary endpoints (transfusion independence, 73.6 vs. 66.1%; LDH normalization, 53.6 vs. 49.4%, with *P* for non-inferiority <0.001) as well as for additional efficacy secondary

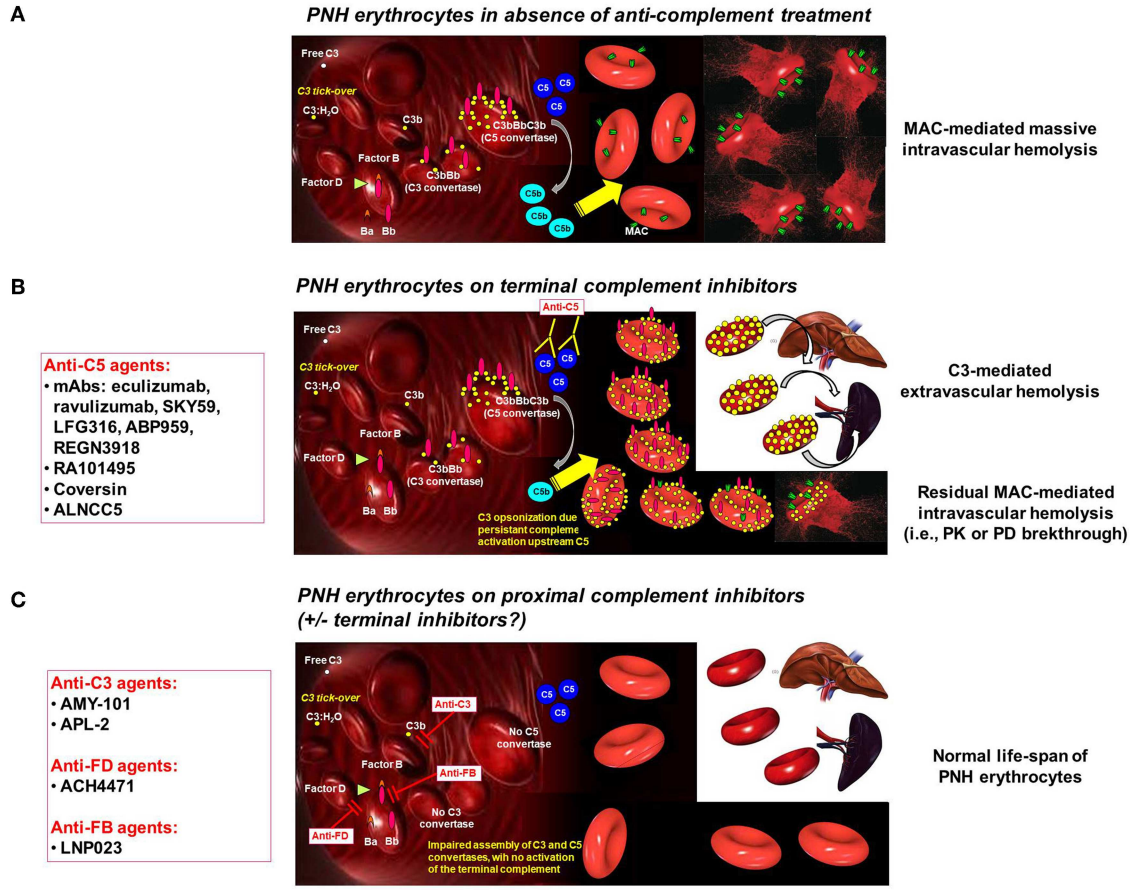

FIGURE 1 | Complement activation on PNH erythrocytes. **(A)** ***PNH erythrocytes in absence of anti-complement treatment***. The complement system may activate due to different triggers through the alternative, classical and mannose/lectin pathway. Spontaneous C3 tick-over continuously generates low-grade activation of the alternative pathway in the fluid phase and possible binding of activated C3 fragments on erythrocytes. Due to the lack of CD55, this leads on PNH erythrocytes to the generation of C3 convertase, with further generation of C3b, which eventually leads to the assembly of C5 convertase. Then, the terminal pathway of the complement cascade is activated, with the generation of the MAC, eventually leading to lysis of PNH erythrocytes lacking CD59. **(B)** ***PNH erythrocytes on terminal complement inhibitors***. Terminal complement inhibitors (i.e., anti-C5 agents) prevent the cleavage of C5 into C5a and C5b, thereby disabling the formation of the MAC. Thus, PNH erythrocytes are largely protected from intravascular lysis. Nevertheless, early phases of surface complement activation remain uncontrolled on PNH erythrocytes due to the lack of CD55; thus, continuous low-grade activation continues leads to opsonization of PNH erythrocytes with C3 fragments. This excess of C3 generates high-affinity C5 convertases, which may account for residual intravascular hemolysis due to pharmacodynamic breakthrough (in addition to possible pharmacokinetic breakthrough due to sub-therapeutic plasma leven of anti-C5 agent). Moreover, C3 opsonization leads to extravascular hemolysis due to C3-specific receptors expressed on professional macrophages in the liver and in the spleen. **(C)** ***PNH erythrocytes on proximal complement inhibitors (± terminal complement inhibitors)***. Proximal complement inhibitors intercept complement activation at the level of its key component C3 (i.e., anti-C3 agents), or even upstream at the level of initial activation of the alternative pathway (i.e., anti-FD and anti-FB agents). All these agents prevent early activation of complement on the surface of PNH erythrocytes, counterbalancing the deficiency of the complement regulators CD55 and CD59. Based on theoretical assumptions and *in vitro* data, proximal complement inhibitors prevent C3 opsonization, thereby preventing C3-mediated extravascular hemolysis. However, by disabling early surface complement activation, proximal complement inhibitors should also prevent intravascular hemolysis. While preliminary clinical data already confirmed that proximal complement inhibitors prevent C3-mediated extravascular hemolysis, ongoing investigation will make clear whether they can adequately prevent intravascular hemolysis even in the absence of terminal inhibitors (as already documented *in vitro*).

endpoints including percent reduction of LDH, stabilized hemoglobin, breakthrough intravascular hemolysis, and quality of life measures (86). No meningococcal infection was observed, with excellent safety and tolerability for the 8-week interval regimen (9). The twin study 302 (NCT03056040) was conceived as a switch-study for PNH patients already on eculizumab, assessing the non-inferiority of ALXN1210 vs. eculizumab in PNH patients in clinically stable conditions on standard-of-care eculizumab treatment (i.e., 900 mg every 14 ± 2 days) (88). A total of 195 patients were randomized 1:1 to switch to ALXN1210 or continue on eculizumab; the primary endpoint was percentage change in LDH (88). Considering the 191 patients who have completed the 6 month treatment, ALXN1210 was non-inferior to eculizumab: percentage change in LDH compared to baseline was minimal in both arms (−0.82% for ALXN1210 vs. +8.39% for eculizumab), with non-inferiority shown also for all efficacy

secondary endpoints (hemoglobin stabilization, breakthrough intravascular hemolysis, and quality of life measures) (88). The safety profile was excellent, with no cases of meningococcal infection recorded in this trial; interestingly, the most common AE was headache (26.8% with ALXN1210 vs. 17.3% with eculizumab). Headache is commonly observed after starting of eculizumab, as a result of inhibition of intravascular hemolysis with reduced release of free hemoglobin and sudden increase of circulating nitric oxide (128); the observation that headache may be seen also upon switching from eculizumab to ALXN1210 may suggest that in some patients a deeper control of intravascular hemolysis has been achieved (88). Based on these data both in untreated PNH patients and as switch-therapy from eculizumab, ALXN1210 has received marketing authorization by the FDA (Ultomiris®, Alexion Pharmaceuticals, New Haven, CT, USA) and approval by EMA is expected soon. Furthermore, an additional trial investigating SC dosing of ALXN1210 has been announced (129).

SKY59/RO711268/Crovalimab

SKY59 (also known as RO711268 or Crovalimab, in development by Roche) is another long-acting anti-C5 mAb, which exploits a pH-dependent binding to the target C5, eventually accounting for profound mAb recycling (130, 131). This mAb has been generated with the same goal of using histidine residues to achieve a pH-dependent binding to the antigen, eventually favoring the dissociation of the immune complex in the low pH lysosome compartment through changes in the surface charge of the mAb (131). This increased dissociation promotes the degradation of the C5 released into the lysosomes (thereby preventing C5 accumulation commonly seen with eculizumab treatment), and the recycling of the mAb to the plasma through its recognition by the FcRn (130, 131). The engineered SKY59 mAb generated with this technology exhibits a much longer half-life *in vivo* in cynomolgus monkeys, accounting for a sustained C5 blockade even after SC administrations (130, 131). Since SKY59 binds C5 epitopes different from eculizumab, SKY59 has been shown to efficiently block even the R885H polymorphic C5variant, at least *in vitro* (130). The clinical development of SKY59 in PNH has been pursued through a complex phase I/II study (NCT03157635) consisting of three sequential parts and an open-label extension (90). In part 1, SKY59 was investigated in healthy volunteers, whereas parts 2 and 3 enrolled untreated and eculizumab-treated PNH patients, respectively; in all three parts safety, tolerability, PK/PD and efficacy of SKY50 was evaluated (90). Data on the first two parts of the study have been recently presented (91, 92). Part 1 was a randomized, placebo-controlled, single ascending dose study to evaluate safety, tolerability, PK/PD of SKY59 in healthy subjects; three dose cohorts of five subjects were investigated: 75 and 125 mg given IV, and 100 mg given SC (91) Single ascending doses of SKY59 were well-tolerated, without severe or serious adverse events; exposure was dose proportional in the two IV dose levels, with a terminal half-life of about 25 days (91). After the SC dosing, bioavailability was estimated around 90%, with peak plasma levels being achieved after 7 days (91). Dose-dependent inhibition of the terminal complement activity was observed, with transient complete inhibition demonstrated in 2 out of 3 subjects receiving 125 mg IV (91). Part 2 of the study consisted in an intra-patient dose escalation, with SKY59 given at the doses of 375, 500 and 1,000 mg on days 1, 8 and 22, followed by weekly SC maintenance doses of 170 mg from day 36 (92). In the 10 eculizumab-naive PNH patients enrolled in part 2, half-life of SKY59 was confirmed as 25 days; complete terminal complement inhibition was observed after all IV and SC dosing (92). All these untreated PNH patients (one carrying the R885H C5 polymorphism) achieved a marked reduction of intravascular hemolysis, as demonstrated by a median LDH reduction of 79%; after 6 weeks of treatment, LDH remained in a range between 0.8 and 1.7 times of the ULN (92). In part 3 of the study PNH patients on eculizumab were switched to SKY59 with an IV loading dose of 1,000 mg (given 2 weeks after the last administration of eculizumab), followed by a randomization for maintenance SC SKY59 at 3 different schedules: 170 mg weekly, 340 mg every 2 weeks and 680 mg every 4 weeks (92). Sixteen eculizumab-treated PNH patients were enrolled; during SKY59 treatment, they all maintained LDH levels similar to those recorded during eculizumab therapy, except patients carrying the R885H C5 variant, who as expected achieved a major reduction (92). Two patients in part 3 developed drug-target-drug complex (DTDC) mediated reactions, with vasculitis-like symptoms similar to serum sickness; they appeared at 9 and 10 days from loading dose of SKY59 due to SKY59-C5-eculizumab complexes generated during the (transient) concomitant presence of the 2 anti-C5 mAbs during the switching period. These adverse events required topical treatment with steroids and resolved within 3 weeks without any sequelae, with no SKY59 discontinuation (92). Taken together, results from parts 2 and 3 demonstrated that SKY59 is an effective C5 inhibitor with excellent bioavailability after SC low-volume dosing (given weekly or even with longer intervals)(92). Hemoglobin levels rose by at least 1 g/dL in untreated patients, and remained stable in patients switching from eculizumab (92). A few episodes of breakthrough intravascular hemolysis were observed, mostly associated with concomitant events triggering complement activation (i.e., PD breakthrough, as confirmed by data on free C5, which remained low in all treated patients). Quite interestingly, in contrast to free C5, plasma levels of total C5 exhibited significant changes during treatment: whereas in untreated patients there was an increase from 140 μg/mL (73.6–184 μg/mL) to 215 μg/mL (109–331 μg/mL), in patients switching from eculizumab a reduction from 295 μg/mL (205–354 μg/mL) to 228 μg/mL (184–305 μg/mL) was observed (92). These findings, although preliminary, appear to demonstrate the specific C5 disposing activity of SKY59 in comparison to eculizumab (92, 131); however it has to be acknowledged that in this study the total C5 levels seen on eculizumab appeared to be quite high compared to what was observed in other studies (132).

LFG316

LFG316 is another anti-C5 mAb in development by Novartis; this agent is currently under investigation in PNH patients within a proof-of-concept phase II study enrolling untreated PNH patients (93). The study exploits LDH change as primary

endpoint, looking for preliminary efficacy of LFG316; since Japanese centers are actively involved, this study aims to address the unmet clinical need for PNH patients intrinsically resistant to eculizumab due to the R885H C5 polymorphism (29). Further details on the study (e.g., PK of LFG316 after systemic injection; this agent has been initially developed for local use in age-related macular degeneration) as well as preliminary results are not yet available.

REGN3918

REGN3918 is an anti-C5 mAb in development by Regeneron, which binds both wild-type and R885H variant of human C5. In a phase I study in healthy volunteers REGN3918 was well-tolerated and resulted in dose-dependent inhibition of the terminal complement pathway, measured as hemolytic activity (CH50) (95). This agent exhibits a favorable PK profile, since it is bioavailable even after SC administration; a single IV loading dose followed by weekly SC dosing resulted in sustained inhibition of C5 activity (95). Further trials in PNH have been announced.

Biosimilars of Eculizumab

In addition to novel anti-C5 mAbs, biosimilars of eculizumab have also been described. For instance, ABP959 is a biosimilar of eculizumab developed by Amgen; this agent is now under investigation in a large Phase III trial (96). Another biosimilar of eculizumab (SB12) has been announced by Samsung Bioepis[1].

RA101495

RA101495 is the lead compound of a new class of small synthetic, macrocyclic peptides developed by Rapharma to inhibit C5 (133); preclinical data have demonstrated efficacy in preventing PNH hemolysis *in vitro* (134). RA101495 was safe and well-tolerated in healthy volunteers after single SC administrations (97); multiple daily SC administrations were confirmed safe, and resulted in complete (>95%) and sustained inhibition of the terminal complement pathway (98). The first study investigating RA101495 in PNH (NCT03078582) enrolled both untreated and eculizumab-treated PNH with evidence of hemolysis (LDH >2 times of the ULN) (99); patients received RA101495 as SC injections, with a loading dose of 0.3 mg/kg followed by 0.1 mg/kg daily (possibly escalated up to 0.3 mg/kg) (99). The study enrolled 10 untreated and 16 eculizumab-treated PNH patients; the latter received RA101495 as a switch therapy from eculizumab (100). All the 10 untreated patients achieved a major reduction in LDH, with median LDH stabilized around 1.5–2 times of the ULN (thus, residual intravascular hemolysis remained evident) (100). In PNH patients switching from eculizumab, LDH response was observed as well, even if transfusion-dependence on eculizumab was associated with subsequent breakthrough hemolysis on RA101495 (100). The authors reported that 16 of the 21 patients completing the 12-week study have continued the treatment with RA101495 within an extension study (102). In addition to this trial, a second study on PNH (NCT03030183) investigated the same treatment regimen as add-on treatment in PNH patients with inadequate response to eculizumab (101). According to the latest update, three patients have been enrolled (100); results from this latter study have not been reported yet. In the meantime, additional anti-C5 macrocyclic peptides have been developed with excellent oral bioavailability (135); preclinical data suggest that exposure levels needed for therapeutic efficacy in humans may be reached (136).

Coversin

Coversin is another recombinantly expressed inhibitor of C5, which originates from the tick *Ornithodoros moubata*; this 16 kDa protein binds C5, thereby preventing its cleavage by all C5 convertases (137). Its potential efficacy in PNH is supported by *in vitro* data showing that coversin may prevent lysis of PNH erythrocytes (103), even in samples from patients carrying the R885H C5 polymorphism (138). Coversin has shown excellent bioavailability after SC administration, without safety concern (103); thus, a clinical program in PNH was started. The first proof-of-concept study was successfully conducted in PNH patients resistant to eculizumab due to the C5 polymorphism (NCT02591862) (104). Then a Phase II single arm, open label trial was conducted in previously untreated PNH patients; this study investigated coversin given SC with a dose-adaptation based on adequate control of hemolysis (105). Indeed, the protocol included an initial loading regimen (single loading dose of 60 mg, followed by 1–3 doses at 30 mg every 12 h), followed by a bi-daily regimen with 15 or 22.5 mg; then from day 29 patients switched to a daily regimen at the dose of 30 or 45 mg (106). In case of suboptimal inhibition patients had the option to increase the daily dose, or split it into bi-daily dosing; after the 3-month treatment, an extension study was made available for all patients willing to continue their coversin treatment (107). Five patients were treated according to the planned schedule, but control of hemolysis during the first month of treatment appeared suboptimal (106); thus the protocol was amended to give 22.5 mg bi-daily to all patients starting from 12 h after the ablative regimen. In general, coversin was very well-tolerated, with mild injection site reaction as the only adverse event (they were self-limiting, and reduced in severity over time); even if anti-drug antibodies were rarely seen, no neutralizing antibodies were detected (106). The primary endpoint of reducing LDH below 1.8 times ULN was achieved (as median value of the full cohort; taken individually, 5 of the 8 patients achieved an LDH value <1.8 times of the ULN); nevertheless, LDH ranged around 1.2–1.8 times of the ULN, without normalization (106). Of the eight patients enrolled, one patient withdrew due to comorbidity unrelated with coversin treatment, and seven decided to continue the treatment within the extension study (107); six are currently receiving a daily dose of 45 mg, and one of 30 mg (106). Self-administration was achieved in all patients, without any need of hospitalization to deliver the treatment.

ALN-CC5

In addition to mAbs and small peptide molecules, another strategy of C5 inhibition was developed aiming to interfere with endogenous C5 production by RNA interference. The

[1]https://www.samsungbioepis.com/en/product/product02.do

first-in-class agent for this strategy is ALN-CC5, a si-RNA duplex specific for C5, that had been shown highly effective in silencing liver C5 production in animal models (139). The clinical program of ALN-CC5 started with a phase I/II trial (NCT02352493) enrolling both healthy volunteers and untreated PNH patients (108). In the 32 healthy subjects enrolled, ALN-CC5 was found to be safe and very effective, leading to >99% reduction of C5 plasma levels (109); this was associated with a profound inhibition (>95%) of serum complement activity (109). Thus, investigation in PNH was started with a high degree of enthusiasm; within the same trial (108) six PNH patients were treated with ALN-CC5 at the weekly SC dose of 200 or 400 mg (110). Among these patients, three were treatment-naïve (and thus were treated in monotherapy), whereas three received ALN-CC5 as add-on treatment to eculizumab (110). The treatment was safe, since no adverse event required treatment discontinuation; results were different in the two patient populations, irrespective of the fact that in all patients C5 production was inhibited by >98% (110). In previously untreated PNH patients C5 knockdown was established slowly, and therapeutic inhibition required about 2 months to appear (110); moreover, inhibition of intravascular hemolysis remained partial, since LDH reduction (37 and 50%) was observed only in 2 of 3 patients (both starting with LDH >5 times ULN), and all patients continued to show LDH stably >1.5 times of the ULN (110).These findings are consistent with *in vitro* data showing that, using C5-depleted sera, complement-mediated activity in PNH erythrocytes is fully restored with small amount of recombinant C5 as low as 0.9 μg/mL (about 1% of normal C5 plasma level) (62, 140). These patients were rescued by the addition of eculizumab at very low dose (600 mg every 4 weeks), supporting the concept that a combined treatment may be more effective. On the other hand, in the study three patients started ALN-CC5 as add-on treatment, for inadequate response to eculizumab; in these patients, combined treatment resulted in LDH normalization (110). This combined effect was seen also in one patient with chronic PK breakthrough, who retained LDH normalization even after reduction of eculizumab to the standard regimen of 900 mg every 2 weeks (110). All together, these data demonstrate that ALN-CC5 monotherapy may result in suboptimal control of intravascular hemolysis in PNH; on the other hand, once used in combination with eculizumab, ALN-CC5 allows for more effective C5 inhibition, which has not been seen so far with anti-C5 mAb. However, data about long-term duration of RNA-based C5 inhibition, as well as on efficacy of re-treatment, are lacking. The clinical investigation of this combined treatment with eculizumab and ALN-CC5 is currently ongoing in a trial enrolling poor responders to eculizmab, aiming to improve their clinical response (111).

Inhibitors of the Proximal Complement

The development of proximal complement inhibitors has not been as intensely investigated as the search for novel anti-C5 agents (at least so far); now, there are only five clinical programs, which have been publicly disclosed, but only four remain active. Before listing and discussing them in detail, it is important to summarize how and why the idea of interfering with the proximal steps of the complement cascade became of interest in PNH. As discussed above, the critical understanding of therapeutic complement inhibition *in vivo* with eculizumab revealed that C3-mediated extravascular hemolysis may limit the hematological benefit of anti-C5 treatment (28, 64, 66). As the initial reporters of this event in PNH, we felt the need to address this problem therapeutically by hypothesizing that blockade of the complement cascade upstream of C5 may represent a promising strategy for treating C3-mediated extravascular hemolysis emerging in PNH during eculizumab treatment (141). Indeed, the field of complement provided several options as alternative targets in the complement cascade (142–144). Our work and that from others provided experimental work supporting the hypothesis that different proximal complement inhibitors may efficiently prevent C3-mediated extravascular hemolysis in PNH, likely also inhibiting concomitantly intravascular hemolysis (**Figure 1C**) (52, 53, 145, 146). The first proximal complement inhibitor to enter clinical development was TT30, a 65 kDa engineered protein which fused the functional domain of complement factor H (FH) with the iC3b/C3dg-binding domain of complement receptor 2 (147). This molecule was designed with the aim of delivering the inhibitory effect of FH at the level of complement activation (i.e., for PNH, the surface of erythrocytes binding C3b); our preclinical work confirmed that *in vitro* PNH erythrocytes were protected against both MAC-mediated lysis and C3 opsonization (52). In 2011, a phase I study (NCT01335165) was conducted to investigate tolerability, PK, PD, and immunogenicity of TT30 given as single IV infusion or SC injection in untreated PNH patients (112). Unfortunately, the results of this study have been published only in abstract form; TT30 was safe and well-tolerated, with no safety concern and no evidence of immunogenicity (113). PK and PD data demonstrated that pharmacological levels of TT30 may be achieved and are associated with inhibition of complement activity (including terminal complement pathway) (113). However, even if biological activity was seen as transient decrease of LDH (after single doses), the program was halted due to the extremely short half-life of the compound (113).

Currently the field of proximal complement inhibitors include broad inhibitors of C3 (with the two compstatin analogs AMY-101 and APL-2) and selective inhibitors of the alternative pathway targeting either complement factor D (FD) and complement factor B (FB).

AMY-101

AMY-101 is an analog of a 13-residue disulfide-bridged peptide named compstatin, discovered in the 90's by Prof. J. Lambris using a phage-displayed random peptide library (148). Compstatin binds to human and non-human primate native C3 and to its active fragment C3b, preventing the convertase activity of compstatin-bound C3bBb, and preventing also the cleavage of compstatin-bound C3 into C3b by pre-formed C3 convertases (148, 149). Compstatin and its analogs are broad inhibitors of C3, which completely disable the activation of the complement cascade along all the activating pathways, including the amplification loop (150). AMY-101 (previously known as Cp40) is the most recent generation analog of compstatin,

which harbors increased affinity and potency and better PK features (151). The effect of AMY-101 in PNH has been initially investigated *in vitro*, where it is able to fully prevent C3 deposition on PNH erythrocytes and they are also protected against MAC-mediated lysis (53). Thus, this analog was identified as the best candidate for clinical development by Amyndas Pharmaceuticals. A single and multiple ascending dose phase I study to investigate safety, PK and PD of AMY-101 in healthy volunteers has been completed (NCT03316521) (114). According to company's announcement, AMY-101 was safe, well-tolerated, and exhibited a PK/PD profile which can support a therapeutic schedule of efficient complement C3 inhibition, via subcutaneous administration every 48 h (152). Phase II trials investigating the efficacy of AMY-101 as monotherapy in both untreated and eculizumab-treated (poor responders) PNH patients have been announced (153).

