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Complementopathies

Andrea C. Baines¹ and Robert A. Brodsky¹

¹Division of Hematology, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, MD, 21205, USA

Abstract

The complement system is an essential part of the innate immune system that requires careful regulation to ensure responses are appropriately directed against harmful pathogens, while preventing collateral damage to normal host cells and tissues. While deficiency in some components of the complement pathway is associated with increased susceptibility to certain infections, it has also become clear that inappropriate activation of complement is an important contributor to human disease. A number of hematologic disorders are driven by complement, and these disorders may be termed “complementopathies”. This includes paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), cold agglutinin disease (CAD) and other related disorders, which will be the focus of this review. A better understanding of the central role of the complement system in the pathophysiology of these disorders may allow for application of therapies directed at blocking the complement cascade.

Keywords

Alternative pathway of complement; paroxysmal nocturnal hemoglobinuria; atypical hemolytic uremic syndrome; HELLP syndrome; cold agglutinin disease; complementopathy

1. Introduction

The complement system is a key component of the innate immune system that provides host defense against a variety of pathogens, including bacteria, fungi and viruses. A wealth of research in complement biology has taken place since the term “complement” was first coined in the late 19th century [1, 2], and we now recognize that complement has diverse roles in a variety of cellular processes, including bridging the innate and adaptive immune responses, maintaining homeostasis and preventing autoimmunity [3, 4]. An overview of the

Corresponding author: Robert A. Brodsky, M.D., 720 Rutland Avenue, Ross Research, Building, Room 1025, Baltimore, MD, 21205-2196, brodsro@jhmi.edu, Phone: 410-502-2546, Fax: 410-955-0185.

First author: Andrea C. Baines, M.D., Ph.D., 720 Rutland Avenue Ross Research Building, Room 1021 Baltimore, MD, 21205-2196, abaines2@jhmi.edu, Phone: 410-614-7521, Fax: 410-955-0185

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normal functions of the complement system will be presented first to provide the reader with a basic context for understanding how dysregulated complement underlies the hematologic disorders that are the focus of this review, including PNH, aHUS and CAD.

2. Role of complement in innate immunity and homeostasis

An effective system for immune surveillance requires mechanisms for detecting harmful pathogens or abnormal cells, while distinguishing them from healthy host cells and tissues. This ability to differentiate between self and non-self (or damaged/altered self) is a hallmark feature of the complement system. As a general paradigm, this is primarily accomplished through pattern recognition – host pattern recognition receptors (PRRs) bind to components unique to microbial surfaces (often consisting of specific carbohydrate structures that are not found on host surfaces), termed pathogen-associated molecular patterns (PAMPs). Interactions between PRRs and PAMPs leads to complement activation and destruction of invading microorganisms. Similarly, host PRRs are also able to recognize abnormal glycosylation patterns on the surface of dead or dying host cells (termed damage-associated molecular patterns, or DAMPs) and immune complexes, activating complement mechanisms that lead to their clearance.

3. Link to inflammation and adaptive immunity

Activation and amplification of complement leads to destruction of pathogens or abnormal host cells through opsonization – a process by which complement ligands coat surfaces, targeting them for phagocytosis – or by lysis induced upon formation of the membrane attack complex (MAC), also referred to as the terminal complement complex (TCC). Other intermediaries generated during this process promote the inflammatory response and modulate the adaptive immune response. Complement components C3a and C5a (known as “anaphylatoxins”), released upon activation of the complement cascade, serve as potent chemotactic agents, leading to recruitment and activation of immune mediators, including neutrophils, macrophages, monocytes and mast cells. Through interactions with B cells and T cells, products of the complement cascade also have an important role in modulating aspects of humoral and cell-mediated immunity.

4. Complement cascade

The complement cascade is a complex network of over 40 soluble and membrane proteins [5], that can be activated by one of three primary pathways: (1) the lectin pathway; (2) the classical pathway; and (3) the alternative pathway (Figure 1). Although the lectin, classical, and alternative pathways are sometimes depicted as three separate pathways, they actually constitute different modes of activation (Table 1) for an interconnected downstream complement cascade. Additionally, there are routes of activation through the coagulation and fibrinolytic systems that bypass these initial activation pathways [6].

4.1 Complement activation via the lectin pathway (LP)

The lectin pathway (LP) is activated when host PRRs, such as mannose-binding lectin (MBL) or ficolins, recognize unique carbohydrate structures (PAMPs) present on the surface

of pathogens or altered glycosylation patterns (DAMPs) on abnormal host cells. This results in activation of C2 and C4 by the mannose-binding lectin-associated serine proteases (MASPs) and subsequent formation of the C3 convertase (C4b•C2a).