APL-2

APL-2 is another compstatin analog which utilizes a first-generation version of compstatin (151), modified through a pegylation aiming to increase its half-life *in vivo*. Two separate double-blinded, placebo-controlled, phase I studies investigated safety, tolerability, PK, and PD of single- and multiple ascending doses of APL-2 in healthy volunteers (115). In total, 24 subjects received single doses (ranging from 45 to 1,440 mg, SC) and 16 subjects received multiple daily SC doses (ranging from 30 to 270 mg) (115). No serious or severe adverse event was reported, nor events leading to study drug discontinuation (115) The exposure to APL-2 increased linearly with increasing doses, and steady-state plasma level were achieved after 28 days of daily dosing (115). PD was measured by *ex vivo* complement functional assay: complement inhibition was demonstrated with single doses of 1,440 mg, and with multiple doses of 180 and 270 mg (115). APL-2 was then investigated both in untreated and eculizumab-treated PNH patients (116, 118). The PADDOCK study (NCT02588833) investigated APL-2 as monotherapy in untreated PNH patients with meaningful intravascular hemolysis (defined as LDH >2 times ULN and Hb <10.5 g/dL) (118). APL-2 was given SC as daily infusions (to limit skin irritation), at doses of 180 (cohort 1) or 270 mg (cohort 2); three patients were enrolled in cohort 1 and 20 in cohort 2 (117). Reduction of LDH was observed in all patients, with 95% of patients achieving LDH normalization by day 29 of treatment; then, LDH remained in the normal range at all time points investigated (117). Similarly, hemoglobin was raised during treatment, increasing from median 8.0 g/dL at baseline to median 10.8 g/dL at day 29, and sometimes higher to a median of 12.2 g/dL when APL-2 treatment was continued (day 85) (117). Only a few patients ($n = 4$) required red blood cell transfusions while on APL-2 (2 prior to APL-2 steady-state, and 1 for concomitant AA), although 17 patients were transfusion dependent at enrollment (117). Other evidence of adequate control of both intravascular and C3-mediated extravascular hemolysis was the normalization of bilirubin, the reduction of absolute reticulocyte counts (which after an initial drop raised again just above the ULN), and even more the proportion of PNH erythrocytes, which started at 32% at baseline and progressively increased to 67% and up to 80% at day 29 and 85, respectively (117). These data demonstrate that systemic inhibition of C3 with APL-2 controls both intravascular and extravascular hemolysis in PNH, eventually leading to substantial hematological benefit. Another study is ongoing in PNH patients with inadequate response (defined as Hb level <10 g/L and/or the need for red blood cell transfusion) to eculizumab (PHAROAH study; NCT02264639); in this phase Ib trial APL-2 is investigated as add-on therapy on top of eculizumab (116). Final data from this trial are not yet available, despite the company announced that in six poor responders APL-2 treatment resulted in mild increase in hemoglobin and reduction of transfusion burden, with concomitant normalization of LDH (154). Globally, more than 5,000 SC doses of APL-2 (of 270 mg or higher) have been administered to PNH patients within these trials, for a systemic exposure >700 patient weeks. So far, no safety concern has emerged, and even the feared higher risk of infectious complications compared with terminal complement inhibition seems controlled (possibly because of the risk-mitigation strategy exploited by extended vaccination schedules and pharmacological antibiotic prophylaxis). Based on these data, Apellis has launched a large phase III trial in PNH patients with suboptimal hematological response to eculizumab, defined as Hb level <10.5 g/dL; in this study, after a short period of concomitant treatment (to reach APL-2 steady-state), patients will be randomized to continue either eculizumab or APL-2 monotherapy (120).

ACH-4471

ACH-4471 is small oral FD inhibitor developed by Achillion which showed inhibitory activity of hemolysis in PNH *in vitro* (155); it was selected among many candidate agents for its better PK profile (156). A first-in-human study was conducted in healthy volunteers as single ascending dose and 14-day multiple ascending doses; no safety issue emerged (121). Therapeutic doses were used in the range of 200–600 mg, which resulted in significant inhibition of the alternative pathway; bi- or tri-daily administration were anticipated to sustain pharmacological levels of the drug (121). The first study in PNH was a phase II trial enrolling untreated patients with clinically meaningful intravascular hemolysis (122). ACH-4471 was used as monotherapy; the primary endpoint of the study was change in LDH level, whereas hemoglobin level and C3 deposition were included as secondary endpoints (122). This study has completed its recruitment (planned for 10 patients) and results are expected before summer 2019; in the meantime, all enrolled patients are continuing ACH-4471 treatment within an extension study (123). In parallel, a second study was started in PNH patients with poor response to eculizumab (defined as the need of red blood cell transfusion); here ACH-4471 was given as add-on treatment on top of standard eculizumab at three different doses (100, 150, or 200 mg thrice a day) (124). In agreement with the actual reasons which led to the clinical development of proximal complement inhibitors in PNH, the primary endpoint of this trial is the change in hemoglobin levels (124).

LNP023

LNP023 is a small FB inhibitor that, together with small FD-inhibitors, constitutes Novartis' pipeline of potent and orally bioavailable selective inhibitors of the alternative pathway. Similarly to anti-FD agents (146), LNP023 prevents lysis and C3 opsonization of PNH erythrocytes *in vitro* (157). LNP023 is currently under investigation in a phase II trial as add-on therapy in patients with poor response to eculizumab, defined as LDH persistently >1.5 times of the ULN (125). This study aims to investigate safety, PK, PD and efficacy of LNP023 used at the fixed dose orally; while the primary endpoints has been set on better control of intravascular hemolysis (based on LDH values), C3 deposition and hemoglobin changes are included as secondary endpoints (125). The estimated enrollment for this study is 15 PNH patients (125); results are expected in summer 2019.

FUTURE ANTI-COMPLEMENT TREATMENT FOR PNH: GOALS, HOPES AND GUESSES

These are exciting days for the field of PNH, and possibly in a few years new anti-complement therapies will change the standard care of this disease and possibly others. As new data are generated, we will better understand the benefits of complement cascade modulation, and what we should aim to do in the near future. Indeed, a couple of lessons are clear even at this stage of investigations.

Hematological Response and Clinical Benefit During Eculizumab Treatment

Eculizumab treatment largely improves survival in PNH patients (23, 24); apparently, this effect is retained irrespective of the different hematological benefits achieved by individual patients. Thus, while a better hematological response is a worthy therapeutic goal, its possible impact on long-term survival cannot be anticipated, and (if any) may require very long follow up to be demonstrated. We emphasize that the categories of hematological response introduced in this manuscript are useful to identify patients who may benefit from novel strategies of anti-complement treatment, but have not been developed to identify patients who may discontinue current eculizumab treatment. While in these circumstances it seems worth looking for a better strategy to improve therapeutic complement inhibition, we have to recognize that discontinuation of current treatment may have catastrophic impact on patient outcomes and must to be avoided. In this context, it is clear that the positive impact of eculizumab on survival is largely due to the prevention of thromboembolic events, which is not necessarily mechanistically associated with hematological response. Thus, even if we can postulate that novel, more effective strategies of complement inhibition will have similar impact on thromboembolism and thus on survival, this will have to be demonstrated in long-term studies. Further investigations are also needed to better understand the reasons underlying "breakthrough thromboembolisms" occurring during eculizumab treatment, which could be associated with suboptimal complement blockade, or being independent of PNH: in the former case, novel strategies of complement inhibition might have a therapeutic role.

A More Effective C5-Inhibition Is Possible

For more than a decade we have thought that C5 blockade by eculizumab (at standard doses) was the perfect C5 blockade, with the exception of patients carrying the R885H C5 polymorphism; long-term clinical outcome is outstanding, (23, 24) and even persistent, mildly increased LDH is clinically irrelevant (42). However, looking to recent data coming from new anti-C5 agents, it is obvious that therapeutic C5 blockade can be improved not only in terms of patient comfort (e.g., administration route, interval between administrations, self-administration). Recent PK/PD data coming from comparison between ALXN1210 and eculizumab in untreated PNH patients (86) (thus, no selection bias based on previous response to eculizumab) show that free C5 (a PK biomarker rather than a PD one, since it reflects anti-C5 mAb concentration in relation with C5 level, instead of C5 cleavage, which is the activity inhibited by anti-C5 mAbs) consistently remains below the threshold of 0.5 μg/mL (about 0.5% of normal C5 plasma level) throughout the 6-month treatment period with ALXN1210, whereas 12.4% of patients treated with eculizumab at the dose of 900 mg every other week exhibited free C5 above this threshold at the time of some administration during the treatment (132). These data support the clinical observation that about 15% of PNH patients are under dosed with the current approved dose of eculizumab (21, 42, 46), while the treatment schedule developed for ALXN1210 (through a formal dose-finding study) (84) eventually results in a "deeper" (or should we say "better sustained") C5 blockade (132). However, this prevention of transient loss of C5 blockade does not result in evident clinical benefit, since no difference even in terms of LDH levels were seen between ALXN1210 and eculizumab (87, 89). Interestingly, even if the rarity of the event precludes robust conclusions, breakthrough episodes seem to be reduced in ALXN1210 as compared to eculizumab: there were five episodes in the ALXN1210 arm vs. 15 in the eculizumab arm (158). Notably, in the eculizumab arm breakthrough episodes were often (7/15) associated with free C5 >0.5 μg/mL, whereas this was never found in the ALXN1210 arm (158). This observation supports the distinction between PK and PD breakthrough intravascular hemolysis (**Table 4**): the former is always associated with free C5 >0.5 μg/mL due to inadequate plasma level of anti-C5, and can easily be prevented by changing the treatment schedule of eculizumab (21, 42, 46). In contrast, PD breakthrough intravascular hemolysis (e.g., infection-associated triggering massive complement activation) may develop even during more effective therapeutic C5 inhibition, at least with anti-C5 mAb (e.g., increased dose of eculizumab, or standard dose of ALXN1210), and seems to not benefit from any intervention on anti-C5 treatment schedule. Finally, we have to acknowledge that, pharmacodynamically speaking, C5 blockade may be even more effective: indeed, when anti-C5 mAb (eculizumab) was combined with an anti-C5 si-RNA the desired LDH normalization was seen (109); perhaps in these circumstances even the excess of high-affinity C5 convertases (57) could not overcome the complete lack of substrate. It remains an open question whether this better control of intravascular hemolysis will be clinically meaningful.

Proximal Complement Inhibition Is Feasible

Inhibitors of the proximal complement have been specifically designed to address the emerging problem of C3-mediated extravascular hemolysis (**Figure 1**) (28, 64, 66); at the moment, they include agents targeting either C3, complement FD and complement FB. Their clinical development was complicated by the concern about a possible increased risk of infectious and autoimmune complications, but this so far has not been observed in studies. Data are obviously less mature as compared with those of anti-C5 agents, but, as anticipated by their distinct mechanism of action, a more profound clinical benefit may be achieved with these newer agents. Indeed, novel in PNH, the use of therapies able to prevent both intravascular and extravascular hemolysis may lead to major improvement in the resolution of anemia. Moreover, the proof that the pathogenesis of PNH (as a hemolytic disorder) is completely disabled comes from the observation that, in addition to hemoglobin normalization: (i) any sign of hemolysis disappears (normal bilirubin and haptoglobin); (ii) no compensatory erythropoiesis is detected (reticulocyte count normalizes); (iii) life-span of PNH erythrocytes is close to normal (the size of PNH erythrocyte population increases close to that of granulocytes). Not all proximal complement inhibitors are the same, but since data are still preliminary at this stage we cannot dissect the therapeutic efficacy of these different strategies thus far; however, some speculations can be done. Anti-FD and anti-FB agents have the obvious advantage of oral administration (even if the short half-life requires 2–3 administrations per day), which is possibly counterbalanced by the fact that they inhibit only the alternative pathway of complement, that anyhow also serves to amplify the activation triggered through the classical or the mannose/lectin pathways. We can speculate that C3 inhibitors are more likely to achieve the best results even as monotherapy, since they disable all complement pathways. Indeed, if therapeutic C3 inhibition is pharmacodynamically complete, and the PK profile allows a treatment schedule not associated with transient sub-therapeutic plasma levels of the agent, breakthrough hemolysis is not expected; in this case, the concomitant treatment with anti-C5 treatment would be unnecessary. On the other hand, proximal inhibitors of the complement alternative pathway (i.e., anti-FD and anti-FB) might fail in fully preventing hemolysis if complement is activated through the classical or the mannose/lectin pathways (which might be the case of infection-driven hemolysis), even if the inhibition of the alternative-pathway mediated amplification loop may result in substantial reduction of hemolysis in these conditions. Thus, with anti-FD and anti-FB inhibitors, it will have to be investigated whether the highest hematological response expected when they are used in combination with an anti-C5 agent will be retained also in monotherapy. While this conservative approach appears initially appropriate in the interest of patients, it is also possible that, since the alternative pathway is the key pathogenic mechanism of PNH, the addition of an anti-C5 agent is not needed with anti-FD and anti-FB. It is important to acknowledge that, due to the significant increase of PNH erythrocyte mass associated with the prevention of C3-mediated extravascular hemolysis, rebound breakthrough hemolysis may be a concern during treatment with proximal complement inhibitors. This risk needs to be mitigated by treatment schedules which prevent any decrease of complement inhibition even in case of missing doses (especially when frequent dosing is used, and self-administration increases the risk of inadequate compliance).

Clinical Endpoints Need to be Redefined

In a chronic, life-threatening disease such as PNH short-term surrogate endpoints are needed for clinical trials. In the eculizumab era, when PNH patients were heavily transfused, transfusion avoidance and hemoglobin stabilization were obvious goals which have been achieved, together with reduction of LDH (which served as a biomarker of disease activity rather than as an endpoint *per se*). Now, it is a matter of debate in identifying the clinically meaningful endpoints that can be exploited in future trials investigating novel anti-complement agents. Logically, LDH is an obvious choice, since it is a marker of intravascular hemolysis (i.e., of disease activity) and it remains slightly increased even during effective eculizumab treatment. Many new anti-C5 agents were designed to deliver more effective C5 blockade, and changes in LDH was used as primary endpoint in their trials; however, it remains debatable if any reduction in LDH (e.g., from the median 1.5 times the ULN seen with eculizumab, to normal levels) by itself may be considered a clinically meaningful endpoint. The frequency of breakthrough hemolysis is another endpoint that has been exploited, but it appears specifically linked to suboptimal dosing of eculizumab; indeed, at least PK breakthrough should not be a problem with novel anti-C5 agents, while PD breakthrough remains possible (at least with anti-C5 agents used in monotherapy). Likely, more robust goals are needed, and the hemoglobin level appears a more proper endpoint given its intimate association with clinical manifestations and a consequence of all the pathogenic events underlying PNH (intravascular hemolysis, extravascular hemolysis and bone marrow function). Notably, improvement of anemia was not the only benefit of eculizumab treatment, since the most important effect impacting long-term survival was the reduction of thromboembolic events (22). Unfortunately, the time-frame to assess the thromboembolic risk is extremely long, and surrogate endpoints would be welcome. At the moment there is no evidence concerning the possible relationship between low-level residual intravascular hemolysis or breakthrough hemolysis and thromboembolic risk, but it is conceivable that a more effective inhibition of the terminal complement pathway (and possibly of the whole complement cascade in general) might have an effect also on thrombophilia of PNH (159). Nevertheless, despite this considerable scientific background, caution should be used and the efficacy of newer anti-complement agents on prevention and treatment of PNH-related thromboembolism needs to be carefully investigated, since it will obviously impact long-term morbidity and mortality.

Toxicity of Standard and Novel Complement Inhibition

Toxicity remains a major concern for any novel treatment, especially in a disease with such a good long-term outcome as PNH treated with eculizumab. Historically, the development of anti-complement treatment raised several concerns about the risk of infectious complications. Indeed, after cases of infection

by *N. meningitidis* seen in initial phases of clinical development of eculizumab (still in indications other than PNH, such as autoimmune diseases), vaccination against this microorganism was mandatory for all patients prior to initiating C5 blocking therapy. Data from post-marketing pharmacovigilance did not lead to any alert concerning the risk of meningococcal infection, nor of any other life-threatening infection (160). Indeed, after more than 10 years of experience with eculizumab it appears evident that the initial concern of infectious toxicity has been largely mitigated, also as a result of prophylactic measures routinely employed in PNH patients on eculizumab. Theoretically, most anti-C5 agents will recapitulate the safety profile seen with eculizumab; however, we must acknowledge that a more effective C5 blockade (which appears to be a clinical goal) might also result in increased risk of infectious complications. In other words, while the low-level residual C5 activity (as demonstrated by slightly increased LDH) seen on eculizumab may be detrimental for hematological benefit, it might be sufficient to eradicate microorganisms at time of infections. Notably, in considering the molecular mechanisms, this low-level residual C5 activity may be due to high-affinity C5 convertases (57) generated on bacteria, which would displace free C5 from eculizumab similarly to what we have described for PD breakthrough. In moving from a more effective C5 blockade to a blockade of the proximal complement the discussion becomes even more complicated: these agents were designed to completely shut down complement (to prevent any residual hemolysis of PNH erythrocytes), but this may eventually lead to higher risk of infectious complications. Some insight may be obtained by analyzing rare families carrying genetic deficiencies of these complement components (161–164). Subjects with inherited C3 deficiency (about 20 families described so far) seem to have an increased risk of infections by encapsulated bacteria (e.g., *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*), which tend to be severe and recurrent (161–164), as well as some risk of autoimmune diseases (165). Recurrent infections have been described also in very rare subjects with inherited deficiencies of properdin (166), complement FD (167), and complement FB (168) even if in this case the clearance of infectious agents may be addressed by the classical pathway, as demonstrated *in vitro* (169). However, all these infections occur usually in childhood, and tend to become infrequent when adaptive immunity has established. These observations, together with the consideration that therapeutic inhibition is not a phenocopy of inherited deficiencies (i.e., the role of complement in the development of innate immunity is fully preserved), support the concept that therapeutic inhibition of proximal complement should be feasible (170). This assumption seems confirmed by recently available data from systemic C3 inhibition, which was not associated with any increased infectious risk, at least with a conservative risk-mitigation strategy based on strict prophylactic measures (117). Nevertheless, the safety of each of these newer agents will require careful monitoring, and possible off-target effects (including any detrimental effect on the bone marrow function) need to be ruled out.

And the Winner Is …

The definition of the best drug for PNH treatment is timely; however, we must think about the best strategy rather than about the best drug. Of course, companies are motivated to demonstrate that one drug is better than another, but this is not necessarily useful for patients: we first need to better understand what is the greatest clinical benefit that may be achieved in PNH patients. We have already discussed available results with each of these novel anti-complement agents, together with their mechanistic goals; interestingly, some agents are used both in monotherapy and in combination with standard anti-C5 treatment, emphasizing that clinical data are essential to prove the initial hypothesis. Novel strategies of C5 inhibition definitely address some patients' needs, such as possible self-administration or extending the interval between IV dosing; however, it is likely that they will not lead to superior clinical benefit, except better patients' convenience. Some novel anti-C5 agents might deliver a more effective C5 blockade, but the actual benefit to patients of a further LDH reduction is uncertain. Indeed, even the best C5 inhibition seen with the combination of eculizumab with the anti-C5 si-RNA will control only residual intravascular hemolysis, with a hematological benefit that for the majority of patients is minor. It is most likely that the next breakthrough in PNH treatment will come from the inhibitors of the proximal complement pathway: anti-C3, anti-FD and anti-FB agents. Preliminary data clearly demonstrate that by interfering with the complement cascade upstream they inhibit MAC-mediated intravascular hemolysis and prevent C3-mediated extravascular hemolysis; but how profound is the inhibition of these targets is unclear. The lesson from the anti-C5 was very instructive: even minimal residual amounts of these complement proteins may be enough to keep complement activity almost intact, likely because they are substrates or very active enzymes generated at time of complement activation. Proximal inhibitors appear to disable all disease mechanisms in hemolysis of PNH; however, we still don't know whether inhibition of C3 is pharmacologically effective enough to prevent possible residual activity (as demonstrated for C5, for instance for PD breakthrough), and whether their targets in the complement cascade may be somehow by-passed in specific clinical circumstances (e.g., for anti-FD and anti-FB in case of complement activation through the classical and mannose/lectin pathways; and for anti-FD also in case of C3 activation by other plasma protease, i.e. FD by-pass) (171). This information is essential to understand whether proximal complement inhibitors can be used as monotherapy, or whether a combination with an anti-C5 agent is needed to achieve a better hematological response. Indeed, the hypothesis of a combined treatment with anti-C5 and proximal inhibitors is also supported by *in vitro* data showing synergy between eculizumab and anti-FD (172). These data may raise the hypothesis that a less than optimal inhibition of two different steps of the complement cascade (e.g., an anti-C5 agent combined with a proximal inhibitor) may result in similar hematological benefit, with mitigation of the infectious risk due to residual, low-level "protective" complement activity.

Thus, we envision a new scenario in the treatment of PNH where inhibitors of the proximal complement (either anti-C3, anti-FD or anti-FB) are essential, and if pharmacologically adequate may be used even in monotherapy to control intravascular and extravascular hemolysis. Alternatively, these proximal inhibitors might require use in combination with

anti-C5 agents, possibly long-acting, to maximize therapeutic efficacy. In this scenario, anemia from both intravascular and extravascular hemolysis would be fully prevented, and normal hemoglobin is expected in absence of bone marrow failure. In this new scenario, we must not forget that the future pricing of these new agents remains a major issue. Unfortunately, national pricing scheme policies on orphan medicine products has allowed exaggerated prices, which seem not always justified by their cost (i.e., manufacturing, research, and development) and their actual clinical value (i.e., impact on life expectancy and quality of life) (82). Indeed, in PNH the very high price of the only approved drug eculizumab has hindered its use in many countries, and even in countries where it is available its prescription was restricted to PNH patients with more symptomatic disease (i.e., severe anemia, or symptomatic hemolysis or life-threatening thrombosis). While the goal of very high profit has driven the interest of pharmaceutical companies, now we hope that this competition will lead to a considerable reduction of drug price. This remains a major need in a world-wide scientific/medical community, since, from the point of view of scientists and physicians working in the field, one additional goal of these exciting developments is to provide effective drugs with reasonable prices, allowing a broad use of effective treatments worldwide.

CONCLUSIONS

In the last decade we have been able to offer PNH patients an almost-normal life-expectancy, irrespective of their disease (23, 24): this was a terrific milestone in medicine. Nevertheless, new challenges and goals are coming, and we are starting to wonder what's in store for PNH patients in the next decade. Thanks to the first therapeutic C5 inhibitor eculizumab we have controlled the most debilitating symptoms of PNH, and prevented the most devastating life-threatening complications, such as thromboembolism. Perspectives of further improvements seem within reach with the introduction of therapies which are less burdensome for patients and may act on their perception of illness: indeed, very long intervals of dosing and/or self-administration of second generation complement inhibitors may help patients become less challenged by their disease. Shall we go beyond this? We think so, and we feel now ready for a real breakthrough in the field coming with the introduction of proximal complement inhibitors. Perhaps the time when PNH patients may live with their aberrant blood cells while keeping normal hemoglobin values and having no signs or symptoms of disease is not too far away. In the next few years this will represent a major achievement in the field, while in the long-term we will continue to pursue our goal of a definitive cure for PNH.

AUTHOR CONTRIBUTIONS

AR, RN, and RP conceived the study and identified the other experts who contributed to the generation of the consensus. AR, AK, RC, PS, RN, and RP wrote the manuscript and together with the other SM, PR, LM, CF, FC, and MS generated the consensus on all the topics discussed in the manuscript. All the authors have critically revised the manuscript and contributed to its preparation in the current version.

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REVIEW
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Therapeutic Modulation of the Complement Cascade in Stroke

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Stroke is a leading cause of death and disability worldwide and an increasing number of ischemic stroke patients are undergoing pharmacological and mechanical reperfusion. Both human and experimental models of reperfused ischemic stroke have implicated the complement cascade in secondary tissue injury. Most data point to the lectin and alternative pathways as key to activation, and C3a and C5a binding of their receptors as critical effectors of injury. During periods of thrombolysis use to treat stroke, acute experimental complement cascade blockade has been found to rescue tissue and improves functional outcome. Blockade of the complement cascade during the period of tissue reorganization, repair, and recovery is by contrast not helpful and in fact is likely to be deleterious with emerging data suggesting downstream upregulation of the cascade might even facilitate recovery. Successful clinical translation will require the right clinical setting and pharmacologic strategies that are capable of targeting the key effectors early while not inhibiting delayed repair. Early reports in a variety of disease states suggest that such pharmacologic strategies appear to have a favorable risk profile and offer substantial hope for patients.

Keywords: complement, vascular disorders, complement activation, cerebral blood flow, complement cascade, stroke therapy

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PUBLIC HEALTH IMPACT OF STROKE

Worldwide, stroke is the second leading cause of death, and the third leading cause of disability-adjusted life-years (DALYs), with a staggering lifetime risk after age 25 of 26.5% (1, 2). Even more concerning is the fact that the global incidence for both major stroke subtypes is increasing (37% for ischemic and 47% for hemorrhagic over the last 20 years). Moreover, despite broad advances in general medical care, stroke-related deaths and DALYs have also increased by 26 and 19%, respectively. It is also important to note that stroke is not merely a disease of the old and wealthy, as those living in low- and middle-income countries and those younger than 75 years of age make up the majority of victims.

CURRENT TREATMENT OF ACUTE ISCHEMIC STROKE

Since 1995, there have been a number of major advances in the prevention and treatment of stroke, most notably, the widespread advent of contemporary anti-platelet therapy, statin therapy, and the introduction of highly effective anti-hypertensive regimes (3). Particularly impactful has been the development of intravenous pharmacologic thrombolysis and intra-arterial mechanical thrombectomy following acute ischemic stroke (4). That said between 25 and 50% of patients who are eligible for intravenous therapy within 9 h of stroke onset still suffer at least moderate disability

(5–7). For those presenting with anterior circulation large vessel occlusions, substantial reperfusion is now achievable for 85% of patients, in some instances as late as 24 h after onset. Yet, 30–50% of patients eligible for this therapy are disabled as well, despite fairly small areas of core infarction (8, 9). Together, these data suggest that therapeutic targeting of post-reflow microvascular failure may have significant clinical utility for a substantial proportion of ischemic stroke patients.

IMPORTANCE OF INFLAMMATORY MICROVASCULAR FAILURE ON STROKE OUTCOME

While post-stroke inflammatory microvascular failure is governed by multiple biological cascades (10), our group long ago implicated the complement cascade as one of the central players that is potentially druggable (11). The first data suggesting that the complement cascade was involved in secondary tissue injury following re-perfused stroke dates to the late 1990s when we showed that soluble complement receptor 1 (sCR1), and its Sialyl-Lewis X glycosylated form (sCR1-sLex), which targeted complement inhibition to sites of activated endothelium, reduced ischemic tissue injury, improved outcome and penumbral blood flow (11). Nevertheless, 20 years of research has yet to result in the development of a clinically relevant anti-complement or even anti-inflammatory therapy for re-perfused stroke. Below we review the existing data implicating the complement cascade in cerebral ischemia reperfusion injury. We will not attempt to reiterate all that has been presented in two recent and exhaustive reviews (12, 13), but rather will attempt to present the data in a manner that facilitates clinical translation. In doing so, we will attempt to address the apparent inconsistencies in the data obtained from different model systems, as well as, the differences in the effect of acute and subacute treatment on cell death, cell survival and tissue regeneration.

IN VITRO DATA EXAMINING THE ROLE OF COMPLEMENT IN CEREBRAL TISSUE HOMEOSTASIS AND ISCHEMIC INJURY

Primary astrocyte, neuronal, and glial cell cultures express many of the complement cascade components (14). Together with resident microglia, these cells generally utilize the complement cascade to respond to infection. In addition, the complement cascade appears to be involved in both central nervous system development and tissue repair, in part by directing synaptic pruning and plasticity (15). Specifically, C1q and C3 tag weaker synapses for microglial removal and C3a and C5a and their receptors appear to be involved in cerebellar development (16, 17). C3a has also been shown to be involved in neuronal differentiation and maturation of neural progenitor cells (NPCs). It additionally guides migration of NPCs using an ERK 1/2 signaling pathway (18) and protects astrocytes from ischemia-induced cell death (19). C5a has also been shown to make microglia more resistant during ischemia by limiting the toxic effects of glutamate (20). Both C3a and C5a appear to be protective in culture against NMDA- and Kainate-induced neurotoxicity, respectively (21, 22). C1q drives neuronal survival in primary cultures and sublytic MAC has a similarly positive effect on oligodendrocyte progenitor cell cultures (23, 24). Complement receptor 2 (CR2) via C3d binding may regulate hippocampal neurogenesis in the adult rodent dentate nucleus (25).

Simulated ischemia via oxygen glucose deprivation (OGD) induces the production of neuronal C1q and C3 which are associated with pro-apoptotic caspase-3 activation (26). Inhibiting C3 expression via siRNA enhances cultured neuronal viability (27). Blocking C5a reduces ischemia-induced apoptosis in culture (28). These results were less dramatic in human neuronal cultures due to the high expression of complement modifying inhibitors CD59, CD46, and CD55 (29).

Together the *in vitro* data suggests that in the absence of ischemic stress, components of the complement cascade are for the most part important to cell survival and appear to be potentially helpful in recovery of function after injury. By contrast, following ischemic stress, complement cascade activation appears to mediate apoptotic cell death, at least acutely.

IN VIVO EXPERIMENTAL DATA EXAMINING THE ROLE OF COMPLEMENT IN CEREBRAL TISSUE INJURY AND REPAIR FOLLOWING ISCHEMIC INJURY

Initiation of Complement Activation Following Cerebral Ischemia Reperfusion

It appears that ischemia-reperfusion results in the upregulation of the complement cascade in a number of ways. Recently, some data suggests that plasmin and thrombin released at the site of thromboembolism may play a role in complement activation independent of the classical, alternative, and lectin pathways (30–32). That said, more recent data in non-human primates suggests that this process is unlikely to be the source of most of the complement generated following cerebral ischemia-reperfusion injury in the context of most reperfused human ischemic strokes (33). Therapeutic treatment with recombinant tissue plasminogen activator also is known to activate the complement cascade via a plasmin-mediated MBL-independent extrinsic/alternative pathway, but the degree to which this is relevant in clinical stroke is similarly unknown (34).

What is clear, however, is that, even in the absence of physiologic or therapeutic clot lysis, the ischemic endothelium expresses neo-epitopes, such as non-muscle myosin, annexin IV, and a subset of phospholipids that are recognized by circulating self-reactive IgM antibodies (35). Following ischemic injury, mannose-binding lectins (MBL) deposit on the activated endothelium. The antibody complexes activate the lectin pathway by reacting with MBL and the ficolins. Carbohydrate-bound MBL then cleaves C2 and C4, forming the C4b2a (C3-convertase), which cleaves C3, and initiates the distal complement cascade (36). Preventing MBL function through genetic deletion or pharmacological manipulation is thought to be protective (37). Furthermore, even in the absence of

thrombin or plasmin-mediated activation of the alternative pathway, amplification of this process appears to be driven by the alternative pathway. Efforts to block either this alternative pathway activation (38) or neo-epitope dependent complement activation (39) have proven extraordinarily effective in improving outcome in rodent models of cerebral ischemia-reperfusion injury (cIRI), as evidenced by reductions in infarct size, reduced apoptotic cell death, reduced neurological deficit scores throughout the recovery period, reduced gliosis, improved neurogenesis and angiogenesis even when employed 24 h post-reperfusion, with the models including aged mice, female mice, and mice exposed to varying severities of ischemic injury (40).

Evidence that the classical pathway is not the primary initiator of complement cascade activation in focal cIRI, at least in adult animals, comes from the observation that C1q deficient mice ($C1q^{-/-}$) are not protected from cIRI and the C1q that is initially expressed appears to be produced predominantly by microglia rather than neurons (37, 41, 42). By contrast, $C1q/MBL^{-/-}$, $MBL^{-/-}$, and factor $H^{-/-}$ mice are all protected from focal cIRI, albeit to differing degrees depending on variations in the model system utilized (43, 44). Moreover, animals treated with a lectin pathway inhibitor, Polyman 2, or, as previously mentioned, an anti-MBL antibody are similarly protected as are those treated with CR2-fH, a targeted alternative pathway inhibitor (44, 45).

Despite the overwhelming amount of data implicating upstream initiation of the complement cascade by the alternative and lectin pathways, there are further data in an alternative model of cerebral ischemia that require mention. Neonatal mice subjected to a global ischemia reperfusion injury are protected by genetic deletion of C1q (46). In this model system, the severity of the ischemia is markedly less and the apoptotic burden is likely more significant than it is in focal stroke in adults (47). Given that this is dissimilar from what is seen in focal cIRI in adult animals including humans, and the fact that C1q deficiency may pre-dispose to epilepsy as a result of uncontrolled synaptogenesis, with post-stroke epilepsy worsening outcomes (48), attempts at therapeutic manipulation in clinical cIRI should likely avoid strategies which include acute C1q blockade. In fact, this hypothetical downside to early C1q blockade, may in part explain the failure of sCR1 treatment to improve cIRI in non-human primates (49).

Downstream Mediators of Complement-Dependent Acute Tissue Injury Following Cerebral IRI

Cleavage of C3 and generation of the anaphylatoxin (C3a), as well as the opsonins (iC3b, C3dg and C3d) and formation of C5 convertase (from C3 and C3b), which in turn generates C5a and C5b which aggregate with C6, C7, C8 and several C9s to form the MAC, could all potentially be involved in tissue injury following re-perfused stroke. However, most of the data suggest that, early following reperfusion, tissue injury is mediated principally by C3a and to a lesser extent C5a while MAC is not pathologically important, but rather, simply, a marker of complement cascade activation (42, 50, 51). The first evidence of this was the finding that $C3^{-/-}$ mice were markedly protected, while $C5^{-/-}$ mice were not protected (38, 42). Similarly, C3aRA treatment early following stroke not only protected mice but enhanced delayed, post-stroke neurogenesis (50, 52). $C6^{-/-}$ mice were also not protected (44), and while C5a receptor antagonist (C5aRA) (53) and C5 anti-body (54) treated mice were protected, the protection was more modest. These data together with the remarkably effective C3 site-directed therapy via CR2-Crry treatment (39), lead one to conclude that most complement mediated tissue injury following focal cIRI occurs as a result of C3 cleavage with the generation of C3a and binding of the C3aR. C5a binding of C5aR likely plays a lesser but collaborative role.

Complement Cascade in Cerebral Repair and Recovery

While C3 depletion or blockade is protective in re-perfused stroke, especially when blockade is acute (52), germ-line depletion or chronic blockade results in loss of functional

TABLE 1 | The Role of the complement cascade in central nervous system cell clearance, viability, and cerebral tissue repair as well as in experimental cerebral ischemia reperfusion injury and human stroke.

Component of the complement cascade	Significant role in cell clearance	Significant role in experimental inflammatory microvascular no-reflow	Significant role in cerebral tissue repair	Significant role in cerebral cellular viability *in vitro*	Implicated in the pathogenesis of adult human stroke
C1q (classical pathway)	Yes (15–16)	No (44–46)	Yes (27–9)	Yes (26)	No
Mannose binding lectin (lectin pathway)	–	Yes (39, 45, 47, 49)	–	–	Yes (70, 72, 74–6, 78)
Factors D/B/H (alternative pathway)	–	Yes (40, 41, 43, 48)	–	–	
C3	Yes (16)	Yes (40, 41, 45)	Yes (23)	Yes (30, 36, 74)	Yes (66, 69, 84)
C3a/C3aR	No	Yes (54, 56–7)	Yes (57, 64)	Yes (17, 21, 24)	Yes (67)
C3b	Yes	–	–	–	No
CR1	Yes	Yes (41)	–	–	No
CR2	–	–	Yes (28)	–	No
C5	–	Yes (63)	–	–	Yes (81)
C5a/C5aR	No	Yes (62)	–	Yes (22, 25, 31)	Yes (71)
C5b–9	Yes	No (48)	Maybe	Yes (27)	Yes (86)

protection at later time points as stroke recovery is impaired by decreased neurogenesis. This impairment of neurogenesis by complement blockade was most pronounced in models of experimental rodent stroke lacking reperfusion where even acute blockade is known to be ineffective (55). In an equally non-reperfused model, intranasal treatment of mice undergoing permanent photothrombotic stroke with C3a in the late subacute period resulted in enhanced functional recovery (56). These data, together with the *in vitro* data presented above, strongly argue for limiting the clinical use of anti-complement therapeutics to the acute period during which reperfusion injury is most active (e.g., <72 h post-reperfusion). **Table 1** summarizes the current knowledge of different components of the complement cascade and their roles in the central nervous system, tissue repair, ischemia reperfusion injury, and human stroke.

CLINICAL DATA FROM HUMANS SUFFERING STROKE SUGGESTING THAT MODIFICATION OF COMPLEMENT CASCADE ACTIVATION MIGHT IMPROVE OUTCOME

Evidence of Complement Cascade Activation in Human Ischemic Stroke and Association With Outcome

Polymorphisms in C3, C5, and factor H are associated with an increased incidence of ischemic stroke as are higher plasma levels of C4 and C5 (57–60). Polymorphisms in MBL are associated with a better outcome after ischemic stroke (43, 61). In patients suffering a stroke, plasma levels of C3a, C3, C4, C5, C5a, factor B, MBL, MASP-1/2, and MAC are all elevated and ficolin-1, ficolin-2, and ficolin-3 reduced (62–66). Increased serum levels of C3, C3c, C4, and MBL are all associated with increased stroke severity, and patients who are MBL-sufficient have higher C3 plasma levels and suffer worse stroke outcomes (43, 67). Reduced early (within 6 h of onset) ficolin-1, a marker of activation of the lectin pathway, is independently associated with unfavorable outcome in adult human stroke (68). Moreover, postmortem studies have identified both complement and IgM deposition in the brain after stroke (69).

In addition, there is data in several other clinical scenarios of cerebral ischemia reperfusion injury where the complement cascade is activated. In cardiopulmonary bypass, glial injury is associated with complement cascade activation (70), and while anti-C5 monoclonal antibody therapy with a problematically long half-life did not improve overall cognition, it did improve outcomes in visuo-spatial functioning both acutely and at 1 month (71). Clinically relevant cerebral reperfusion injury is also experienced by a quarter of patients undergoing carotid endarterectomy for carotid stenosis (72). Interestingly MBL, C3, factor H, and C5 polymorphism correlate with both early and late cognitive dysfunction, and C3a levels are not only increased in those injured, but also correlate with duration of cross-clamp (a surrogate of severity of ischemia), and are predicted by MBL polymorphisms (73–75). In patients with

TABLE 2 | Complement inhibitors in clinical development with potential utility in clinical stroke (94).

	C1-INH berinert cinryze	OMS-721	ACH-4471	LPN-023	ACH-5228	Novartis oral agent	Small molecule anti-fD	APL-2/9	AMY-101	APT-070 mirococept	Eculizumab	ALN-CC5 cemdisiran	Coversin	Avacopan
Classical pathway	×													
Lectin pathway	×	×												
Alternate pathway (factor B)				×		×								
Alternate pathway (factor D)			×		×		×							
C3								×	×	×				
C3a/3aR														
C5										×	×	×	×	
C5a/5aR														×
Company	Pharming behring shire	Omeros Achillion		Novartis Achillion		Novartis	RaPharma	Apellis	Amyndas	Inflazyme	Alexion	Alnylam	Akari	Chemocentryx

carotid stenosis, the degree of plaque burden, the severity of the stenosis and the instability of the plaque is correlated with elevated circulating levels of MAC and reduced circulating levels of ficolin-2 (76, 77). Finally, in comatose post-cardiac arrest patients, who suffer global cerebral ischemia reperfusion, C3a/C3 ratios independently predict survival (78).

Evidence of Complement Cascade Activation in Hemorrhagic Stroke and Association With Outcome

While less common than ischemic stroke, hemorrhagic stroke is associated with even worse outcomes and also exhibits complement cascade activation. This is important given that clinical efforts to revascularize ischemic stroke patients can result in either intracerebral or subarachnoid hemorrhage. In subarachnoid hemorrhage, C3a and M-ficolin levels are elevated in serum, cerebrospinal fluid and in the brain parenchyma and they are correlated with secondary ischemic injury and functional outcome (79–81). In patients suffering intracerebral hemorrhage, M-ficolin levels are similarly elevated and polymorphisms in factor H as well as iC3b levels are associated with functional outcome and mortality (51, 82). These observations support the experimental intracerebral hemorrhage data from rodents showing that inhibition of C3a and C5a receptors improve outcome with the effect at least in part due to amelioration of IL-1 dependent perihematomal edema formation (83, 84).

Blood-Brain Barrier Dysruption Following Human Stroke and Its Implication for Systemic Intravascular Complement Blockade vs. Intracerebral Blockade

There has been considerable speculation regarding the importance of the blood brain barrier in developing therapeutic strategies for central nervous system disease. Cerebral ischemia reperfusion injury differs from most of these other diseases in two important aspects. First, the blood brain barrier is opened to some degree following cIRI (85–87). Moreover, this opening is most profound in the time frame where anti-complement strategies are likely to prove most protective (39) prior to when regenerative and repair mechanisms appear most important. Secondly, it appears that even if anti-complement strategies fail to cross the blood brain barrier, much, if not most, of their beneficial effect is likely to be mediated intravascularly at the level of the cerebral arteriole and capillary. In this respect, anti-complement therapies are distinct from most traditional neuroprotective strategies aimed specifically at increasing the tolerance of neurons to ischemia and the downstream intracellular cascades that result. That said, even anti-complement therapies with large molecular sizes, such as Eculizumab, appear to be able to cross even a moderately impaired blood brain barrier in neuromyelitis optica and improve clinical outcome (88). This portends even better results for smaller anti-complement therapies.

Complement Blockade in the Setting of Intravenous Thrombolytic Therapy

The complement cascade has been implicated in cross-talk between the inflammatory cascade and the thrombotic cascade. While in some settings complement inhibition has been shown to prolong bleeding times, the use of a variety of anti-complement therapies together with intravenous thrombolytic therapies in cIRI has consistently shown enhanced outcomes with no increase in bleeding and in some instances a reduction in hemorrhagic conversion, a complication of ischemic stroke, possibly due to stabilization of the blood brain barrier (34, 89). This is additionally important for use with pharmacologic thrombolytic therapies that can exacerbate hemorrhage and edema (34).

Clinically Available Complement Cascade Modifying Therapies for Clinical Translation

While it has widely been reported that anti-inflammatory strategies shown to be effective in experimental model systems fail to improve outcome following human stroke, most of this failure can be explained by the fact that these strategies are only useful in limiting reperfusion injury. Unfortunately, the patients included in these largely underpowered phase II trials do not have documented reperfusion and less than a third would even be expected to have a penumbra sufficient to demonstrate protection. Scientifically unjustified administration schedules based on incompletely studied pharmacokinetics has only further hindered efforts. Even so, despite all of these deficiencies, the most recent trials have shown some modest signal of benefit in both functional outcome and quality of life (90).

To date, anti-complement strategies have not been trialed in human stroke. Given the data presented above one would expect that either inhibition of the lectin and, or, alternative pathway might prove useful, as might inhibition of C3 convertase or the downstream anaphylatoxins, C3a and C5a. While recombinant C1-INH is clinically available as Cinryze, Ruconest, and Berinert, an important, but fairly rare side effect is pro-thrombotic events which would be potentially disastrous in the setting of ischemic stroke (91, 92). While anti-C5 Eculizumab, is well-studied and has proven beneficial for a variety of diseases, both in and outside of the central nervous system, its inability to block C3a binding of C3aR and its extremely long half-life (>10 d) makes its development less attractive. Selective alternative pathway inhibitors might also prove somewhat beneficial but the anti-C3 compstatin analogs seem to hold the most promise. Mechanistically, the latter block C3a and C5a generation, regardless of the upstream pathway responsible for activation of the cascade. They also appear to be safe in a variety of human diseases and have been designed with short half-lives that allow for ischemic protection without negatively impacting repair and recovery (93). The complement inhibitors currently in clinical development are listed in **Table 2**.

AUTHOR CONTRIBUTIONS

EC, AC, BC, AK, and JS contributed meaningfully and to the research and acquisition of data for this review, along with the subsequent analysis, drafting and revising of the paper. EC was the primary author due to his substantive drafting contributions, but all other authors provided equal contributions for the paper's creation.

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REVIEW
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Complement Inhibitors in Clinical Trials for Glomerular Diseases

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Defective complement action is a cause of several human glomerular diseases including atypical hemolytic uremic syndrome (aHUS), anti-neutrophil cytoplasmic antibody mediated vasculitis (ANCA), C3 glomerulopathy, IgA nephropathy, immune complex membranoproliferative glomerulonephritis, ischemic reperfusion injury, lupus nephritis, membranous nephropathy, and chronic transplant mediated glomerulopathy. Here we summarize ongoing clinical trials of complement inhibitors in nine glomerular diseases and show which inhibitors are used in trials for these renal disorders (http://clinicaltrials.gov).

Keywords: inhibitors, clinical trials, glomerular disease, C3 glomerulopathy, complement, ANCA, aHUS

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INTRODUCTION

Defective complement action is a cause of several human glomerular diseases including atypical hemolytic uremic syndrome (aHUS), anti-neutrophil cytoplasmic antibody mediated vasculitis (ANCA), C3 glomerulopathy, IgA nephropathy, immune complex membranoproliferative glomerulonephritis, renal ischemic reperfusion injury, lupus nephritis, membranous nephropathy, and chronic transplant mediated glomerulopathy (1–3).

Pathology of these kidney disorders are caused or modified by (i) genetic alterations in complement genes that lead to impaired protein expression and/or function (4), (ii) by autoantibodies that target complement components or regulators, (iii) by autoantibodies which recognize specific surface structures, DNA and IgGs, and upon binding initiate complement or (iv) as recently shown by altered plasma levels of FHR-modulators (5). Understanding how the gene variants deregulate complement, or how disease relevant autoantibodies interfere in the cascade help to understand the underlying pathomechanisms of these renal disorders and allow to pinpoint for each disease which step and which level of the cascade is compromised or modified. This provides a rationale for treatment with complement inhibitors (5), and allows to address the questions, where and at which level the complement cascade should be targeted and for how long a complement inhibitor should be used.

The complement inhibitors Eculizumab (Soliris), Berinert, or Cinryze are currently approved by the Food and Drug administration (FDA) in the US and the European Medicines Agency (EMA). A new generation of complement inhibitors is currently evaluated in clinical trials and new inhibitors are being developed and tested in preclinical settings. Different types of inhibitors exist, including humanized monoclonal antibodies, small proteins binding to specific complement components, and furthermore recombinant proteins allowing substitution of defective or absent proteins, as well as a small interfering RNA.

Here we summarize the ongoing clinical trials of complement inhibitors in nine glomerular diseases and show which inhibitors are used in trials for these renal disorders (http://clinicaltrials.gov).

COMPLEMENT: INITIATION, MAJOR ENZYMATIC CHECK POINTS, AND MULTIPLE EFFECTOR LEVELS

To appreciate the many steps of complement activation and the fine balanced regulation an overview of this important homeostatic system is presented. The complement cascade has three activation routes, acts via two major enzymatic levels, which provide central checkpoints for the control of effector response and initiate the pore forming terminal complex (**Figure 1**). Overactive effector pathways drive and enhance specific responses that cause kidney pathology (6). The progression of each step and specific activation pathways are regulated by selectively acting endogenous inhibitors and this control provides the rational to where and at which level activation and pathway progression can be influenced by therapeutic agents.

Initial spontaneous activation of the complement cascade by the alternative pathway (AP) occurs in the fluid phase, activation proceeds and propels on surfaces. The lectin (LP) and the classical pathway (CP) are initiated on target surfaces by different recognition and initiator proteins (7, 8). Upon activation two central enzymatic levels are formed, both of which cleave a soluble complement compounds C3, C4, and C5, and generate small, soluble and cell attracting inflammatory mediators, i.e., C3a, C4a, and C5a and also form the surface acting effector compounds, C3b, C4b, and C5b. The first enzymatic level is mediated by two newly formed C3 convertases with overlapping activity (9). Both the AP and the LP/CP convertase (C3bBb and C4bC2b, respectively) cleave the central complement protein C3 and generate the soluble inflammatory mediator C3a and surface acting C3b which opsonizes target surfaces (10). In addition this level can generate a potent, self-amplifying loop which enhances and propels C3 conversion, generates more effector products and thereby increases the density of deposited C3b can subsequently be processed by specific proteases which are assisted by cofactors and regulators. The processed variants, iC3b, C3dg, or C3d are recognized by different receptors and mediate important effector functions (6).

Subsequently when activation proceeds a second enzymatic level is formed. Two C5 convertases, i.e., the AP pathway (C3bBbC3b) and the LP/CP pathway (C4bC2bC3b) convertases, which use C5 as substrate are generated. C5 cleavage produces the potent soluble anaphylatoxin C5a and C5b is deposited on the target surface (10). Surface deposited C5b initiates the third major effector part and forms the pore forming terminal complex C5ab-9, also termed TCC (terminal complement complex), which inserts in the target membrane, forms a pore and causes cell lysis (**Figure 1**).

Thus, complement when activated generates several effectors. The major and primary soluble effectors are the anaphylatoxins C3a, C5a, and C4a that by recruiting and activating immune cells, induce complement inflammation. Recently PAR1 (protease-activated receptor) and PAR4 were identified as non G-coupled receptors for C4a and a FHR1 receptor in form of EMR1 was reported (11, 12). The major surface acting effectors are C3b, the C3- and the C5 convertases and C5b-9. C3b deposition results in opsonophagocytosis and C5b-9 deposition induces the pore forming, membrane damaging C5b-9 complex, and soluble C5b-9 likely has pro-inflammatory activity (1, 2).

Complement action can initiate in the fluid phase and on surfaces and a large panel of regulators controls cascade progression at many sites and in specific steps. Regulators which are distributed in the fluid phase or are expressed on cellular surfaces control initiation of each activation pathway, formation and action of the C3 convertases, density and type of deposited C3b, the fate of C3b, formation and action of the C5 convertase, fate and half-life of the anaphylatoxins and formation of the terminal complement complex.

An important challenge is to understand complement regulation, define how the activators and the many regulators cooperate with each other, understand which inhibitor acts at which site and furthermore how and when the absence of one single regulator, or a single defective regulator affects the concerted action and disturbs the cascade. Understanding of the homeostatic role of this evolutionary old system, and characterizing the precise action of each single regulator and their interplay is highly relevant to elucidate the pathological principles of complement associated disorders. This ultimately allows to design precisely acting complement targeting therapeutic agents.

DISEASES

Hemolytic Uremic Syndrome (HUS)

HUS, hemolytic syndrome (HUS) is characterized by an over activated complement system. This disease which has different triggers is defined by hemolytic anemia, thrombocytopenia and acute renal damage resulting in thrombotic microangiopathy (TMA). The majority of HUS cases are caused by infections, with enterohemorrhagic, shiga toxin producing *Escherichia coli* (EHEC*)*, inducing Shigatoxin HUS. Similarly, infections with *Streptococcus pneumoniae* can cause pneumococcal HUS. HUS induced by infections is more frequent in children (13, 14).

Genetic alterations and autoantibodies cause very similar or even the same clinical symptoms. Genetic-atypical HUS, accounts for about 15% of total HUS cases and is more frequent in adults. About 60–70% of these patients have genetic defects in one or several genes, which include nine complement genes (15–17) and a gene coding for a cytoplasmic signaling protein (18). The affected genes encode proteins, that (i) form the alternative pathway C3 convertase (C3, Factor B), (ii) regulate the activity of this central complement enzyme (Factor H, MCP/CD46, Factor I), (iii) act as complement modulators (FHR1, FHR3, FHR4, thrombomodulin) (18), or (iv) the cytoplasmic signaling protein (DAGKε) (19). This puts local AP C3-convertase induced complement and C3 regulation in the focus of genetic aHUS

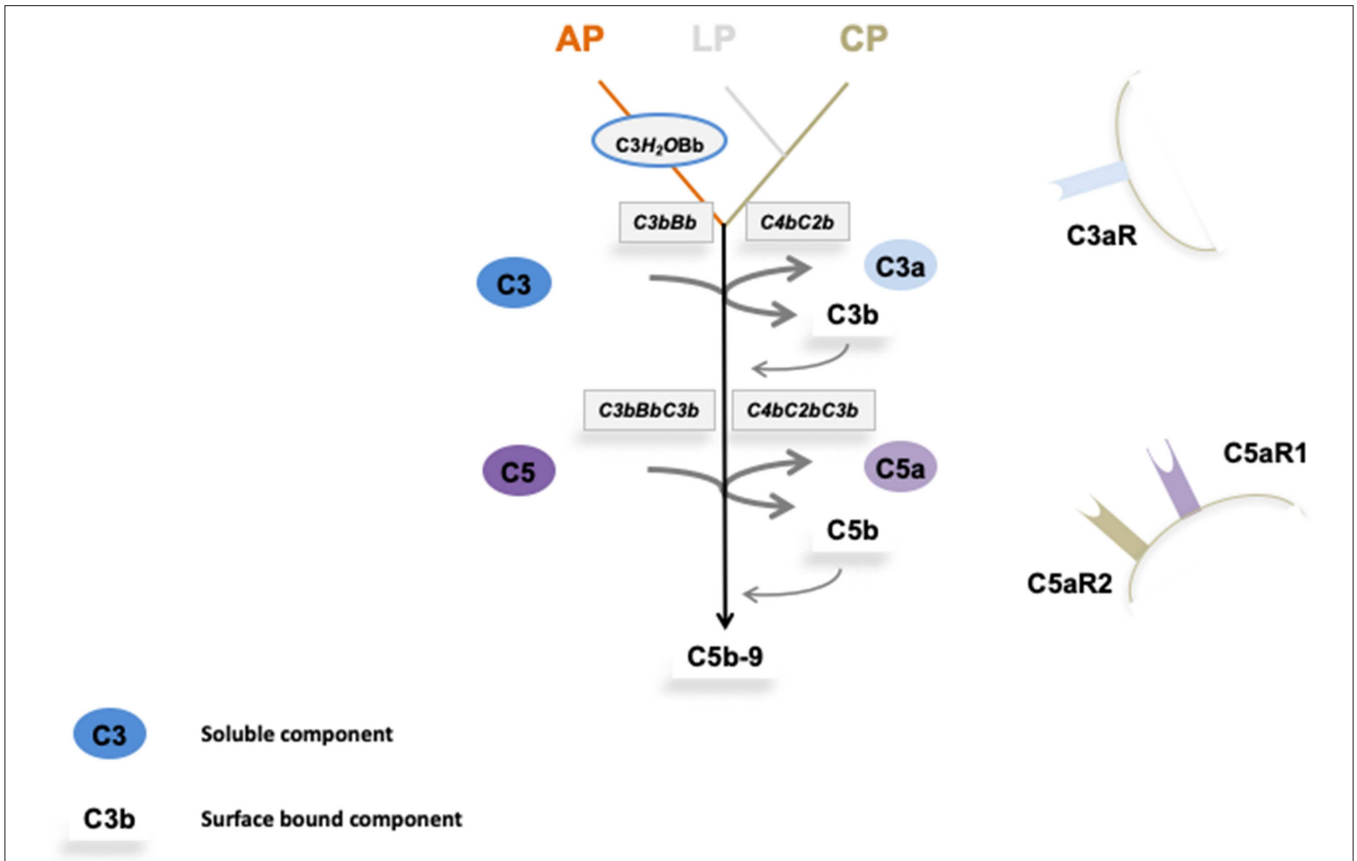

FIGURE 1 | Overview on complement activation and cascade progression. Complement activation is mediated by the alternative pathway in the fluid phase and on surfaces, and by the lectin and the classical pathway on surfaces. Multiple regulators determine and adjust cascade progression and subsequence effector action. Thereby discriminating the action between intact self, altered self, and non-self. Three initial reactions activate the complement cascade and have different initial triggers. The alternative pathway (AP) which is initiated spontaneously and continuously in the fluid phase and in the absence of regulators is amplified on surfaces. Both the lectin (LP) and the classical pathway (CP) are initiated on surfaces. The type of surface influences activation and the regulator repertoire decides on cascade progression or inhibition. Different regulators control cascade progression in the fluid phase and on surfaces. The three pathways form surface bound convertases, the AP allows generation of the AP-C3 convertase and the LP/CP trigger CP convertase. The AP C3 convertase also triggers a potent amplification loop. The general role of both C3 convertase is to cleave the abundant plasma protein C3 (concentration 1,000–1,500 μg/ml) into the anaphylatoxin C3a and the opsonic C3b. The enzymatic response on the first enzymatic levels is frequently enhanced by the potent self-amplifying amplification loop. If activation C5 convertases are generated and again C5 convertases of the AP and of the LP/CP pathways do exist. The major role of the C5 convertase is to cleave C5 (plasma concentration 350 μg/ml) into the powerful anaphylatoxin C5a and to generate C5b. Surface bound C5b initiates terminal complement and formation of the terminal complex, C5b-9, also termed membrane attack complex which can form lytic pores. Thus, complement acts on two major enzymatic levels, each of which generates a unique set of effector components with rather diverse functions. The complexity of this cascade is mediated by regulators and inhibitors, which control activation in the fluid phase (AP) and which ensure that activation mainly occurs on non-self surfaces or modified surfaces. In the physiological setting this coordinated action allows to direct the toxic and clearance power to the foreign/modified particles. In case of any dysbalance this action can be targeted toward self-structures and this can cause pathology at specific sites.

mediated pathology and shows the important role of the lytic branch of complement.

An autoimmune form of HUS is observed in about 15% of HUS patients (20, 21). Most autoantibodies have the same binding profile; they bind to the C-terminal recognition region of Factor H (SCRs19-20) and block Factor H surface binding. Most HUS patients with autoantibodies are of young age and have a homozygous deletion of a ca 24 kb chromosomal segment which encompasses the *CFHR3-CFHR1* genes. Therefore, this form is also termed DEAP-HUS patients (**DE**ficient for FHR1-FHR3 and **A**utoantibody to Factor H **P**ositive).

Due to the over activation of the complement system the endothelial cells of glomerular capillaries are damaged or destroyed (**Figures 2A,B**) resulting in the formation of the typical microscopic thrombi of acute TMA cases (**Figures 2A,C**). As a result of multiple thrombus formation in TMA glomerular sclerosis might arise. After a possible resolution of the initial TMA episode, the glomerular capillaries can be remodeled. These activated endothelial cells might synthesize a new glomerular basement membrane (GBM) resulting in a GBM duplication or multilayering, typically found in chronic or relapsing TMA. An overview of typical histological

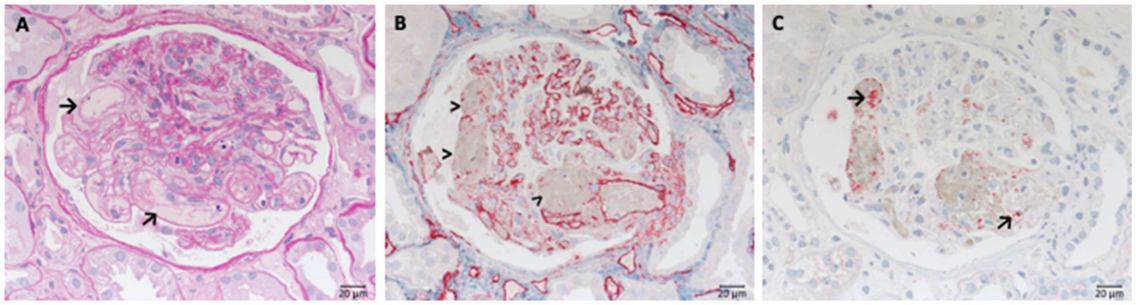

FIGURE 2 | Morphological changes in atypical uremic hemolytic syndrome (aHUS) resulting in thrombotic microangiopathy (TMA). **(A)** Periodic acid Schiff (PAS) reaction: focal loss of endothelial cells and fibrin precipitates in dilated glomerular capillaries (→). **(B)** Loss of CD34-positive endothelial cells stained red (>). **(C)** Accumulation of platelets stained red in CD61 immunohistochemistry (→).

features of genetic aHUS induced acute TMA is displayed in **Figure 2**.

Genetic aHUS is a prime example of a complement mediated disease and the terminal complement inhibitors Soliris/Eculizimab is already approved and on the market for therapy. This monoclonal antibody binds to C5, blocks C5 activation and formation of the terminal complement complex, i.e., C5b-9. Eculizumab was approved for treatment of genetic aHUS by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2011. Currently a new version of this C5 binding drug is in clinical trials for genetic aHUS. Ravulizumab (or ALXN 1210), is a pH dependent, C5 binding antibody which releases C5 at low pH. This drug is already approved for PNH (2018) (22–25) (**Table 1**). For genetic aHUS additional C5 targeting complement inhibitors are evaluated in clinical studies. These include the lectin pathway inhibitor OMS721, which binds and blocks MASP2 action, is evaluated in phase III, coversin, a tick (*Ornithodoros moubata)* derived C5 inhibitor and CCX168 the C5aR1 antagonist in phase II studies (**Table 1**). Studies with ALN-CC5 (Cemdisiran), a siRNA which blocks hepatic C5 production were performed, but apparently this trial was withdrawn.

The overall experience with Eculizumab is very good and positive results have been reported for long-term therapy. However, also incomplete inhibitory activities are reported e.g., for patients with C5 mutations (26). It will be of interest to compare how different targeting strategies, e.g., directly targeting C5, the lectin pathway, or the C5a receptor effector pathway influence the outcome and if genetic aHUS patients with different gene mutations or autoantibodies respond differently to the various inhibitors.

ANCA Associated Glomerulonephritis

ANCA (anti-neutrophil cytoplasmic antibody-) associated vasculitis (AAV) describes a collection of related disorders, which include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA), also called Churg-Strauss syndrome (27). A characteristic feature of these diseases are autoantibodies which cause complement inflammation and cell infiltration in blood vessels and which result in necrosis. The pathological principle of the autoantibodies is targeting neutrophil proteins myeloperoxidase and proteinase 3. AAV affects approximately 40,000 people in the US and approximately 4,000 new cases are identified each year. In Europe more than 75,000 people are affected and at least 7,500 new cases emerge each year (28).

Complement inflammation triggered by the alternative pathway is associated with ANCA. Autoantibodies induced C5a generation leading to neutrophil infiltration and activation, which initiates a vicious cycle: more neutrophils are attracted to local sites and the activated cells release their granule contents. The damage occurs in small blood vessels, mostly in the kidney but also other organs like lungs, nerves and sinuses can be involved (24).

Because of the damage caused by the degranulation of granulocytes necrosis and subsequent rupture of small vessels arise (**Figure 3**), particularly inside glomerular tufts. This is followed by proliferation of parietal cells and influx of macrophages, leading to crescent formation, and later segmental (partial) or complete scarring of the affected glomeruli occurs. Due to the almost complete lack of immune deposits inside of the glomeruli the renal manifestation of ANCA associated diseases is frequently called pauci-immune necrotizing glomerulonephritis. However, contrary to the assigned name complement molecules have been detected inside glomeruli affected by AAV in immunohistochemical and mass spectrometry-based studies. The typical picture of a fresh necrosis of glomerular capillaries in AAV is shown in **Figure 3**.

These pathological aspects make the C5a–C5aR1 axis of particular interest for therapeutic intervention in order to block attraction of neutrophils to local sites, inhibit cell activation and vascular destruction. Ongoing clinical trials approach the inflammatory complement C5a–C5a receptor 1 (C5aR1) axis with the soluble C5a peptide binding mAb (IFX-1; InflaRx) in phase II and the C5a receptor antagonist (Avacopan, Chemocentryx) in phase III trials. A clinical trial with Eculizumab was initiated and but enrollment of patients was terminated (**Table 1**).

TABLE 1 | Complement inhibitors being evaluated in clinical trials of glomerular diseases.

Disease	Inhibitor name	Alternative name	Inhibitor type	Inhibitor target	Company	Phase	Clinical trial code	Comments
aHUS	OMS721	OMS 00620646	Antibody	MASP2	Omeros	III	NCT03205995	
	Eculizumab	Soliris	Antibody	C5	Alexion	**Market**		NCT02574403; Phase 4, duration of Eculizumab treatment
	Ravulizumab	ALXN1210	pH-dependent Antibody		Alexion	III	NCT03131219 NCT02949128	Ravulizumab is already approved for paroxysmal nocturnal haemoglobinuria
	Coversin (rVA576)	Nomacopan	Peptide		Akari Therapeutics	III	NCT03829449	
	CCX168	Avacopan	Small molecule	C5aR1	ChemoCentryx	II	NCT02464891	
ANCA-associated vasculitis	IFX 1	CaCP 29	Antibody	C5a	InflaRx	II	NCT03712345	Granulomatosis with Polyangiitis; Microscopic Polyangiitis
	CCX168	Avacopan	Small molecule	C5aR1	ChemoCentryx	III	NCT02994927	
C3 Glomerulopathy	OMS721	OMS 00620646	Antibody	MASP2	Omeros	II	NCT02682407	DDD
	AMY-101		Antibody	C3	Amyndas	I	NCT03316521	
	APL-2		Peptide		Apellis	II	NCT03453619	DDD and C3 glomerulonephritis
	ACH 4471	ACH-0144471	Small molecule	FD	Achillion	II	NCT03459443	DDD and C3 glomerulonephritis
							NCT03369236	DDD and C3 glomerulonephritis
							NCT03124368	DDD and C3 glomerulonephritis
	LNP023		Small molecule	FB	Novartis	II	NCT03832114	C3 Glomerulonephritis
	Eculizumab	Soliris	Antibody	C5	Alexion	I	NCT01221181	DDD and C3 glomerulonephritis
						II	NCT02093533	C3 glomerulonephritis
	CCX168	Avacopan	Small molecule	C5aR1	ChemoCentryx	II	NCT03301467	DDD and C3 glomerulonephritis
IgA nephropathy	OMS721	OMS 00620646	Antibody	MASP2	Omeros	III	NCT03608033	
	APL-2		Peptide	C3	Apellis	II	NCT03453619	
	LPN023		Small molecule	Factor B	Novartis	II	NCT03373461	
	Cemdisiran	ALN-CC5	RNAi	C5	Alnylam	II	NCT03841448	
	CCX168	Avacopan	Small molecule	C5aR1	ChemoCentryx	II	NCT02384317	
Immune complex membranoproliferative glomerulonephritis	ACH 4471	ACH-0144471	Small molecule	Factor D	Achillion	II	NCT03459443 NCT03124368	
Ischemic reperfusion injury	C1INH	Berinert	Protein	C1r and C1s	CSL Behring	I	NCT02134314	C1INH is already approved and on the market for hereditary angioedema
Lupus nephritis	OMS721	OMS 00620646	Antibody	MASP2	Omeros	II	NCT02682407	
	APL-2		Peptide	C3	Apellis	II	NCT03453619	
Membranous nephropathy	OMS721	OMS 00620646	Antibody	MASP2	Omeros	II	NCT02682407	
	APL-2		Peptide	C3	Apellis	II	NCT03453619	
Transplant	C1INH	Cinryze	Protein	C1r and C1s	Shire	III	NCT02547220	Acute Antibody-Mediated Rejection(for patients with kidney transplant)
		Berinert			CSL Behring	I	NCT02134314	ESRD
							NCT01134510	Kidney transplant - therapy to prevent organ rejection
	AMY-101		Antibody	C3	Amyndas	I	NCT03316521	
	Eculizumab	Soliris	Antibody	C5	Alexion	II	NCT02145182	Prevention of delayed graft function
	LFG-316	Tesidolumab	Antibody		Novartis	I	NCT02878616	ESRD

C3 Glomerulopathy With Membranous Nephropathy and Dense Deposits Disease

C3 glomerulopathy is an umbrella term for a spectrum of related diseases. The diagnosis is primarily based on evaluation of renal biopsies showing prominent immunofluorescent or immunohistochemical staining for C3, which should be two orders of magnitude more intense than staining for immunoglobulins like IgA, IgG, or IgM (29, 30). The major subtypes of C3 glomerulopathy are C3 dominant membranoproliferative glomerulonephritis (C3 MPGN) (**Figure 4**) and dense deposit disease (DDD) (**Figure 5**). DDD is identified by electron microscopy showing thickened glomerular basement membranes with very electron dense material within the membrane and in the mesangium accompanied by mesangial proliferation (31) (**Figure 5**). Due to historic reasons, DDD is sometimes still referred to as MPGN II. In C3 glomerulopathy cases displaying MPGN patterns, double contours of the GBM as well as endocapillary and mesangial hypercellularity arises (32) (**Figure 5**).

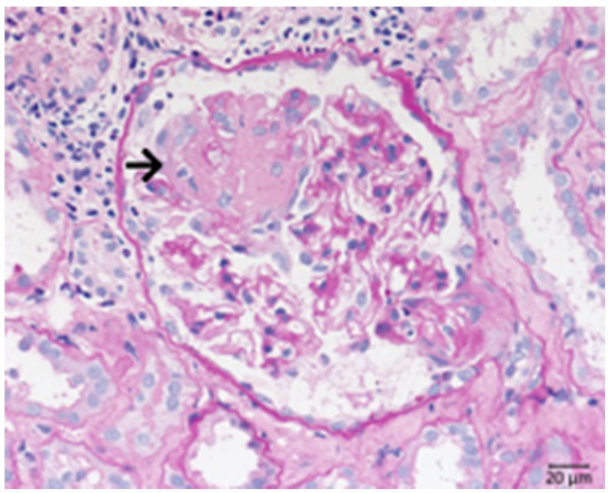

FIGURE 3 | Morphological changes in ANCA associated pauci-immune necrotizing glomerulonephritis. PAS-reaction of a case of ANCA-associated Glomerulonephritis, displaying a rupture of the glomerular basement membrane. See fresh fibrin precipitates inside the bowman space at the side of the necrosis (→).

C3 glomerulopathy is caused by defective complement regulation with genetic, as well as autoimmune causes. Often defective complement regulation occurs in plasma in the fluid phase and correlates with C3 consumption and low C3 plasma levels. Other forms of this disease develop on basis of normal plasma C3 levels. Genetic causes of C3 glomerulopathy include mutations in the genes coding for *Factor H, C3, CFHR1, CFHR2, CFHR3, CFHR5,* and Factor B. *Factor H* gene mutations are mostly homozygous or compound heterozygous. In C3 glomerulopathy multiple complex patterns of *CFHR* gene variations are reported. Alterations include single nucleotide variations in one of the five *CFHR*-gene, and structural variations in the *CFHR* gene cluster with duplications and deletions of gene elements or of larger chromosomal segments. These genetic alterations generate FHR variant proteins, FHR hybrid proteins and furthermore alter the FHR plasma levels.

Autoimmune factors in C3 glomerulopathy include autoantibodies in form of C3-Nephritic Factor, C4-Nephritic Factor or C5-Nephritic Factor. Most autoantibodies bind to neoepitopes exposed in these central complement enzymes, however some autoantibodies also bind to the single components, i.e., C3, C3b, Factor H or Factor B. These different autoantibodies show that complement action on the level of the C3 convertase, as well as C5 convertase are relevant for this disease spectrum (33–35).

Investigator initiated trials with C5 targeting by Eculizumab were reported for C3 glomerulopathy. First treatments showed favorable outcome, however studies with large patients cohorts revealed positive effects of Eculizumab in a fraction, about 40% of the patients, but not in all patients (36–38).

Currently seven complement inhibitors are evaluated in clinical phase I/II trials: OMS721, the MASP2 inhibitor (Omeros), Amy 101 (Amyndas), APL2 a C3 targeting peptide (Apellis), ACH-4471, a Factor D binding antagonist (Achillion), LNP023 a Factor B blocking compound (Novartis), Eculizumab (Alexion), and the C5a receptor 1 targeting Avacopan (Chemocentryx) (**Table 1**).

ACH-4771 is a small Factor D inhibitor that is applied orally and that blocks the catalytic side of Factor D. In presence of

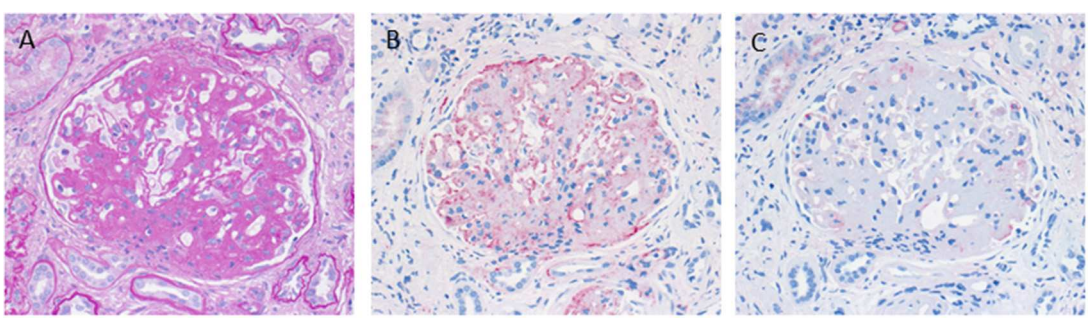

FIGURE 4 | Morphological appearance of C3 glomerulopathy. **(A)** PAS reaction reveals a membranoproliferative pattern with double contours of the GBM. **(B)** Strong immunohistochemical positivity for C3 at the GBM and in the mesangium. **(C)** Only very scant and segmental positivity for IgG.

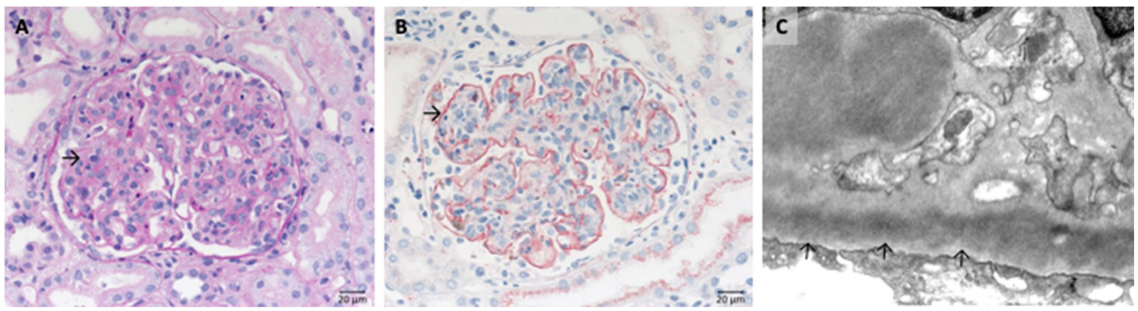

FIGURE 5 | Morphological changes in dense deposit disease (DDD). **(A)** PAS–reaction of a dense deposit disease case. Note the mesangial and endocapillary hypercellularity without prominent double contours of the glomerular basement membrane (→). **(B)** Note a strong red positivity for C3 inside the glomerular basement membrane (→). **(C)** The name giving electron dense deposits within the thickened glomerular basement membrane (GBM, →).

inactive Factor D the alternative pathway convertase C3bBb, is not formed and complement activation does not proceed. The other orally administered inhibitor LPN023, binds to the active site of Factor B, inhibits the alternative pathway C3 convertase and blocks C3 cleavage. Thus, different inhibitors are currently evaluated which target different levels of the complement cascade, the activation level, the lectin pathway, the C3 convertase of the AP, C3- and C5 cleavage. In this regard, C3 glomerulopathy has the potential to develop to an example for a disease where complement therapy will be approached based on personalized gene or autoimmune profile. Based on the different action sites of the inhibitor, it will be of interest to see which compound or which targeted pathway is most effective and which subform responds or benefits from which inhibitor.

IgA Nephropathy

IgA nephropathy (IgAN) is a leading cause of chronic kidney diseases with a complex disease pathology and with several factors involved (39). Genome–wide association studies identified the *CFHR*-gene cluster as a susceptibility locus and opposing effects were reported for individual *CFHR* genes (40). Homozygous *CFHR1/CFHR3*-deficiency resulting in the absence of FHR1 and FHR3 in plasma is protective and *CFHR5* is an IgAN susceptibility gene (41–44). Rare FHR5 protein variants with altered C3b binding represent risk factors (44). Current work focuses on FHR1- and FHR5-plasma levels (45, 46). Elevated FHR1- plasma levels and higher FHR1::Factor H-ratios influence alternative pathway regulation and correlate with disease severity (45–47). In addition also variations of FHR-plasma levels are of pathological relevance (48–50) and enhanced FHR5 plasma levels is an independent risk factor (46). Altered FHR1 and FHR5 plasma levels, or FHR1/Factor H ratios are disease relevant are related to diseases severity and correlate with alterations in glomerular filtration rates (49–51).

Autoimmune factors include galactose deficient IgA immune complexes or autoantibodies against O-glycans and C3. Genetic and clinic studies link complement and the alternative pathway to immune pathogenesis of IgA nephropathy. But also environmental factors play a role for diseases development. In addition properdin, a complement activator and complement Factor H are identified in the immune deposits.

IgA nephropathy is marked by the deposition of galactose-deficient IgA1 in the glomerular mesangium (**Figure 6A**). As a result, mesangial matrix increase and some proliferation of mesangial cells is often seen (**Figure 6B**), moreover, endocapillary hypercellularity, and eventually rupture of glomerular capillaries with subsequent formation of crescents might occur (extracapillary component). Depending of the severity of the disease glomerular and interstitial injury arises. However, clinical course of IgA nephropathy varies from almost absent clinical symptoms in many cases to fulminant renal failure resulting in dialysis dependent loss of renal function within weeks. Numerous studies suggest that the histological pattern and the deposition of complement components such as C1q, C3c, and lectin are predictors of worse outcome. The classical appearance of mesangioproliferative IgA nephropathy is depicted in **Figure 6**.

Clinical studies addressing the complement system in IgA Nephropathy include targeting the MASP-2 by OMS721 (Omeros) (phase III), C3 by APL-2 (Apellis), Factor B by LPN023 (Novartis), C5 by Cemdisiran, and C5aR1 by CCX168 (Chemocentryx) (**Table 1**).

Immune Complex Membranoproliferative Glomerulonephritis

Immune complex membranoproliferative glomerulonephritis (MPGN) is marked by dominant deposition of immunoglobulins (**Figure 7**) and to a lesser extent of complement components inside the mesangium and along the inner side of the GBM. As a result, mesangial, and endocapillary hypercellularity as well as GBM duplication arise (**Figure 7B**). Additionally, necrosis and crescent formation might occur. Furthermore, in cryoglobulin associated cases hyaline pseudothrombi can be observed inside glomerular capillaries. Several putative causes for the development of immune complex MPGN are known, such as chronic infections, like endocarditis, monoclonal gammopathy, hepatitis C and the formation of cryoglobulins. An example of the typical histological pattern of immune complex MPGN is displayed in **Figure 7**.

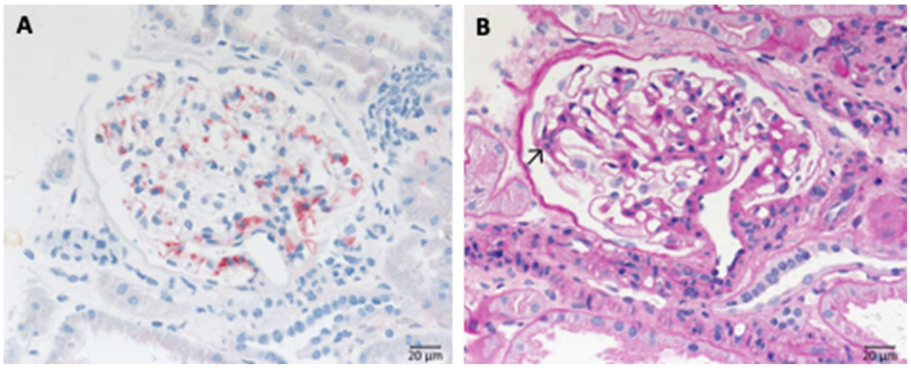

FIGURE 6 | Morphological changes in IgA nephropathy: **(A)** IgA immunohistochemistry of a case of IgA nephropathy, displaying a noticeable mesangial positivity. **(B)** PAS reaction of the same glomerulus: note the mesangial matrix increase and the focal mesangial hypercellularity (arrow).

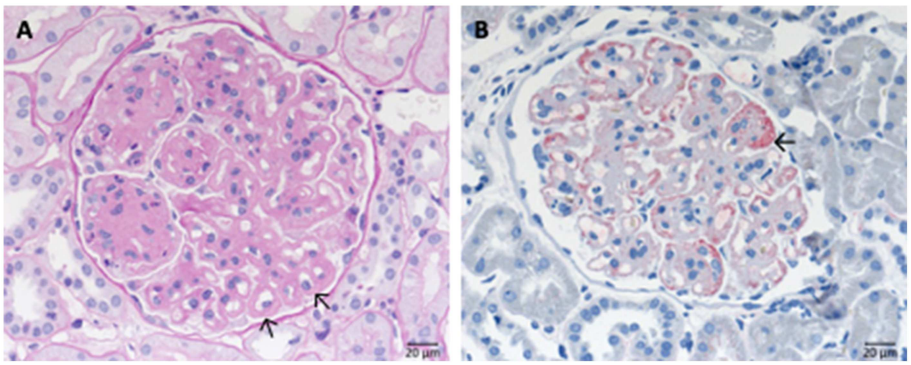

FIGURE 7 | Morphological changes in immune complex membranoproliferative glomerulonephritis (MPGN). **(A)** PAS-reaction of a case of membranoproliferative glomerulonephritis due to a chronic hepatitis C infection. Note the lobular appearance and double contours of the GBM (→). **(B)** Note positivity for IgG at the side of double contours of the GBM (→).

Clinical trials in Immune complex membranoproliferative Glomerulonephritis are with ACH 4471 (Achillion) in phase II (**Table 1**).

Lupus Nephritis

Lupus nephritis is an inflammatory kidney disease occurring in patients suffering from systemic lupus erythematosus (SLE) and active complement is important for the pathogenesis. The classical complement pathway, in particular the components C1, C4, and C2 were for a long time associated with this common form of kidney damage, in particular by defective removal or clearance of damaged self-cells, debris material, and immune complexes. Accumulated self-material initiate complement. In addition a role of the lectin and alternative complement pathway are shown in lupus nephritis (52, 53). Inflammatory complement initiated via C5a results in neutrophil infiltration, and autoantibodies to intracellular proteins induce the cascade further.

Systemic lupus erythematosus is an autoimmune disease usually involving multiple organs and as a possible complication the kidneys can be involved. The most important renal consequence of SLE is the development of immune complex glomerulonephritis. However, other possible damage patterns are TMA, interstitial nephritis, podocytopathy, and amyloidosis (**Figure 8**). SLE associated glomerulonephritis is separated according to the histologic appearance into six different classes (RPS/ISN). Class I lupus nephritis resembles glomerular IgG deposition without further morphological findings at the light microscopic level. Class II shows deposition of immune components, particularly IgG inside the mesangium. A mesangial proliferation is visible by light microscopy. Class III lupus nephritis shows additionally duplication of the GBM and/or endocapillary hypercellularity and/or crescent formation in a minority of the affected glomeruli. Class IV (**Figure 8**) is characterized by an MPGN like pattern with duplication of the GBM and/or endocapillary hypercellularity and/or crescent formation in the majority of glomeruli. Class V lupus nephritis resembles membranous glomerulonephritis and can occur in combination with any of the other mentioned classes or alone. As an end stage of lupus nephritis class VI is defined as scarring of over 90% of the sampled glomeruli inside a renal biopsy.

Genetic studies showed that the *CFHR1-CFHR3* deletion presents with increased risk for SLE (54). Homozygous deletion of this 24 kbp chromosomal *CFHR1-CFHR3* containing fragment

has kaleidoscope features. First the deletion is common in the healthy population and shows different frequencies in ethnic groups, i.e., being present in ca 30% of the healthy African, 18% of the healthy Asian and 5–6% of the healthy European population (55). Second this homozygous setting confers risk for two renal diseases, SLE and DEAP-HUS and for infections with the pathogenic bacterium Neisseria meningitides. Third the same deletion has protective roles in IgAN, and in the retinal disease Age related macular degeneration (AMD).

In lupus nephritis two phase II clinical trials are ongoing with the complement inhibitors OMS 721 (Omeros) which targets the lectin pathway via MASP2 protein and APL2 (Apellis) which interferes with complement activation at the level of the central complement component C3 (**Table 1**).

Membranous Nephropathy

Membranous nephropathy results from binding of IgGs to antigens expressed at the surface of podocytes. Membranous nephropathy is grouped into primary cases (80% of cases) without causative autoimmune disease (56, 57). In primary membranous nephropathy cases about 70% show autoantibodies directed against phospholipase A_2 receptor 1 (PLA_2R1), display antibodies directed against thrombospondin type 1 containing 7A (THSD7A) in serum (1–2%) and to complement Factor H (3%) (57–63). PLA_2R and THSDA7 are podocyte antigens. The autoantibodies are directed to the receptors exposed on the surface of podocytes. Animal studies show that proteinuria is caused by the terminal complement complex. Also other mechanisms have been proposed.

Also secondary cases (20% of cases) are known with causative diseases like SLE (Lupus nephritis class V). Complexes formed by the autoimmune IgG and the proper antigen are deposited along the outside of the GBM at the anchoring side of the foot processes of the podocytes (**Figure 9**).

Most scenarios of primary membranous nephropathy are mediated by autoantibodies to M type Phosphoplipase A_2 receptor (PLA_2R) (95%) and Thrombospondin type 1 domain containing 7a (THSD7a) receptor, a podocyte antigen (3–5%). Recently an additional third autoimmune form was described, where autoantibodies developed which target complement Factor H (3%) (63).

Most PLA_2R and THSD7A autoantibodies are of the IgG4 subtype, a subtype which does not activate complement, however additional autoantibody forms are identified (56). Components of the classical and the alternative pathways are prominently localized at the site of the IgG-antigen deposits. The IgG-antigen complexes can be found along the outside of the GBM at the anchoring side of the podocyte foot processes (**Figure 9B**). The GBM expands and overgrows during disease development (**Figure 8**). Also older deposits inside the GBM are resorbed over longer time periods. As a result foot process retraction arises and nephrotic range proteinuria develops. The typical morphological changes in membranous nephropathy are shown in **Figure 9C**.

About 3% of MN patients have Factor H binding antibodies which target the C terminal recognition region of the human regulator and block surface binding (63). In the autoimmune form DEAP-HUS a related pathologic principle,

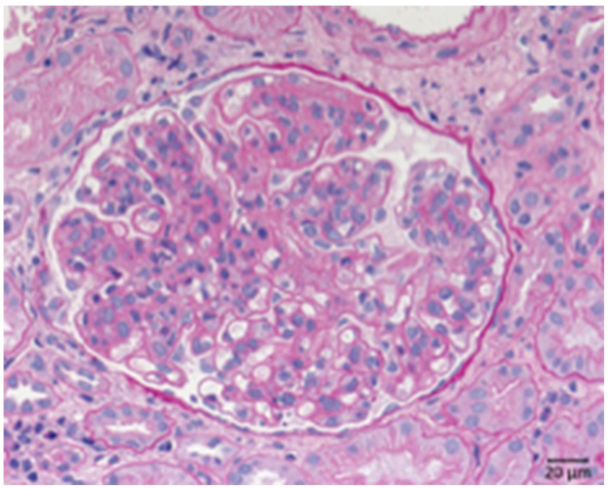

FIGURE 8 | Morphological changes in lupus nephritis, class IV. PAS reaction of a lupus nephritis class IV. Note lobular pattern with pronounced endocapillary and mesangial hypercellularity as well as thickened GBM.

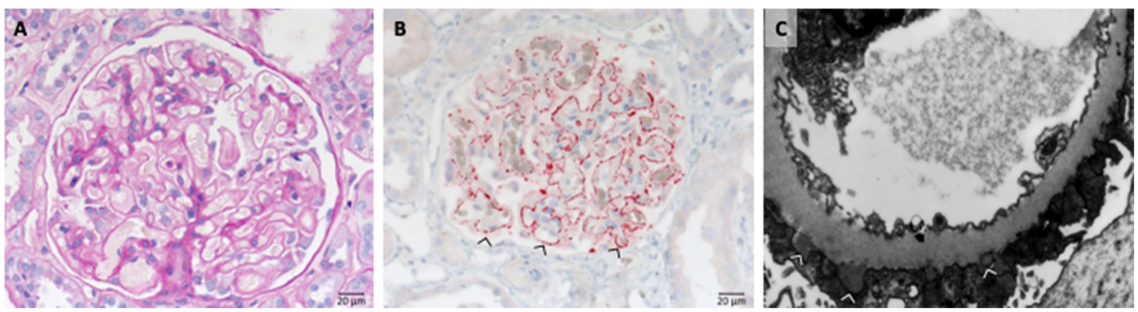

FIGURE 9 | Morphological changes in membranous glomerulonephrits nephropathy. **(A)** PAS-reaction with slightly thickened GBM. **(B)** PLA_2R1 immunohistochemistry of a PLA_2R1-antibody associated case of membranous glomerulopathy. Note strong granular positivity for PLA_2R1 at the GBM (>). **(C)** Electron microscopic depiction of subepithelial electron dense deposits (>).

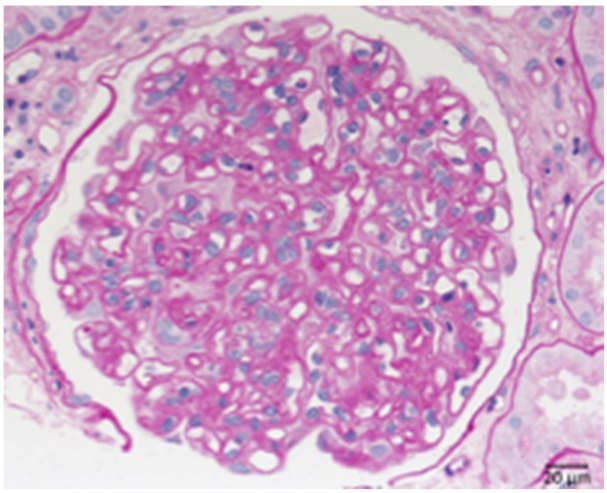

FIGURE 10 | Chronic transplant glomerulopathy. PAS: Chronic transplant glomerulopathy displaying pronounced double contours of the GBM and slight endocapillary hypercellularity.

with autoantibodies targeting the C -terminal binding region Factor H block Factor H surface action (64, 65), These features indicate that complement regulation and furthermore cell damage mediated by the terminal complement pathway is a disease relevant mechanisms at the podocyte surface.

Two complement inhibitors OMS 721 (Omeros), which targets the lectin pathway protease MASP2 and APL2 (Apellis), which binds to C3 component, respectively are currently evaluated in clinical trials of membranous nephropathy. Both are in phase II (**Table 1**).

Renal Transplant

Complement is activated in transplanted organs and in particular a transplanted kidney can be challenged by activated complement. Numerous approaches are being used to limit complement activation in the transplant in order to block complement inflammation and complement terminal pathway action (66). Involved in renal transplant as the new, foreign surface and under resourced tissue provide a platform for complement activation (67).

Two primary immunological damage mechanisms occurring in transplant kidneys are humoral rejection, with the formation of antibodies against structures of the transplanted organ and cellular rejection with the sensitization of T-cells against donor kidney antigens. The role of the complement system is particularly prominent in cases of humoral rejection. In these cases C4d, an indicator of acute humoral rejection can be deposited alongside peritubular capillary walls. In case of chronic humoral rejection the steady binding of antibodies leads to the activation or loss of endothelial cells particularly in glomerular and peritubular capillaries. This is followed by the formation of new GBM material (**Figure 10**). Therefore, a membrane duplication or multilayering of the basement membranes arise in glomerular and peritubular capillaries. The typical morphology of chronic transplant glomerulopathy is displayed in **Figure 9**.

Different clinical conditions are evaluated in form of acute antibody mediated rejection and also in end stage renal diseases and kidney transplantation to prevent organ rejection. Currently one phase III and two phase I studies are ongoing which evaluate efficacy of the C1 Inhibitors (Cinryze and Berinert). In addition the C5 inhibitor Eculizumab (Alexion) is tested in phase II, and the C5 monoclonal antibody LFG-316 developed by Novartis is tested in a phase I studies (**Table 1**).

Renal Ischemic Reperfusion Injury

Ischemic reperfusion injury is a common cause for acute kidney damage which can follow transplantation and which can damage the transplanted organ (68). Acute tubular damage is a common cause for kidney failure and the complement system which is activated on damaged self-cells can propel and increase such local damage (69). Ischemic reperfusion injury is marked by damage to the tubular epithelium. The tubular epithelial cells appear flattened or swollen and the cells suffer a total or partial loss of the brush border.

Complement inhibition in this form of kidney damage is being pursued with the C1 Inhibitor (**Table 1**).

SUMMARY OUTLOOK

As outlined here, the current development shows that complement inhibition in renal disease is actively pursued in several clinical studies. Initial proof of concept comes from the inhibitor Soliris/eculizumab which is approved for treatment of genetic aHUS, as well as for two other complement disorders paroxysmal nocturnal hemoglobinuria (PNH) and myasthenia gravis (MG). The expanding list of trials and the increasing number of complement inhibitors, which are being developed and are tested in preclinical studies demonstrate that complement inhibition is an option for therapy of glomerular disorders.

The inhibitors which are tested in clinical trials for glomerular diseases target the activation pathways i.e., the lectin pathway via MASP-2, the central component C3 (APL1 and Amy101), the alternative pathway convertase via Factor D (ACH4471), Factor B (LPN 023), target the terminal pathway via C5 (Eculizumab, LFG-316), by blocking C5 synthesis in the liver via C5-siRNA (Alnylam), or directly interfering with the inflammatory C5a—C5aR1 axis (IFX1; InflaRX, and Avacopan; Chemocentryx). Thus, these complement inhibitors target different proteins in different activation pathways or effector levels of complement (**Figure 11**). This provides the option to block complement at different levels and to interfere with different effector pathways. Given that complement is involved in many renal (and also other) diseases the existing inhibitors allow interfering with complement at different stages.

These inhibitors target complement at different levels and address the various effector pathways. A detailed understanding of the pathological mechanism for each single disease and also of the subforms, combined with an understanding of the mode of action of each inhibitor and a better understanding of the

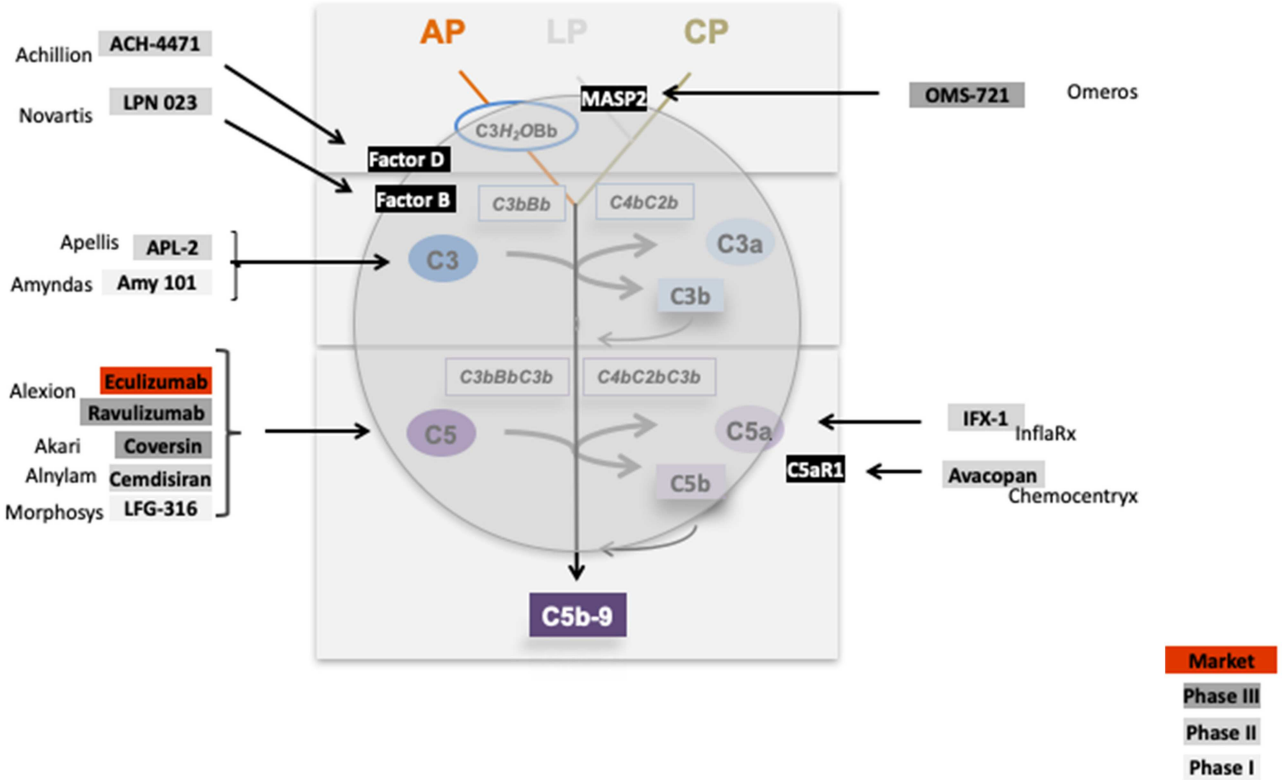

FIGURE 11 | Complement inhibitors target different levels and steps of the complement cascade. Complement inhibitors which are evaluated in clinical trials for various kidney diseases bind to different complement proteins and inhibit the cascade at different levels. C1 inhibitor binds to C1 and blocks C1 activation. OMS 721 binds to the lectin pathway protease MASP2. C3 targeting proteins include APL2 (Apellis) and AMY 101 (Amyndas). The Factor D inhibitor ACH-4471 (Achillion) binds to Factor D, a protease which cleaves in its active state Factor B. LPN023 (Novartis) a small Factor B binding protein blocks formation of the enzymatically active AP C3 Convertase. Several compounds target complement at the level of C5. Eculizumab, and the new version Ravulizumab (both Alexion) bind to C5 and block activation of the protein. Coversin is a tick derived C5 binding protein (Akari) and C5 inhibitor. C5 synthesis is blocked by Cemdisiran as an RNAi targeted strategy (Alnylam), and by LFG-316 (Novartis). The complement inflammatory C5a—C5aR1 axis is inhibited by IFX-1 (InflaRx) and Avacopan (Chemocentryx).

regulatory loops, regulatory networks and feedback pathways of the complement cascade, as well as the crosstalk with other immune systems like the coagulation cascade will allow to use the inhibitors for the clear benefit of the patient. It will be of interest to see which of the various inhibitors is most effective for the outlined renal diseases and given the heterogeneity of the diseases and their existing subforms it will be of interest to evaluate the different causes and responses of the inhibitors. In total, many clinical trials and the emerging list of additional new inhibitors show that complement inhibition in glomerular diseases has a very promising future.

AUTHOR CONTRIBUTIONS

PZ, TW, and CS designed the concept and planned the work. RR, SA, and FP performed the work. All authors wrote the manuscript.

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REVIEW
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Therapeutic Modulation of the Complement System in Kidney Transplantation: Clinical Indications and Emerging Drug Leads

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The complement system is integral to innate immunity, and it is an essential deterrent against infections. The complement apparatus comprises of >30 fluid-phase and surface-bound elements that also engage with the adaptive immune system, clear harmful immune complexes, and orchestrates several salutary physiological processes. An imbalance in the complement system's tightly regulated machinery and the consequent unrestrained complement activation underpins the pathogenesis of a wide array of inflammatory, autoimmune, neoplastic and degenerative disorders. Antibody-mediated rejection is a leading cause of graft failure in kidney transplantation. Complement-induced inflammation and endothelial injury have emerged as the primary mechanisms in the pathogenesis of this form of rejection. Researchers in the field of transplantation are now trying to define the role and efficacy of complement targeting agents in the prevention and treatment of rejection and other complement related conditions that lead to graft injury. Here, we detail the current clinical indications for complement therapeutics and the scope of existing and emerging therapies that target the complement system, focusing on kidney transplantation.

Keywords: kidney transplant, complement–immunological term, HLA, monoclonal Ab, antibody mediated allograft rejection

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INTRODUCTION

Complement proteins account for 3 g/l of plasma and make up ∼15% of the globulin fraction (1). The complement system is activated via three canonical pathways: (**Figure 1**) (a) the classical pathway, triggered by recognition of subclasses of surface-bound IgG and IgM antibodies by complement component C1q; (b) the lectin pathway, triggered by recognition of bacterial surface sugars by mannose-binding lectin (MBL); and (c) the alternative pathway that is constitutively active due to spontaneous hydrolysis of C3, a phenomenon that has been christened C3 "tickover" (2). These three pathways lead to the formation of critical enzymes complexes called C3 convertases, which trigger events that culminate in the generation of the cell-killing membrane attack complex (MAC) (1, 3). We direct you to recent reviews on complement biology for a detailed description of the pathways and mechanism of complement activation and regulation (3–6).

The approval of the complement-targeting anti-C5 antibody, eculizumab, by the United States Food and Drug Administration (FDA) for the rare disease paroxysmal nocturnal hemoglobinuria (PNH) in 2007 has led to a renaissance in complement therapeutics (7). PNH is a hematological

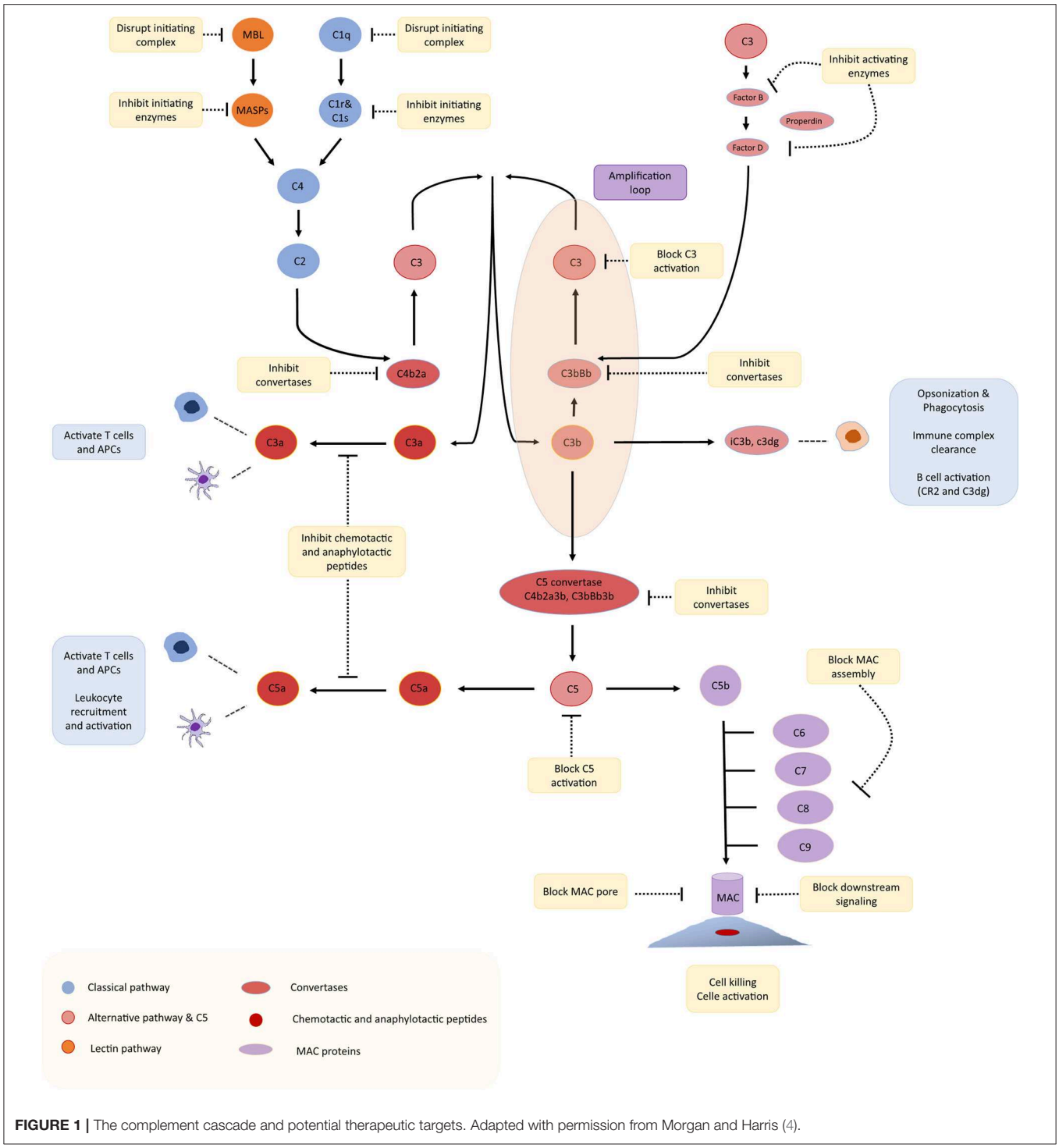

FIGURE 1 | The complement cascade and potential therapeutic targets. Adapted with permission from Morgan and Harris (4).

disease characterized by transfusion-dependent hemolytic anemia and life-threatening thrombosis. PNH results from the clonal expansion of hematopoietic stem cells that have a somatic mutation in a gene that is critical to the synthesis of glycosylphosphatidylinositol (GPI) (8). GPI serves as an anchor for many proteins to the cell surface, including CD59, a MAC regulatory protein (8, 9). Therefore, red cells deficient in GPI are susceptible to complement-mediated intravascular hemolysis. PNH is an uncommon disease that affects 10,000 people in North America and Western Europe (8). Untreated, it is associated with a dismal median survival of 10 years after diagnosis (8). Eculizumab (Soliris, Alexion) is a recombinant humanized anti-C5 monoclonal antibody that prevents the cleavage of C5 by C5 convertase. In a phase 3 clinical trial published in

2006, eculizumab stabilized hemoglobin levels in transfusion-dependent patients with PNH (9). Following this trial, the FDA approved the use of eculizumab for PNH in March 2007.

In atypical hemolytic uremic syndrome (aHUS), complement overactivity leads to systemic thrombotic microangiopathy involving the kidney, central nervous system, heart, and gastrointestinal tract. In 2011, the FDA approved eculizumab for use in aHUS following a clinical trial demonstrating significant improvement in renal function with its use (10). Hereditary angioedema (HAE) is an inherited disorder in which a deficiency of C1-inhibitor (C1-INH) leads to dysregulation of the complement cascade and the kallikrein pathway leading to recurrent episodes of life-threatening angioedema. C1-INH is a serine protease inhibitor (SPI) that removes activated C1r and C1r from C1q (4). Two nano-filtered C1-INH products derived from human serum, cinryze (Takeda/Shire Pharmaceuticals) and berinert (CSL Behring), were approved for the treatment and prophylaxis of attacks of HAE after randomized trials demonstrated their efficacy (4, 11). The clinical success of these two drugs has fueled interest in complement and sparked the development of complement targeting therapeutics for numerous other conditions like age-related macular degeneration (AMD), C3 glomerulopathy, and an array of conditions related to kidney transplantation.

Kidney transplantation is the therapy of choice for eligible patients with end-stage renal (kidney) failure. However, mismatches in human leukocyte antigens (HLA) between the donor and the recipient can cause the recipient's immune system to reject the transplanted allograft. The mechanism of immune injury in rejection has been the subject of intense research over the last few decades. This has led to a detailed understanding of the molecular processes involved in rejection, and it is now clear that the complement system plays a central role, especially in the pathogenesis of antibody mediated rejection (AMR). Several groups have recently published results of clinical trials exploring the role of complement blockade in AMR of transplant kidneys.

ROLE OF COMPLEMENT IN KIDNEY TRANSPLANTATION

Kidney transplantation is the treatment of choice for end-stage renal disease (ESRD) due to the superior long-term survival, cost savings to the health care system, and the greater quality of life it offers relative to dialysis (12, 13). However, the prevention and treatment of rejection is still a major impediment to successful transplantation (14). The transplantation of tissues from a donor who is genetically disparate from the recipient elicits an immune response in the recipient against alloantigens that, if uncontrolled, can lead to allograft destruction (13).

Preformed circulating anti-HLA antibodies are present in up to 30% of patients awaiting kidney transplantation as a result of previous exposure to allo-HLA antigens from blood transfusions, pregnancies, and previous transplants and may cause immediate graft failure due to hyperacute rejection (15). The incidence of acute allograft rejection has declined significantly since the emergence of calcineurin inhibitors (CNIs) in the 1980s (16). However, this has not resulted in a concurrent improvement in long-term allograft survival (17). Late-onset AMR has emerged as a leading cause of allograft loss and is increasingly recognized as the reason for the poor long-term graft survival (18). In AMR, donor-specific antibodies (DSAs) bind to mismatched HLA molecules and can trigger classical complement pathways leading to allograft vascular injury (19). This injury is mediated by the MAC as well as C3a and C5a driven inflammation (19). Histologically, this is characterized by the demonstration of infiltrating polymorphonuclear leukocytes (PMNs) and macrophages in renal glomerular and peri-tubular capillaries (**Figure 2**). Complement split product, C4d, is prominently deposited in the microvasculature of renal allografts undergoing AMR (**Figure 3**) (20). C4d remains covalently attached to the endothelium and is detected in peritubular capillaries in renal allograft biopsies by immunohistochemistry. It acts as a footprint of HLA-binding by DSAs and resultant complement activation (21).

The advent of sensitive solid-phase Luminex platform bead-based assays for the detection of HLA antibodies has enhanced our ability to detect DSAs and diagnose AMR (22, 23). In this flow-cytometry based assay, fluorochrome impregnated micro-beads coated with HLA proteins are incubated with patient serum. Anti-HLA antibodies bind the cognate HLA antigens on the beads and are detected by a dual- laser instrument (24). In 2003, standardized diagnostic criteria for AMR that featured C4d microvascular deposition and the presence of circulating DSAs were incorporated into the classification schema for kidney transplant pathology set forth by the Banff group (25). Spurred by the success of complement therapeutics in diseases mediated by complement dysregulation like PNH and aHUS, the role of pharmacologic complement blockade is being explored through clinical trials in a variety of kidney transplant-related settings (26).

The presence of preexisting DSA increases the risk of AMR and may adversely impact the graft survival even in recipients of negative lymphocytotoxic cross-match kidney transplants (23). C1q-binding DSA appear to be associated with significantly lower long-term graft survival than non-complement fixing DSA, indicating that the complement activation by DSA is critical to their pathogenic potential (25). Characterizing the pathogenic potential of DSA detected by modern sensitive assays is valuable in the risk stratification of patients with respect to the perils of AMR. This will allow transplant physicians to adopt a more nuanced approach to the utilization of potent and frequently expensive immunosuppressive agents, and thereby balance the risk of rejection with that of debilitating infections and malignancies.

COMPLEMENT THERAPEUTICS IN KIDNEY TRANSPLANTATION

Complement Modulation in Treatment of AMR

Enhanced understanding of the etiology of AMR and the prominent role played by the complement system implies

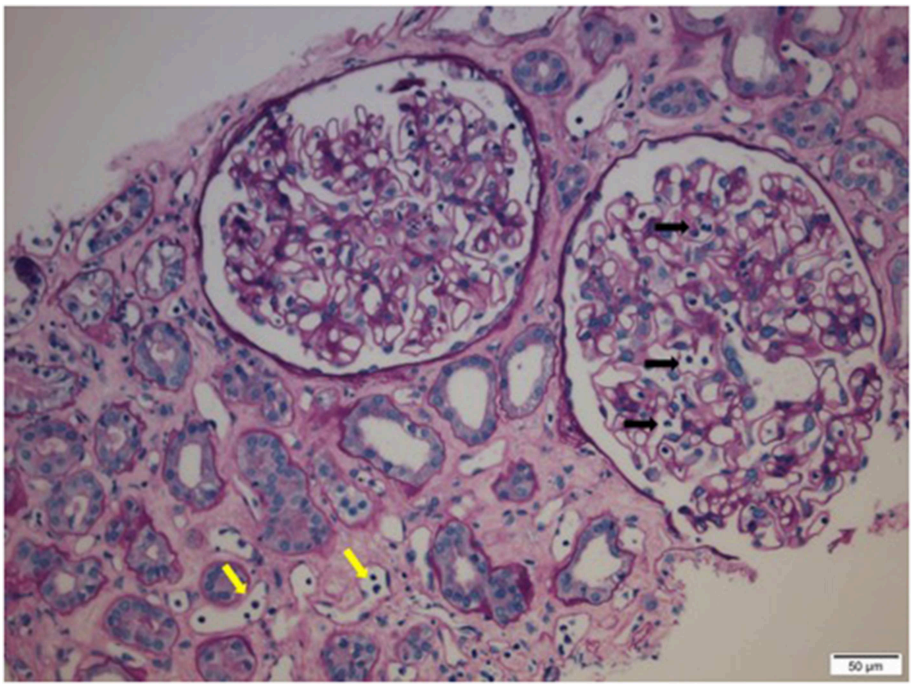

FIGURE 2 | Infiltrating polymorphonuclear leukocytes (PMNs) in renal glomerular capillary loops (black arrows) and peritubular capillaries (yellow arrows) in a renal allograft undergoing acute antibody mediated rejection (AMR).

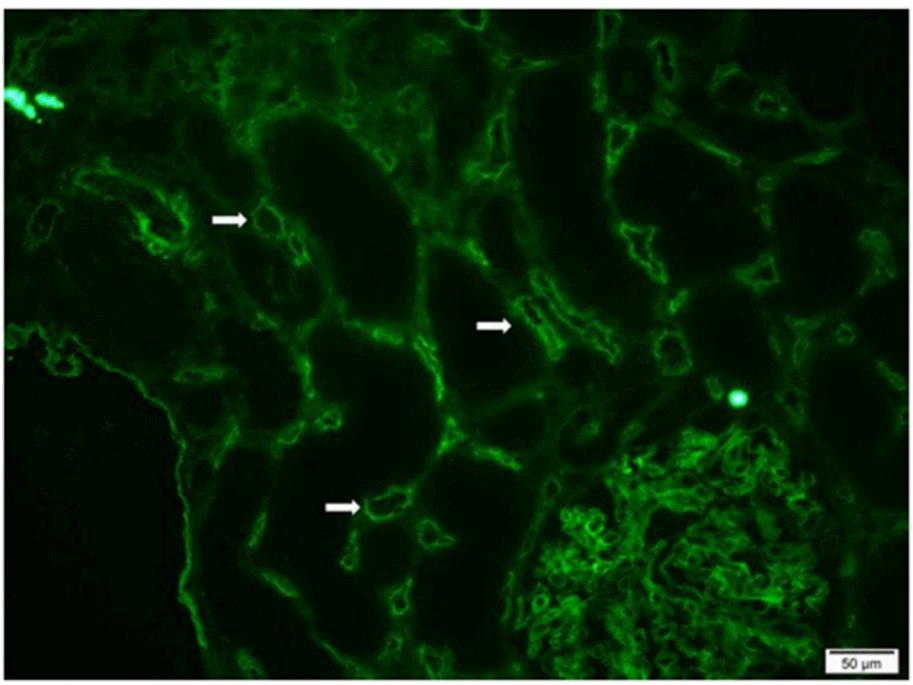

FIGURE 3 | Immunofluorescence microscopy demonstrating diffuse and prominent deposition of C4d on the endothelium of peritubular capillaries (white arrows) in a renal allograft undergoing acute antibody mediated rejection (AMR).

a possible role for pharmacologic complement inhibition in clinical transplantation. The terminal complement inhibitor, Eculizumab, and C1 esterase inhibitor (C1-INH) have been the subjects of recent clinical trials exploring the potential of targeting the complement cascade in the prevention and treatment of AMR (19, 27, 28).

DSA depletion by plasmapheresis combined with intravenous immunoglobulin (IVIg) is currently the mainstay of treatment for AMR (29). However, regimens reported in literature differ broadly with respect to the duration of therapy, dosing of IVIg, and the role of B-lymphocyte and plasma-cell targeting agents like rituximab and bortezomib (26, 30). Based on promising single center experience treating acute AMR, a multi-center trial that began enrollment in 2013 evaluated the role of eculizumab in the treatment of biopsy-proven AMR and acute graft dysfunction (31–33). Patients in the standard of care (SOC) arm received three sessions of plasmapheresis with IVIg administered after each session. Those in the treatment arm received a first dose of 1,200 mg of eculizumab followed by 4 weekly doses of 900 mg and a dose of 1,200 mg at week 5 (31). Patients received additional doses of 1,200 mg at weeks 7 and 9 if DSA levels at week 6 remained >50% of baseline (31). However, recruitment for this trial was terminated in 2017 due to the study drug's failure to improve allograft function assessed by estimated glomerular filtration rate (eGFR) 3 months after transplantation (31).

In AMR, activation of the classical pathway is triggered by ligation of the C1 complex to HLA-antigens that are bound by DSA. Therefore, complement inhibitors targeting C1 can be leveraged to treat AMR while sparing the alternative pathway, thereby potentially averting infectious complications (34). Additionally, complement inhibition upstream of C5 with C1 inhibition would be predicted to block the generation of complement activation products of C3 that contribute to neutrophil and monocyte recruitment and tissue injury in AMR. Two nano-filtered C1-INH products derived from human serum, cinryze (Takeda/Shire Pharmaceuticals) and berinert (CSL Behring), are approved for the treatment and prophylaxis of attacks of HAE. The role of C1-INH in the treatment of AMR was explored by two recent studies (27, 28). Viglietti et al. treated six patients who had AMR that was not responsive to SOC therapy with C1-INH (berinert, CSL Behring) and high-dose IVIg for a duration of 6 months (27). At the end of the 6-month follow-up period, the authors reported significant improvement in mean eGFR. Fewer patients demonstrated C4d deposition on allograft biopsies and circulating C1q fixing DSAs. However, other histologic features, like chronic glomerulopathy, were unchanged (27). Montgomery et al. conducted a randomized phase 2 trial to evaluate the role of a 2-week course of C1-INH (Cinryze, Shire Pharmaceuticals) as an add-on therapy to SOC plasmapheresis and low-dose IVIg, compared to SOC alone. Nine patients were randomized to each arm. While there was no significant difference in graft survival and histologic findings of AMR after 20 days, 6-month allograft biopsies showed an absence of transplant glomerulopathy in the C1-INH treated patients, whereas three out of seven patients in the placebo arm were found to have this histologic feature that is associated with chronic antibody mediated injury and premature allograft loss (28). It is to be noted that the above two studies differed in study design and the C1-INH product used (27, 28). Both of these C-INH products are also the subject of recent phase 3 trials. A double-blind, randomized, placebo-controlled study (NCT03221842) of human plasma-derived C1-INH, berinert (CSL Behring), as an add-on to SOC for the treatment of refractory AMR in adult kidney transplant recipients, is currently underway. The investigators aim to enroll 90 study subjects and measure the primary outcome of time to loss of treatment response (35). However, another multicenter, randomized, placebo-controlled study (NCT02547220) to evaluate the efficacy and safety of the other C1-INH product, cinryze (Takeda/Shire Pharmaceuticals), for the treatment of acute AMR in kidney transplant recipients, was terminated early because interim analysis showed that the study met the pre-specified criteria for futility (36). Therefore, the jury is still out on the utility of C1-INH in the treatment of AMR.

C1 complex is a multi-molecular cluster constituted by the PRP, C1q, and two serine proteases, C1r, and C1s (4). Upon activation, C1s proceeds to cleave C4 and C2 and results in the formation of C4b and C2a, respectively (4). These activation fragments associate to form the C4b2a complex, which is the classical pathway's C3 convertase (4, 26). An anti-C1s murine monoclonal antibody TNT003 and its humanized variant BIVV009 have been shown to block chemotaxis of macrophages in experimental models of HLA antibody-triggered classical pathway activation (37, 38). Eskandary et al. published the findings of a phase 1 cohort study of 10 kidney transplant recipients with acute or chronic active AMR (median of 4.3 years post-transplant) who received BIVV009 (34). Patients enrolled had features of classical pathway activation like complement-fixing DSA or C4d staining in peritubular capillaries in allograft biopsies. No severe adverse events were reported in 7 weeks of follow up. C4d staining turned from positive to negative in five patients and a significant improvement in C4d deposition in biopsies of two other patients. However, microvascular inflammation, gene expression profiles, DSA strength, and renal allograft function were unchanged (34). While BIVV009 appeared to be well-tolerated, it needs to be studied in future trials to determine its role in AMR therapy.

Complement Modulation in Highly Sensitized Kidney Transplant Candidates

Sensitization to HLA is often a significant challenge to kidney transplantation (39). The calculated PRA (CPRA) score estimates the population prevalence of potential donors with HLA antigens against whom a transplant candidate is sensitized (24, 40). Approximately one-third of kidney transplant candidates awaiting a deceased donor transplant in the US have a CPRA >30%, and this decreases the likelihood that they will receive an organ offer (41). These patients have a prolonged wait time for transplantation and a reduced transplant rate (42). While

policies that preferentially assign allocation points to patients who are highly sensitized, and innovative strategies like kidney paired donation may facilitate transplantation in this patient population, many–especially those with a CPRA >99%–are unlikely to receive a transplant. In such patients, transplantation across HLA barriers by "desensitization" may be their only chance at emancipation from dialysis (39). Living donor kidney transplantation after desensitization by the depletion of DSA is associated with a greater survival rate than waiting for an HLA compatible kidney transplant or staying on dialysis (42, 43). However, there is a high incidence of AMR in recipients of HLA incompatible kidney transplants following desensitization by the current approaches that employ plasmapheresis and IVIg, with some centers reporting an AMR rate as high as 40%. AMR, in this setting, can be difficult to treat and result in graft loss (44).

Eculizumab's role in the prevention of AMR in HLA-incompatible kidney transplants was tested in an observational study of 26 highly sensitized patients by Stegall et al. (45). The rate of AMR in the study group was compared to 51 historical controls. Study participants received plasmapheresis before transplant if the strength of the B-cell flow-cytometry crossmatch with their respective living donors was >300 mean channel shifts (MCS) in order to lower their DSA to acceptable levels for transplantation. The eculizumab regimen consisted of two doses given peri-operatively, followed by weekly doses for 4 weeks. Further dosing depended on assessment of DSA levels thereafter. In contrast, those in the historic control group had received pre-transplant plasmapheresis and an additional 4–14 post-transplant plasmapheresis treatments. In this study, recipients who received preemptive treatment with eculizumab, had a 7.7% incidence of AMR by 3 months post-transplant, whereas the historic control group had a significantly higher incidence of AMR (41.2%) (45). However, long-term follow-up demonstrated that histologic findings such as microvascular inflammation, transplant glomerulopathy, and C4d deposition were no different between the two groups. Among subjects who had sustained levels of DSA, eculizumab treatment did not prevent transplant glomerulopathy. This may be representative of events upstream of the activation of the terminal complement pathway, like anaphylatoxin C3a and iC3b, C3dg opsonins which drive chronic injury and inflammation. Additionally, injury can occur through complement-independent mechanisms such as Fc-mediated antibody dependent cellular cytotoxicity by NK cells, or the direct activation of endothelial cell signaling pathways by DSA AMR (46).

Contrary to the encouraging findings from these non-randomized studies reported by Stegall et al., a randomized trial of eculizumab in the prevention of AMR in HLA-sensitized recipients of living-donor kidney transplants initially failed to show any benefits (47). In this multicenter study, 102 HLA-incompatible candidates were desensitized prior to transplant with SOC therapy (plasmapheresis and intravenous immunoglobulin). At the time of transplant they were randomized to either receive post-transplant eculizumab or additional plasmapheresis. There was no difference in the primary composite outcome which included histologically-proven AMR, graft loss, patient death, or loss to follow-up 9 weeks after transplant (5/51 vs. 7/51, $P = 0.76$) (47).

Post-hoc analysis demonstrated that the failure to show benefits in the treatment arm was likely related to trial design, inasmuch as there was discordance between the central and local pathology assignment of the diagnosis of AMR. When the central pathologists re-analyzed the biopsies with the clinical data that had been available to the local pathologists, the discordance went away, and the difference between the treatment and control groups became significant. Likewise, if all categories of AMR severity were assigned the diagnosis of AMR, the results were also significant. Most clinicians believe that the diagnosis of AMR is binary, and the grading system used in the study was not clinically relevant, again reinforcing a positive result to the study (48). Another *post-hoc* analysis, showed the peril of changing the DSA strength inclusion criteria mid-study which was done to boost enrollment, allowing patients with relatively low immunologic risk for AMR (including patients with C1q negative DSA) to enter the trial. This had the effect of lowering rates of AMR in both study arms below expected based on prior reports in HLA incompatible transplant recipients (49).

C1-INH is a promising therapeutic target in transplantation. Its role in the prevention of AMR in HLA-incompatible transplantation was evaluated in a phase 1/2, placebo-controlled study by Vo et al. (50). The study randomized 20 patients who underwent kidney transplantation following desensitization with a regimen consisting of IVIg, rituximab, and plasmapheresis into two arms. The C1-INH group received one dose intraoperatively, followed by a twice-weekly regimen for a total of seven doses (50). Delayed graft function (DGF) occurred in only one patient treated with C1-INH and in four patients in the placebo group, suggesting that C1-INH may offer protection against ischemia-reperfusion injury. AMR occurred in none of the C1-INH treated patients, and in one of the placebo-treated patients during the study period. There appeared to be a lower incidence of C1q-binding DSA in the C1-INH group. No serious treatment-related adverse effects were reported in either group (50). The question of whether complement inhibition has a role in the prevention of acute or chronic AMR in highly sensitized patients undergoing HLA incompatible transplantation remains an open question which can only be answered by well-designed large randomized trials. Complement inhibition upstream of C5 with C1-INH is indeed an attractive, logically sound target, since it prevents the formation of anaphylatoxins and opsonins (4).

Complement Modulation to Prevent Recurrence of Disorders of Complement Dysregulation Post-transplant

Alternative complement pathway dysregulation underlies the etiopathogenesis of two ultra-rare kidney diseases, aHUS, and C3 glomerulopathies, both of which can lead to ESRD (51). Genetic abnormalities implicated in the causation of these conditions include mutations in complement factor H, complement factor I, membrane cofactor protein (MCP), complement factor B,

and C3. In 2011, the FDA approved eculizumab for aHUS therapy following a clinical trial that demonstrated that it significantly improved kidney function (10). While there is no approved therapy for the treatment of C3 glomerulopathy, in a retrospective study conducted by Avasare et al., a combination of anti-proliferative agent mycophenolate mofetil (MMF) and corticosteroids induced remission in 67% of patients (52). At this time, the global nephrology professional society guidelines state that there is not enough evidence to support eculizumab's role as primary therapy for rapidly progressive C3 glomerulopathy (53). An unfortunate aspect of the natural history of both of these diseases is that they recur even after successful kidney transplantation (54). Apart from its well-established role in the treatment of aHUS in native kidneys, terminal complement blockade with eculizumab is increasingly recognized as an effective therapy for prevention and treatment of aHUS after renal transplantation (51, 55). In the case of C3 glomerulopathy, however, the role of eculizumab has not been clearly established. Evidence from small case series does not support the use of eculizumab in this condition, either in native or transplanted kidneys, except perhaps in cases where soluble or serum MAC (sMAC) is elevated (56). Small phase one clinical trials have been initiated to test the safety of C3 inhibitors in the treatment and prevention of C3 glomerulopathy (57).

COMPLEMENT IN XENOTRANSPLANTATION

Outcomes in transplantation are hindered by drug-related adverse effects, chronic allograft rejection, and a scarcity of organs (58). Currently, more than 110,000 individuals are awaiting a transplant in the US; only a third of these receive a transplant, and many die waiting for an organ (59). Xenotransplantation entails transplantation of organs across species, and when combined with induction of tolerance, it could be the answer to the above issues (60, 61).

Transplantation of organs derived from pigs is an option that has attracted the greatest interest in the field (60). However, pigs express an epitope called α-1,3 Gal (62). This is a terminal carbohydrate modification of many glycoproteins and glycolipids that is present in most species and is made by an enzyme called α-1,3-Gal transferase (58). Old world monkeys and humans lack this epitope due to a mutation in the 1,3-Gal transferase gene. Since many species like bacteria and other microbes do have the α-1,3-Gal epitope, humans are exposed to it, and we have naturally occurring antibodies circulating that recognize α-Gal (62). When organs are transplanted from pigs to primates, these antibodies bind the donor vascular endothelium to activate complement and may cause hyper-acute rejection (HAR) or delayed xenograft rejection (DXR) (62). In the early 2000s, the development of technology to knock out the gene for α-1,3-Gal transferase accelerated the tempo of research in the field of xenotransplantation (63).

With advances in CRISPR (clustered regularly interspaced short palindromic repeat)/CRISPR-associated 9 (Cas9) technology, various groups have created genetically altered pigs to facilitate xenotransplantation (58). In addition to knocking out pig glycan genes, human complement regulatory proteins have been expressed from transgenes in some genetically engineered pigs, and is seen as an important strategy to prevent xenograft injury. In 1998, White et al. reported a median graft survival of 40 days in hearts of transgenic pigs expressing human decay accelerating factor (hDAF/CD55) that were transplanted into cynomolgus monkeys or baboons. These primates received potent immunosuppression with cyclosporine, cyclophosphamide, and steroids. This group expressed hope at the time that the use of organs from transgenic pigs may help to solve the problem of donor shortage in clinical transplantation (64). Pigs expressing CD46, CD55, and CD59 on the surface of vascular endothelial cells have been used in preclinical primate studies and appear to extend the xenograft survival (65, 66).

More recently, progress has been made by combining multiple glycan knockouts with human complement and coagulation regulatory gene knock ins (67). In 2016, Mohiuddin et al. published findings describing prolonged graft survival following pig to primate heart transplantation using immunosuppression and genetic engineering (68). Their study involved α-1,3-Gal transferase knockout pigs expressing human complement regulatory protein CD46 and human thrombomodulin. Pig hearts were heterotopically transplanted in the abdomen of baboons. Immunosuppression included induction with anti-thymocyte globulin and anti-CD20 antibody, maintenance with mycophenolate mofetil, and high dose anti-CD40 antibody. The authors reported a median graft survival of 298 days and a longest graft survival of 945 days in five consecutive recipients using this regimen. However, lowering the dose of anti-CD40 antibody at day 100 or after 1 year led to a rebound of anti-pig antibodies and graft loss (68). More recently life-supporting function of baboon orthotopic heart xenografts have been shown to consistently exceed 195 days, and this has been seen as a major breakthrough (69).

While currently available, transgenic knockout pigs may provide a solution to the problem of potent and immediate humoral responses due to pre-formed anti-Gal antibodies and allow transplantation. However, agents utilized in most of the successful preclinical primate studies do not have a track record of safety in humans. Therefore, to realize the goal of clinical xenotransplantation with sustained graft function in humans, tolerance induction is seen as a key element.

CHALLENGES AND FUTURE DIRECTIONS

Severe infections of *Neisseria meningitidis* and other encapsulated bacteria like *Streptococcus pneumoniae* have been well-documented in people with inherited deficiencies in complement components (7). The nature of the deficiency determines the specific infection to which affected individuals are susceptible. For example, abnormalities of the terminal

complement pathway predispose individuals to *N. meningitidis* infections, whereas C3 deficiency leads to a broad array of infections (7). Given its crucial role in immunity, prolonged pharmacologic complement blockade will likely involve an incremental increase in infectious risk above what we have become accustomed to with traditional immunosuppression. Another important consideration is the remarkably high cost of complement-targeting therapeutics that are currently approved. A market analysis of therapeutic agents available for the treatment of PNH conducted in 2007 estimated the average annual cost per patient receiving eculizumab, a drug with orphan disease designation, is $389,000 (8). This puts pressure on health care systems and makes the drug inaccessible in many regions in the world (7).

Several complement inhibitors are currently under development for a variety of disorders involving complement dysregulation. Compstatin is a peptide agent that prevents cleavage of C3 by C3 convertase (4). The FDA granted orphan drug status to Compstatin analog, AMY-101 (Amyndas Pharmaceuticals SA) for use in PNH and C3 glomerulopathy[1,2]. In the future, the availability of targeted therapeutic agents that interfere with the complement cascade at various levels will provide valuable opportunities to mitigate allograft injury, not just due to AMR, but also from ischemia-reperfusion injury and recurrent complement mediated glomerulopathies. Experience in kidney transplantation with these agents can also be potentially translated into successful therapies for heart, lung, and liver transplant recipients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

[1]https://www.fiercepharma.com/pharma/amyndas-lead-candidate-amy-101-receives-orphan-drug-status-from-fda-and-ema-for-treatment-c3

[2]https://www.fiercebiotech.com/biotech/fda-grants-orphan-drug-designation-for-pnh-to-amyndas-pharmaceuticals-novel-complement

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REVIEW
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Current and Future Approaches for Monitoring Responses to Anti-complement Therapeutics

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Aberrations in complement system functions have been identified as either direct or indirect pathophysiological mechanisms in many diseases and pathological conditions, such as infections, autoimmune diseases, inflammation, malignancies, and allogeneic transplantation. Currently available techniques to study complement include quantification of (a) individual complement components, (b) complement activation products, and (c) molecular mechanisms/function. An emerging area of major interest in translational studies aims to study and monitor patients on complement regulatory drugs for efficacy as well as adverse events. This area is progressing rapidly with several anti-complement therapeutics under development, in clinical trials, or already in clinical use. In this review, we summarized the appropriate indications, techniques, and interpretations of basic complement analyses, exemplified by a number of clinical disorders.

Keywords: clinical trial, laboratory investigation, immunoassays, functional assays, CV%

INTRODUCTION

Physiological and Therapeutic Regulation of Complement Activation

The complement system consists of more than 50 soluble and membrane-bound proteins that function in both innate and adaptive immunity. Excessive complement activation contributes to the pathogenesis of many inflammatory diseases and numerous disease processes (summarized in **Table 1**). The underlying mechanisms include (1) increased and persistent activation, such as that resulting from the presence of soluble or solid-phase immune complexes as in systemic lupus erythematosus (SLE), myasthenia gravis, and related autoimmune disorders; (2) altered expression or function of various complement regulators as in paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), and C3 glomerulopathies (C3G); or (3) a combination of the two. Furthermore, complement activation is a part of reactions resulting from activation of all cascade systems of the blood, e.g., during ischemia reperfusion injury (IRI).

TABLE 1 | Complement-related diseases and disease processes.

Disease	Analyte
Age-related macular degeneration	FH, FI, CD46 (MCP)
aHUS	FH, FI, CD46 (MCP)
Alzheimer's disease	C1q, C3, CR3
ANCA-associated vasculitis	C5a
Angioedema	C1-INH
C3 glomerulopathies	C3, FH, FHRs
Diabetic nephropathy	CD59
Encapsulated bacterial infection	C3
PNH	DAF, CD59
SLE	C1q, C1r, C4 or C2, FH, FCN3
Transplant	C3a, C5a, C5b-9, C4d

MCP, membrane cofactor protein; FH, factor H; FI, factor I; aHUS, atypical hemolytic uremic syndrome; ANCA, anti-neutrophil cytoplasmic antibody; C1-INH, C1-inhibitor; FHRs, factor H-related proteins; PNH, paroxysmal nocturnal hemoglobinuria; DAF, decay accelerating factor; SLE, systemic lupus erythematosus; FCN3, ficolin 3.

The complement system is regulated at distinct levels as illustrated in **Figure 1** and **Table 2**.

(1) The first level of regulation occurs at the initiation level where the recognition molecules within two of the complement system's three activation pathways form complexes with proteases. In the classical pathway (CP), this complex is the C1 complex consisting of C1q, $C1r_2$, and $C1s_2$ molecules. In the lectin pathway (LP), the complex contains MASP-1 and/or MASP-2 and one of several recognition molecules, such as mannose binding lectin (MBL); Ficolin-1,−2,−3; or Collectin 10/11. All proteases in these pathways are regulated by C1 inhibitor (C1-INH), a serine protease inhibitor with broad specificity that also is active toward other cascade systems, such as the kinin/kallikrein system.
(2) The second level involves the generation of C3 convertases that cleave C3 into C3a and C3b. The initiating complexes of the CP and the LP cleave C4 and C2 and generate the LP/CP convertase C4bC2a. In the alternative pathway (AP), a convertase is generated from C3, factor B, and factor D in conjunction with properdin, and in a self-perpetuating process, the AP convertase C3bBb is formed.
(3) In the third level, the C5 convertases, which are derived from the C3 convertases, switch their specificity from C3 to C5, thereby cleaving C5 into C5a and C5b. The C5 convertases are regulated in the same way as the C3 convertases. C4b and C3b are regulated by the plasma protease factor I in three steps, mediated by one of several co-factors. The two first cleavages generate iC4b or iC3b, which lose their ability to generate either the C3 or the C5 convertases but which promote phagocytosis via interaction with complement receptors CR1 (CD35), CR3 (CD11b/CD18), CR4 (CD11c/CD18), and/or CRIg. The third factor I-mediated cleavage splits the molecule into the target-bound C4d and C3d,g (a ligand for CR2 or CD21) fragments and C4c/C3c, which is released from the activating surface.

At this level, there are a number of regulators that protect autologous cell surfaces against complement attack. These include membrane-bound molecules, such as CR1, decay acceleration factor (DAF; CD55), and membrane cofactor protein (MCP; CD46), all of which disrupt the C3 convertases by different mechanisms (1). Additional regulators, including C4b-binding protein (C4BP, which regulates the CP/LP convertase) and factor H (the main regulator of the AP), are recruited from the plasma via glycosaminoglycans and/or deposited C3 fragments to the cell surface, thus providing further protection.

The anaphylatoxins C4a, C3a, and C5a, which are generated by the cleavage mediated by C1s and the convertases, respectively, attract and activate mainly leukocytes via their receptors C3aR, C5aR1, and C5aR2. Recently, C4a was also shown to activate endothelial cells via the thrombin receptors PAR1 and PAR4 (2). Anaphylatoxins are regulated by carboxypeptidases (e.g., B and N) that desarginate the polypeptide in the C-terminus, leading to a significant, but not complete, loss of activity (3).

(4) The final stage in the sequence is the formation of the C5b-9 complex (either the fluid-phase sC5b-9 or the membrane attack complex, MAC), which may insert into the cell membrane, thereby inducing cell lysis at high concentrations, or alternatively trigger inflammation and upregulation of tissue factor at sub-lytic concentrations (4). Terminal pathway (TP) regulators, such as cell-bound CD59 or vitronectin and clusterin in the fluid-phase regulate the formation and binding of C5b-9 to autologous cell surfaces.

Mechanisms of Therapeutic Regulation

Substances intended for therapeutic regulation of complement in human disease have a number of different mechanisms of action (5). Antibodies against functional sites in the molecule are commonly used and best exemplified by eculizumab and ravulizumab, which are antibodies that prevent the cleavage of C5 by the C5 convertases. Alternatively, small molecules, aptamers (6), or peptides are used, which can either block the active site of serine proteases or prevent the interaction of proteins in the complement cascade. Examples in this class of complement inhibitors include factor D inhibitors and peptides of the compstatin family that prevent the substrate C3 to be cleaved by particularly the AP C3 convertase, as well as the CP C3 convertase. A final group and probably the largest one consist of recombinant proteins in either full-length or truncated forms of physiological regulators of complement. The first example is soluble complement receptor (sCR1 [CD35]; TP10), which is a receptor and regulator of the convertases that acts as a cofactor for factor I, or by increasing the decay of the convertases. CR1 belongs to a large family of complement regulators, which consists of varying numbers of homologous domains, so-called short consensus repeats (SCR). CR2 (CD21), MCP (CD46), DAF (CD55), factor H, and C4BP are found in this family. Many of those, in full-length or in truncated recombinant forms, have been engineered to regulate complement activation for therapeutic purposes. Serpins, such as C1-INH, are another type of regulators with broader specificity and have been employed

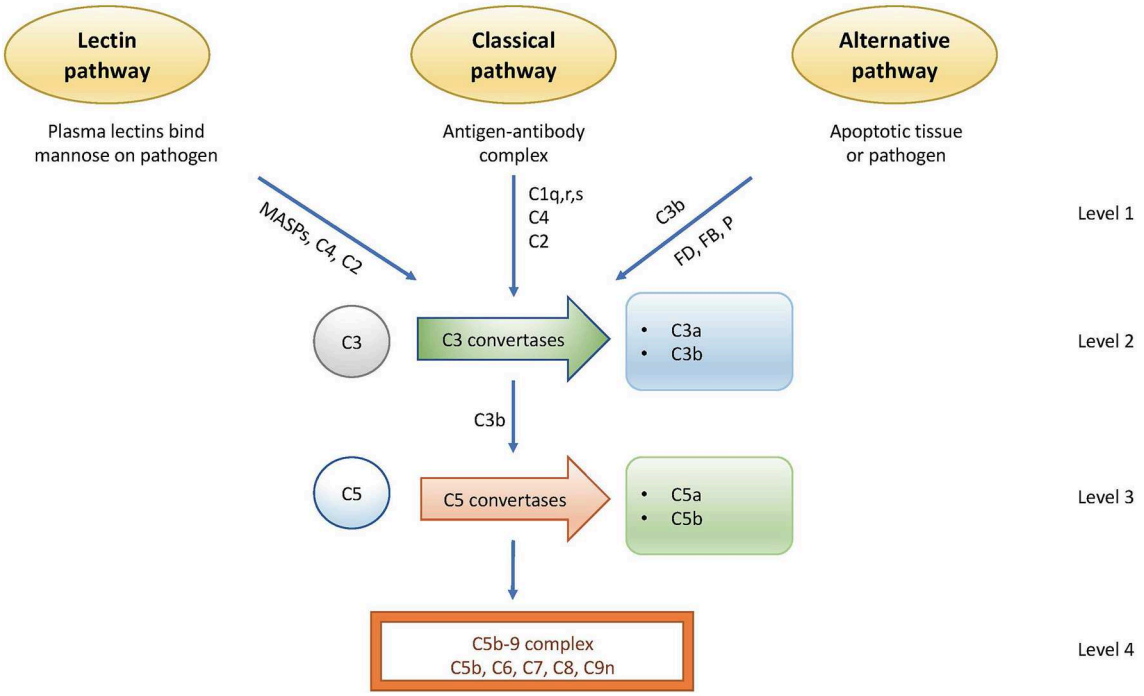

FIGURE 1 | Activation and regulation of the complement system. Activation occurs via the lectin pathway (LP), the classical pathway (CP), and the alternative pathway (AP). Regulation occurs at distinct points. Level 1: inhibition of proteases generated by the LP and AP; level 2: control of the C3 convertases; level 3: control of the C5 convertases; level 4: control of the formation of the C5b-9 complex of the terminal pathway (TP).

TABLE 2 | Regulatory targets of the complement system.

Regulators	Function
Carboxypeptidase-N	Removal of terminal arginine to degrade C3a and C5a
C1-INH	Inhibits C1r, C1s, MASPs
C4BP	Accelerates decay of LP/CP convertases Cofactor for FI
CD46 or MCP	Cofactor for FI
CD55 or DAF	Accelerates decay of convertases
CD59 or Protectin	Binds to C8 and C9, prevents assembly of terminal complement complex
FH	Recognizes self surfaces, accelerates convertase decay, cofactor for factor
FHL-1	Accelerates convertase decay, cofactor for factor I
MAP-1	Binds to MBL/ficolins, inhibits C4 deposition
Type 1 complement receptor (CD35/CR1)	Dissociation of C3 convertase subunits, cofactor for factor I-mediated cleavage of C3b and C4b

C1-INH, C1 inhibitor; MASPs, mannan-binding lectin serine proteases; C4BP, C4 binding protein; FI, factor I; MCP, membrane cofactor protein; DAF, decay accelerating factor; FH, factor H; FHL-1, factor H like protein 1; MAP, MBL/ficolin associated protein; MBL, mannose-binding lectin.

as therapeutics. Purified or recombinant C1-INH is one of two complement-targeting drugs (together with anti-C5; eculizumab and ravulizumab) that have been approved for clinical use.

Antisense strategies to silence the gene expression of a drug target are a more recent development in drug discovery, where targeting at the DNA or RNA level may be a way to overcome high target concentrations. This strategy has also been applied to the complement field. Alnylam Pharmaceuticals has developed a C5-directed RNAi therapeutic that is liver-targeted through GalNAc conjugation and silences intrahepatic expression of C5 (5, 7). Hence, this strategy leads to a systemic reduction in C5 levels and terminal pathway activity, and is currently being trialed as a therapy for PNH and aHUS. Likewise, the company Ionis Pharmaceuticals has developed a systemically administered factor B targeting antisense oligonucleotide that has also entered clinical trials with the aim of reducing AP activity in AMD and IgA nephropathy (5, 8). While some antisense therapies are directed systemically, this strategy also offers an opportunity to target complement factors in a specific tissue or at the site of disease through delivery systems, such as antibodies.

ANALYTICAL METHODS

Robust and accurate measurement of complement proteins and activation products is required, in order to monitor patients treated with drugs that result in detectable changes in the complement status, such as treatment with compstatin variants or anti-C5. Standard complement evaluation includes three main categories: (1) complement function, (2) quantification of individual complement protein, and (3) quantification of activation products (9).

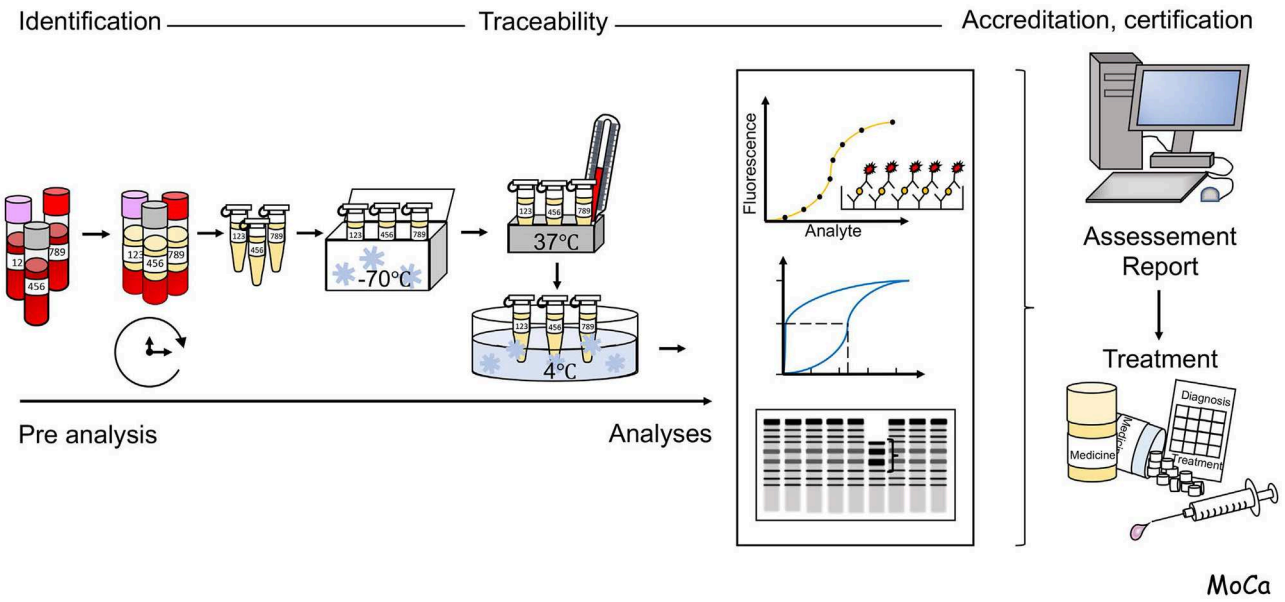

FIGURE 2 | Schematic overview of the flow of events in the process for analysis of clinical patient samples. Reliable analysis of samples requires the control of pre-analytical handling of and choice and validation of appropriate analytical techniques, Detailed information is given in the section "Pre-analytical handling and methodological considerations".

Pre-analytical Handling and Methodological Considerations

A schematic overview of the process for analysis of clinical patient samples is illustrated in **Figure 2**.

A major issue to take into consideration for laboratory analysis of complement activation is the choice of biological sample and, if applicable, the use of anticoagulants. EDTA-plasma is suitable for quantitation of individual complement factors and for assessment of activation products, while serum is suitable for analysis of complement function. Serum can be substituted by plasma that is anticoagulated with FXa or thrombin inhibitors (e.g., Dabigatran, or lepirudin, i.e., recombinant hirudin, respectively), which do not disturb complement function. As a general recommendation, the samples should be frozen at −80°C within 120–240 min. It is important not to freeze the specimen at −20°C, which creates a slow freezing rate and further activation/inactivation of individual components. The optimal transportation method is using dry ice containers. In addition, measurement of split products with enzyme-linked immunosorbent assay (ELISA) requires cautious handling, such as collecting the blood in EDTA-containing tubes, immediately placing samples on ice, and storage at −80°C after 30 min of centrifugation. The issue of sample handling in relation to generation of complement activation *in vitro* has been discussed extensively elsewhere [e.g., (9–11)].

Functional Assays

The proper functioning of the individual complement activation pathways depends on the integrity of each of its participating component(s), and therefore a functional test that monitors a whole activation pathway from initiation to the effector phase (formation of the C5b-9 complex, i.e., lysis) can detect both therapeutic-induced deficiencies in complement components and consumption-related decreases in complement activity, thus combining the information obtained from the various types of assays described above.

Traditionally, complement function by the CP is assessed by hemolytic assays that use sheep erythrocytes coated with rabbit antibodies (preferably IgM but sometimes combined with IgG). When serum (or lepirudin-anticoagulated plasma) is added, C1q binds to the immunoglobulins, leading to the assembly of the C5b-9 complex of the terminal pathway, thereby lysing the sheep erythrocytes (12, 13). Complement activation by the AP is monitored by the same assay principle with the exception that rabbit or guinea pig erythrocytes are used instead, as these are spontaneous activators of the human AP.

Hemolytic assays can be performed in various ways; the original assays, the so-called CH50 and AH50 assays, are based on titration of the amount of serum needed to lyse 50% of a fixed limited amount of cells during a certain time interval (12, 14). The considerably less laborious, and more rapid, one-tube assays give similar results and is based on the fact that the "dose" of complement is proportional to the number of cells lysed and the assay is therefore performed in an excess of erythrocytes (12).

Hemolytic assays are quite sensitive to the specific individual-derived erythrocytes that are used in the assays. Probing the erythrocytes before use is necessary in order to choose the right preparation. Most functional assays are linear in their dose-response except for the functional ELISAs, since there is no standard curve applied in these assays.

As an alternative to erythrocytes, liposomes coated with an activator are used in some tests and the assays are otherwise performed in a similar manner to CH50 assays. An important advantage with using artificial liposomes as activators is that results are no longer dependent on the source of animal of the RBCs used, which should improve reproducibility over time.

More recently, a method was introduced that made use of three separate ELISAs, for the first time enabling the simultaneous determination of all three activation pathways (including the LP). The assay can best be described as a "solid-phase functional test," since it incorporates recognition structures specific for each pathway (IgM for the CP, mannan or acetylated bovine serum albumin [BSA] for the LP, and LPS for the AP). These molecules are coated onto microtiter plate wells, and then serum is added and incubated under conditions in which only one pathway is operative at a given time, and the other two pathways are blocked. For each ELISA, the final step is the detection of the resulting C5b-9 complex by monoclonal antibodies (mAbs) against a neo-epitope in complex-bound C9 (15). One can here expect that the assay for AP activation will differ from the hemolytic assays in that the ELISA depends on LPS activation and properdin while the hemolytic assay lyse the cells because of an insufficient regulation of the AP on the cell surface.

Individual Components

The concentration of individual complement proteins is determined by various quantitative immunoassays. The most common employed methods are immunoprecipitation techniques, today mainly nephelometry and turbidimetry, where polyclonal antibodies against the protein of choice, e.g., C1-INH, C4, C3, or factor B, are added to the sample to form immune complexes that will distort the detection of light beams passed through the sample. Turbidimetry measures antigens based on changes in the light transmission. These techniques are accurate and fast, and have a large capacity and low variance. Also, C1q is commonly analyzed by nephelometry but is an inappropriate analyte for this technique due to its antibody binding properties (16). However, one of the main challenges for these methods is antibody reactivity with breakdown components or parent proteins particularly in C3, factor B, and C4 assays. For components with a low plasma concentration, ELISAs are more appropriate (17). This technique is also applied in measurement of activation products and autoantibodies against complement factors.

Recently, multiplex assays for complement components have been introduced and are now commercially available. The advantage of such assays is the simultaneous determination of several components, thereby saving both time and sample volume. To date, the analytes in the available kits have been restricted to components with high plasma concentrations, and no LP-specific panels are available yet on the market. There is no standardized regulatory guideline for validation of these tests. On the other hand, cross reactivity of reagents and inter- or intra- CV% are challenging aspects of multiplex immunoassays (18).

Activation Products

Complement activation generates activation fragments and protein–protein complexes, which can be quantified to assess the magnitude of complement activation. Two principles are used in assays for activation products: (1) mAbs specific for epitopes that are hidden in the native protein but exposed upon activation (so-called neo-epitopes). Most available assays for C3a, C3b/iC3b/C3s, C4a, C4b C4d, Ba, Bb, and aC5b-9 are based on mAbs to neo-epitopes (15, 19). A potential issue is that supposedly neoepitope–specific antibodies are polyreactive and also recognize the native protein. Even a low level of cross-reactivity can disturb the assay as the native protein typically is present in a much higher concentration than its activation product. This is the case using mAbs against C4d for monitoring CP activation in the fluid phase or in biopsies that also detect C4b, iC4b, as well as intact C4 (20). (2) The other option is to use polyclonal antibodies that often require fractionation of the native protein from its activation fragment or product. This principle is used for quantification of C3d,g by nephelometry. Polyclonal (and monoclonal) antibodies can also be used in sandwich ELISAs for quantitation of protein complexes, such as C1s-C1-INH, C3bBbP, sC5b-9 complexes, etc. The aforementioned assays are based on immune reactivity and antibody specificity.

Complement Analysis in Tissues or Body Fluids Other Than Serum/Plasma

In addition to analyses of plasma/serum, which measures systemic complement activation, it may, in many cases, be more informative or even required to determine the local activation state in a particular compartment or tissue. Increased levels of complement activation products during inflammatory processes can be found, e.g., in body fluids, such as cerebrosinal fluid (CSF) (21) or synovial fluid. Here, the assays outlined above can generally be used, whereas the rate-limiting factor may be the availability of material as sampling is more complicated compared to a peripheral venipuncture.

Lastly, staining of tissue sections or biopsy material for deposition of complement activation products will give information of the degree of complement activation at the local site of the organ. This is performed in clinical routine for, e.g., complement-mediated glomerulopathies.

A potential future methodology not yet in clinical practice would be to combine non-invasive imaging methods with a complement specific probe, e.g., a neo-epitope specific antibody coupled to a tracer. This method has been applied to antibodies specific for the C3 activation products C3d,g, with a recent example of an animal model of tuberculosis infection (22).

Analysis of Complement-Induced Receptor Signaling

The anaphylatoxins C3a and C5a, formed as a result of an activating cleavage of C3 and C5, respectively, are potent biological molecules that exert diverse biological effects on cells and tissues via their cognate receptors. The activating C5a receptor C5aR1 (CD88) is the target of ongoing drug

development programs, where ChemoCentryx has a small-molecule inhibitor (Avacopan/CCX168) in trials for aHUS, ANCA-associated vasculitis, and hidroadenitis suppurativa (23). Using an alternative strategy, Innate Pharma has developed a C5aR1 blocking antibody (IPH5401) currently in a phase I study as an adjuvant to cancer immunotherapy, testifying to the diverse biological effects of C5a signaling (24). To directly assess C5aR1 receptor blockade by CCX168, Bekker et al. performed an *ex vivo* assay after drug administration to healthy volunteers, with inhibition of CD11b upregulation on circulating neutrophils by exogenous C5a as endpoint (25). Such an *ex vivo* assay design will likely also be of value when designing assays to evaluate blockade of the receptor for C3a, which currently are in preclinical development (26) and may hold promise as a drug to enhance the efficacy of cancer immunotherapy (27).

Performance of Various Techniques

Most assays have either high specificity and low sensitivity or low specificity and high sensitivity. The conclusion is that one often needs more than one assay for diagnosis of a disease, i.e., determination of the degree of activation and which pathway that is activated.

Of the techniques above, most are easy to perform and can be used on many samples. One exception is the CH50 and AP50, which require serial dilutions of the samples, and if low levels are obtained, there may be a requirement that the samples must be reanalyzed (13, 14). Other assays are therefore warranted.

The coefficient of variation (CV%; standard deviation/mean value) is an extremely important property of an assay, which affects the power of the trial, i.e., the probability to reject a false null hypothesis. High CV% is associated with poor discrimination between various populations, while a low CV% will allow the opposite and is associated with a successful trial. The CV can be calculated either as an intra-assay (within one run) or inter-assay (between several runs) CV. The intra-assay CV is relevant in small trials when all samples can be run at the same time in one batch. If the samples are analyzed consecutively over time, the inter-assay CV is of great importance, since it will reflect differences between different runs performed on different days. For instance, one study demonstrated an intra-assay CV% for alternative pathway components ranging from 3% (factor D) to 8% (factor B) and inter-assay CV% ranging from 5% (factor D) to 15% (factor B) (28).

The CV% depends on the pre-analytical handling, the type of assay that is used and the performance of the laboratory. When considering the CV%, it can be as low as 5% or less when nephelometric or turbidimetric assays are applied. For other immunochemical assays including ELISAs, 10% and more are common. The CH50 and AP50 are poised to have high CV% if they are not run in the same batch of tests, due to the inborn variation of the assay and the different erythrocyte preparations. The one-tube hemolytic assay is much better in this respect and has a CV of $<$10% in the normal range (12) (Nilsson B, unpublished data). The intra-assay CV% for alternative and classic pathway function were also determined by more advanced ELISA techniques as 3.2 and 5.7%, respectively (29).

A laboratory may also have more than one device to analyze the samples and here it is important to make sure that these devices have the same precision and variation. Also, it is not recommended to use more than one laboratory for sample analysis to limit additional confounding from site to site. There is only one international standard (the IFCC international reference preparation [CRM470]) that is available for C4 and C3. Despite this, there is a huge difference in the precision between different turbidimeters and nephelometers (30, 31). Despite accurate measurement methods, it is usually difficult to distinguish between the exact activation mechanism by only measuring activation products, since more than one pathway is often involved in the activation in many conditions (32).

To illustrate this, a group of researchers compared three different assays to measure immune complexes by ELISA, including C1q binding assay, deposition in solid phase C3 binding glycoprotein (CIF) and anti-C3 antibodies in sera from patients with SLE, rheumatoid arthritis, and systemic sclerosing. All three tests showed specificity over 95%, but various sensitivity (C1q-ELISA−28.97%, CIF-ELISA−19.63%, anti-C3-ELISA−17.29%), which indirectly affects the correlation coefficient for each disease category (33).

Emerging Techniques for Measuring Complement Function, Components, and Fragments

The advent of the Human Genome Project greatly accelerated subsequent developments in transcriptomics, proteomics, metabolomics, and other "omic" technologies (34). Platforms to study genome-wide DNA variants have advanced from genome-wide genotyping arrays targeting hundreds of thousands to millions of single-nucleotide polymorphisms (SNPs) to whole exome sequencing (WES) and whole genome sequencing (WGS) with wet-lab and analyses pipelines as well as pricing continuing to improve. Transcriptomics has advanced from array-based RNA/cDNA nucleic acid probes for genome-wide RNA expression studies, to "whole-transcriptome" sequencing of RNA molecules (often termed bulk "RNAseq") to single-cell RNAseq where RNAs can now be interrogated at single-cell resolution [reviewed in (35)].

Proteomic and metabolomic approaches often overlap in sample preparation techniques and separation of molecules by mass/size, charge, and hydrophobicity. The analytic processes and platforms have also advanced significantly on a number of fronts including sample preparation processes, chemistry, and analytical instruments (36–38). One of the most commonly used analysis pipelines uses liquid chromatography and mass spectrometry (LC-MS) of peripheral blood leukocytes, blood/plasma/serum, or CSF preparations in which the proteins have been digested with trypsin into peptides. Peptides from multiple time points are typically tagged using isobaric mass tags (TMT labeling) in order to perform relative quantification (36, 38, 39).

Tagged peptides from each time point are often separated using a reverse-phase HPLC gradient directly coupled to a standard mass spectrometer instrument. Peptide sequences can

be determined by tandem mass spectrometry (MS/MS) coupled to database searching, such as the Sequest algorithms and Proteome Discoverer software. The peak intensity for each identified peptide across all experimental/clinical time points can then be calculated from the LC-MS data. Integrating genome-wide genotyping or sequencing with transcriptomics, as well as proteomic, metabolomic, and other omic datasets from blood and other tissues from samples of individuals selected for phenotypes of interest and appropriate controls over extended time periods can be performed using approaches such as integrative Personal Omics Profiling (iPOP). Such longitudinal multi-omic iPOP studies over prospective health and disease time points have revealed major insights into dynamic biological processes including multiple significant infections, and the development of a range of diseases (36, 38, 39).

A number of specific considerations should be made when analyzing such studies in the cascade characteristics of the complement system. The peripheral, dynamic, and temporal nature of the complement cascade means that in order to maximize insight, samples have to be taken at the most appropriate time points—and special consideration needs to be paid to sample storage and preservation as these platforms are particularly sensitive to oxidation—so samples need to be stored rapidly in liquid nitrogen or at −80°C with care to displace oxygen for long-term storage using liquid nitrogen or argon. Multiomic analysis can be performed to discover "omics" signatures related to complement related primary or secondary outcomes, using approaches such as ANOVA-based differential methods (40) or more complex machine learning methods. Sets of genes, proteins, metabolites, and other omic datasets can be tested for enrichment in *a priori* defined molecular pathways using standard bioinformatics tools (41).

Proteomic-based approaches have been used to investigate the potential involvement of complement in numerous human diseases and pathological conditions, both systemic and involving different specific organs. Here, we present a few examples from a rapidly growing body of analytical data.

The complement system plays an important role in the protection of the eye and complement components and regulators have been identified in most parts of the eye, using immunohistochemical, mRNA-based, or, more recently, proteomic approaches. However, dysregulated complement activation is implicated as a driving force in a number of ocular diseases, mostly studied in age-related macular degeneration (AMD), but also in glaucoma, uveitis, and neuromyelitis optica as reviewed in (42).

Formation of extracellular retinal deposits called drusen along Bruch's membrane in the submacular space is a risk factor for developing AMD, but drusen is also found in non-AMD individuals. Unbiased proteome analysis by LC-MS iTRAQ (isobaric tags for absolute quantification) technology of human drusen has identified 129 proteins, out of which one third were found only in AMD. Complement components that were elevated in AMD included C3, C5, C6, C7, C8γ, C9, and the regulators vitronectin and clusterin (43, 44).

Immune defense proteins, including complement proteins, were also quantitated in the macular Bruch membrane/Choroid complex in a study of human post-mortem eyes comprising 24 AMD eyes (10 early/mid stage, six advanced dry AMD, and eight wet AMD) and 25 normal control eyes. A total number of 901 proteins were identified, most of which did not differ in concentration between AMD and controls and were therefore concluded to reflect the proteome of normal macular tissue at the age of 81 years (the average age of the eye donors included in the study). Fifty-six proteins were increased and 43 decreased in AMD compared to controls. Approximately 60% of the elevated proteins were related to immune response and/or host defense, including C3, C4, C5, C6, C7, C8a, C9, factor B, factor D, and the regulators factor H and clusterin (45). The elevated protein constituent has proven to be variable in different stages of AMD, which is indicative of various mechanisms of disease progression, suggesting that a tailor-made complement-modulating treatment is needed (45).

The complement system also has a known role in diabetes and related complications. A recent iPOP study assessed the early biological processes impacting the transition to clinical type 2 diabetes (T2D). Multiomic profiling from healthy and prediabetes individuals ($n = 106$ total) took place over 4 years. Extensive host and microbial changes were observed to occur during respiratory viral infections, and insulin-resistant participants responded differently from insulin-sensitive participants. Furthermore, specific host–microbe interactions were observed to differ between insulin-resistant and insulin-sensitive individuals. Interestingly, these xenobiotics were also tightly associated with expression of host factors involved in the complement system (C4B, C4BPB, and C4BPA), which is associated with the development of diabetes (38).

The Integrative Human Microbiome Project (iHMP) followed 100 adult pre-diabetic participants for several years (46) and subjected a subset of 23 individuals to weight perturbation, where they consumed an additional 1,000 kcal per day for 30 days. All individuals were subjected to WES at baseline and multi-omic profiles were generated at all time points comprising RNAseq, proteome LC-MS of PBMCs, Proseek multiplex analytes from plasma, metabolomics (LC-MS), circulating cytokines (Luminex), and 16S rRNA sequencing and whole metagenome shotgun sequencing of microbiota. These data represent the largest integrative profiling project ever conducted on a cohort of humans. Despite the modest weight gain induced in this perturbation study, a wealth of biomolecular changes was evident across omic data types. Integrating proteomic and transcriptomic information revealed significant differences between pre-diabetics and healthy controls even at baseline, with many of these indicative of autoimmune responses (37). After weight gain, participants showed significant changes in pathways related to inflammation including complement pathways.

Additional studies looking at more limited combinations of omics have been attempted in complement-related diseases. These included combining transcriptomics and genotyping datasets in abdominal aortic aneurysms (AAAs) (47). Additional integration of expression data from bladder, breast, colon, lung, and lymphoma cancers with genomic datasets from the same individuals have also been investigated and have revealed that

Complement C1q -Binding Protein (C1QBP) showed association with patient survival, and identified C1QBP-involved pathways as potential targets for therapeutic intervention (48).

Defining the characteristics of the complement system in an individual can also help tailor individualized treatment with complement-targeted therapeutics. A well-known example that illustrates this concept well is the case of eculizumab resistance, where the genetic basis underlying a poor response to the drug was originally described in Japanese PNH patients (49). It was shown that poor responders harbored a C5 coding polymorphism that abrogated eculizumab binding while C5 still retained hemolytic activity. The mutation was subsequently confirmed to coincide with the eculizumab binding epitope in C5 (50). Akari therapeutics has launched clinical trials with the tick-derived peptide Coversin that blocks C5 cleavage via a binding site that is non-overlapping with eculizumab. Notably, Coversin is explored as a treatment option in patients with proven eculizumab resistance (51). This being one particularly illustrative example, next-generation sequencing methods or other omics techniques will undoubtedly be valuable in identifying other variants in an individual's complotype that limit or influence the response to a complement-targeting drug.

THERAPEUTIC REGULATION OF COMPLEMENT IN HUMAN DISEASE

Examples of Therapeutic Complement Regulators

Currently, there are only two types of complement inhibitors available in the clinic: C1-INH preparations and C5-targeting antibodies. C1-INH, either purified from plasma or produced in recombinant form, inhibits proteases generated by the CP and LP (C1r, C1s, MASP-1, and MASP-2) in addition to FXIIa, FXIa, and KK, which are activated by the contact system. The clinical use of C1-INH preparations is as substitution therapy in hereditary angioedema, and not as a complement inhibitor *per se*, although this possibility is explored in extension trials with, e.g., transplantation as indication. Eculizumab is a humanized anti-C5 mAb that prevents activation of C5 and thereby both the generation of C5a and the initiation of C5b-9 formation. Indications for eculizumab are aHUS, PNH, and refractory myasthenia gravis, and it is currently in clinical trial for, e.g., ABO-incompatible kidney transplantation. Ravulizumab is a further development of this antibody that has a more prolonged half-life (52).

In addition to these two inhibitors, a large number of compounds that act at different control points within the complement cascade are under development for various indications. Some compounds that are currently in clinical trials are anti C1s mAbs that inhibit CP activation, peptides within the compstatin family that all bind to C3, thereby making it resistant against activation by both C3-convertases, and APT070, which blocks downstream complement activation by inhibiting the C3-convertases. An intriguing example is Omero's inhibitory antibody to the LP protease MASP2, currently in trials as a treatment for aHUS, which has been considered a prototype of an AP-driven disease (**Table 3**).

Drug candidates in preclinical development are also expanding the potential use of these drugs beyond "classical" complement-driven diseases to neurological disorders like multiple sclerosis and Alzheimer's disease. There are a number of ongoing Phase I, II, and III clinical trials across a variety of disease spectra reviewed in detail [e.g., (83, 84)].

Principles for Monitoring the Effect of a Therapeutic Drug *in vivo*

Monitoring of the effects of complement therapeutics can be achieved using assays described above, measuring either the functional capacity of a certain pathway or the circulating levels of a specific component or activation product. When a complement component is activated *in vivo* either by proteolytic cleavage and/or other types of conformational changes triggered by, e.g., protein–protein interactions, the individual component is taken up by cells, e.g., leukocytes and Kupffer cells, leading to decreased levels of the component. This results in decreased levels (consumption) of complement components. If a whole pathway (CP + TP or AP + TP) is activated, all components are consumed along the activation sequence, and the function is reduced in the affected pathway while activation products derived from the individual affected components will be increased. If a therapeutic drug affects the complement activation at the level of an individual component, then the specific component and the downstream complement factors will be affected.

We conducted a systematic survey of registered clinical trials of complement-targeting drugs (83) to illustrate how complement activation is monitored during the clinical phase of drug development. Thirty trials were found where a biochemical measure of complement activation was among the endpoints (**Table 3**). Generally, in trials where information on complement monitoring was available, patient samples (typically serum) were subjected to well-established assays, such as CH50/AH50 or functional ELISA. When combined with measurements of activation products and individual complement components, these assays provide a fair assessment of the complement inhibitory activity of a drug *in vivo*. However, as outlined above, drawbacks in current methods for complement testing include limited sensitivity and susceptibility to errors in sample handling. We argue that novel methods could be of value, in order to precisely probe the efficacy and specificity of a drug and understand the physiological consequences of complement inhibition. In addition, determining a patient's complement-related genotype prior to starting a new drug may help to individualize treatment and select the complement-targeting drug that is most likely to be efficient in a given individual. Such improvements will in turn aid investigators in the development of the coming generations of complement therapeutics. Likewise, improved methods are needed to achieve a deeper physiological understanding of the consequences of complement inhibition in patients.

In this context, it is also important to note that drug levels and by-products should be quantified for pharmacological

TABLE 3 | Monitoring of complement activity in clinical trials of complement therapeutics.

Target	Drug candidate	Company	Entity	Indication	Status	Assay for monitoring complement activation	References
C1r, C1s, and MASPs	Cinryze	Shire	Protein	Transplantation	Phase I	Classical pathway and MBL pathway activity	(53)
	Cetor	Sanquin	Protein	Trauma or sepsis	Phase III	C1 inhibitor concentration	(54)
	Ruconest (conestat alfa)	Pharming	Protein	Contrast-induced nephropathy	Phase II	C1 inhibitor serum levels	(55)
C1s	BIVV009	Bioverativ	Antibody	Cold agglutinin disease	Phase 1	Classical pathway Wieslab® assay CH50	(56)
MASP2	OMS721	Omeros	Antibody	Thrombotic microangiopathies	Phase II	Lectin pathway activation	(57)
				aHUS	Phase III	C3 activity C4 activity	(58)
C3	AMY-101	Amyndas	Peptide	C3G	Phase 1	CH50 AH50 C3 plasma levels C4 plasma levels	(59)
	APL-9	Apellis	Peptide	PNH	Phase 1	CH50 AH50 C3 serum levels C3a serum levels	(60)
FD	Lampalizumab	Genentech	Antibody	AMD and/or GA	Phase III	Complement factor I profile biomarker (genotype)	(61)
	ACH-4471	Achillion	Small molecule	PNH	Phase II	Alternative pathway Wieslab® assay Factor D Factor Bb	(62)
FB	LNP023	Novartis	Small molecule	C3G	Phase II	Circulating C3 levels Circulating Bb levels Circulating sC5b9 levels	(63)
				PNH	Phase II	C3 fragment deposition on RBCs	(64)
				PNH	Phase II	C3 deposition on RBCs	(65)
				C3G	Phase II	C3 deposit score in kidney biopsies C3 levels Bb levels	(66)
				IgA nephropathy	Phase II	Bb levels sC5b-9 levels	(67)
Convertases	Mirococept	MRC	Protein	Transplantation	Phase III	Complement activity in serum C3a levels in urine	(68)
C5	Soliris (Eculizumab)	Alexion	Antibody	Membrane proliferative glomerulonephritis	Phase II	sC5b-9 levels	(69)
				Guillain-Barré syndrome	Phase II	Hemolytic complement activity in serum	(70)
				STEC-HUS	Phase III	CH50	(71)
	Tesidolumab (LFG316)	Novartis and MorphoSys	Antibody	AMD and/or GA	Phase II	C5 concentration in blood	(72)
				Uveitis and/or panuveitis	Phase II	C5 serum levels	(73)
	SKY59 (RG6107, RO7112689)	Chugai and Roche	Antibody	PNH	Phase I/II	*Ex vivo* liposome lysis in serum C5 serum levels	(74)

(Continued)

TABLE 3 | Continued

Target	Drug candidate	Company	Entity	Indication	Status	Assay for monitoring complement activation	References
	REGN3918	Regeneron	Antibody	PNH	Phase I	CH50	(75)
	ABP959	Amgen	Antibody	PNH, aHUS	Phase I	CH50	(76)
	Coversin	Akari	Protein	PNH	Phase II	CH50 ELISA	(77)
	Cemdisiran	Alnylam	Oligonucleotide	PNH	Phase I/II	Complement activity in serum C5 serum levels	(78)
C5a	IFX-1	InflaRx	Antibody	Sepsis	Phase II	C5a plasma levels	(79)
				SIRS, complex cardiac surgery	Phase II	C5a plasma levels CH50	(80)
				Hidradenitis suppurativa	Phase II	C5a plasma levels	(81)
C5aR1	Avacopan (CCX168)	ChemoCentryx	Small molecule	aHUS	Phase II	C3 serum levels C4 serum levels C5 serum levels Factor H C5a *Ex vivo* C5b-9 deposition on microvascular endothelial cells	(82)

Adapted from Ricklin et al. (83). Only trials with specified methodology for complement monitoring were included.
AAV, anti-neutrophil cytoplasmic-antibody-associated vasculitis; aHUS, atypical hemolytic uremic syndrome; ABOi, ABO incompatible; AMD, age-related macular degeneration; APS, antiphospholipid syndrome; CNV, choroidal neovascularization; COPD, chronic obstructive pulmonary disease; GA, geographic atrophy; GVHD, graft vs. host disease; IPCV, idiopathic polypoidal choroidal vasculopathy; LN, lupus nephritis; MN, membranous nephropathy; PNH, paroxysmal nocturnal hemoglobinuria; SIRS, systemic inflammatory response syndrome; wAIHA, warm autoimmune hemolytic anemia; CH50, classical pathway hemolytic assay; AH50, alternative pathway hemolytic assay; sC5b9, soluble C5b9 complex.

and toxicological purposes. Each drug requires a specific assay that can be based on various techniques, e.g., immunoassays (proteins, antibodies), HPLC, mass spectrometry, etc. In the case of eculizumab, quantification of the specific antibody is possible by ELISA (29). However, two populations of antibodies are circulating, one in complex with C5 and one that is not bound to the antigen (due to the long half-life). Two assays are necessary if one wants to keep track on both populations. The antibodies not bound to the antigen can be detected using a direct-binding assay with solid-phase bound C5 detecting bound IgG, while the other assay needs to pull down C5–IgG complexes using anti-C5 (other epitopes than eculizumab) and detecting IgG. Using these assays, it is possible to follow the pharmacokinetics of the drug.

DISCUSSION

A large number of clinical trials evaluating complement regulatory drugs have been completed or are currently ongoing. It has become evident that there is a pressing need to improve monitoring of the complement status in patients receiving treatment with these drugs; a need that is only expected to increase in the future given the extensive list of complement-targeting drugs that are in clinical trials. Poor laboratory assessments can obviously lead to inconclusive results, and the limitations of the currently available complement assessments are the CV of the assays and the selection of the complement specific tests. The CV results from not only the specific test that is used but also the pre-analytic handling of the samples before analysis. All these parameters are under the influence of the laboratory that performs the analyses.

As indicated above, the samples need to be processed as soon as possible (preferably in 4 h or less) including centrifugation at +4°C and storage at −80°C to avoid damage to the sample, particularly if the analyte is an activation product. Transportation should be on dry ice after freezing at −80°C. For instance, some of the split products have very short half-lives, making robust reproducible measurements very difficult. Poor handling will inevitably lead to variations. Also, the assay selection is of great importance since the difference in CV can be as much as 5–10 CV% between different assays. For instance, the difference between CH50 and a functional ELISA of the CP or the one-tube assay can be substantial, with CV% up to 20% often observed, particularly if inter-assay coefficient variations are being considered. Similar differences in CV% can occur if the protein concentrations are assessed by either radial immunodiffusion or nephelometry. The variation will lead to poor discrimination between individual comparisons as well as wide reference intervals and therefore blunt discrimination between normal and pathological values. So which level of CV% is acceptable? This is totally dependent on the parameter that is supposed to be evaluated. The lowest possible level is recommended since high CV% will lead to less power in the trial and the need of additional test individuals.

What is the desired or appropriate level of complement inhibition to seek with therapeutics in order to achieve a clinical response? Targets within the complement system are often plasma proteins with high circulating concentrations, which in turn necessitate high drug concentrations. A well-described phenomenon in PNH patients during anti-C5 therapy with eculizumab is breakthrough hemolysis. Defined as either pharmacokinetic or pharmacodynamic breakthrough, it results from insufficient dosing or massive complement activation exceeding the inhibitory capacity of eculizumab. To overcome this problem, there are strategies to increase the half-life of C5 antibody preparations, e.g., to promote its recycling from endosomes as in the case of Alexion's ALXN1210 (Ravulizumab). Several trials also evaluate additional C5 targeting drugs as add-on therapy to eculizumab. Hence, a very high degree of inhibition appears to be necessary to completely block complement-mediated hemolysis in this condition; on the other hand, the clinical benefit of complete complement inhibition remains controversial. For example, a low degree of residual intravascular hemolysis may not be considered be a clinically relevant issue (85).

The term "complotype" has been coined to describe an individual's genetic setup of common complement polymorphisms that determine complement activity on the genetic level (86). Assessing the genetic basis for complement activity in an individual, e.g., with targeted genotyping or next-generation sequencing, will undoubtedly facilitate individual dosing of complement-targeting regimens. Another potential implication is that the minimal required degree of inhibition may vary between patients depending on the genetic setup of their complotype.

In order to get a full view of the effect of an anti-complement drug, many times not only one parameter is sufficient. Analysis of all three complement categories (function, single component, and activation product) is necessary in order to get the full picture. With the advent of novel omics analyses and multiplex assays, detailed analyses of individual complement components and activation products are possible. This will help to follow pharmacokinetic events and possible side effects. It will also allow detailed analyses of other types of side effects, such as metabolic changes by metabolomic analyses.

AUTHOR CONTRIBUTIONS

MM, OE, BK, KE, and BN wrote the article. MM and CM prepared the figures. BP, KS, and MH-L edited the manuscript. All authors approved the final manuscript.

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