4.2 Complement activation via the classical pathway (CP)

Typically, the classical pathway (CP) is initiated by immune complexes through binding of complement protein C1q, a PRR, to immune complexes containing IgM or IgG, in solution or bound to antigens on the cell surface. The CP can also be activated by direct binding of C1q to PAMPs on microbial surfaces. Upon binding, the C1 complex undergoes a conformational change, activating the C1r and C1s protease subunits, which leads to cleavage of C2 and C4 and formation of the C3 convertase (C4b•C2a).

4.3 Complement activation via the alternative pathway (AP)

The alternative pathway (AP) of complement is unique among the three primary activation pathways in that it is constitutively active, allowing it to be primed for surveillance and rapidly activated when pathogens are encountered. This is accomplished through the slow spontaneous hydrolysis of complement component C3, referred to as “tick-over.”

4.4 Complement activation via the coagulation cascade and fibrinolytic system

Extensive interplay exists between the complement system and coagulation/fibrinolytic systems. In light of their shared evolutionary origins and homeostatic functions, it follows that coordinate activation of coagulation and complement occurs in response to injury and infection. Indeed, it has been shown that certain activated factors in the coagulation and fibrinolytic cascades and complement components can cleave and activate one another [6–10]. These connections also explain why many complement driven diseases (e.g. PNH and aHUS) include pathologic thrombosis as a hallmark clinical manifestation. Under normal conditions, the complement and coagulation cascades are activated locally at the site of injury/infection. However, disruption of normal regulatory mechanisms can result in widespread systemic activation of both cascades with detrimental effects to the host.

4.5 Proximal complement cascade and convergence on C3 activation

Regardless of the mode of activation, the three major pathways converge on the central complement component C3 (Figure 1). Although the LP and CP have different initiating factors, activation through either pathway leads to formation of a shared C3 convertase (C4b•C2a). In the alternative pathway, there is slow spontaneous activation of C3 to form C3b, which binds to complement factor B (FB) to generate the AP C3 pro-convertase (C3b•B). Under normal conditions, in the absence of a harmful pathogen or other trigger, C3b generated by this low level constitutive activity is inactivated, halting further downstream activation and amplification of the complement cascade. When activation proceeds, C3b•B is processed by complement factor D (FD) resulting in formation of the active AP C3 convertase (C3b•Bb). Further cleavage of C3 by the C3 convertase leads to formation of C3a and additional C3b. Apart from its role in these initial activation steps, C3b has three additional important roles in the proximal portion of the complement cascade: (1) it binds to cell surfaces in complex with FB, triggering a positive feedback loop; this results

in the generation of even more C3a and C3b, leading to rapid amplification of the AP (termed the “amplification loop”) [11], (2) it complexes with C3b•Bb to form the C5 convertase (C3b•Bb•C3b), and (3) it serves as an opsonin, coating foreign particles and marking them as targets for phagocytosis.

4.6 Terminal complement

The first step in the terminal portion of the complement cascade is cleavage of complement component C5 by the C5 convertase, yielding C5a and C5b. Like the C3a generated in the proximal portion of the cascade, C5a serves as an anaphylatoxin, a substance released into surrounding fluids and tissues to recruit/activate inflammatory cells [12, 13]. C5b triggers assembly of the membrane attack complex (MAC), also referred to as the terminal complement complex (TCC), a pore-forming structure made up of C5, C6, C7, C8 and multiple C9 molecules [14]. The MAC forms a channel once inserted into the lipid bilayer, leading to lysis of cells or infecting organisms.

5. Regulation of complement

Effective defense against harmful pathogens and maintenance of homeostasis requires a careful balance between activation and regulation of complement. If not inactivated immediately by regulators, C3b molecules that are generated assemble into the C3 convertase (C3b•Bb) and a potent amplification loop is triggered within the AP. A number of regulatory factors, including both soluble (or serum) proteins and membrane proteins (Table 2), act at different steps of the complement cascade to control its activation (Figure 1) [15]. The genes encoding many of these regulatory proteins are part of a gene cluster on chromosome 1 (1q32), termed the regulators of complement activation (RCA) locus, indicating a common evolutionary history [16]. The majority of the regulatory factors function by exerting decay accelerating activity (DAA) and/or cofactor activity (CA), impacting the formation and stability of the convertases, which are inherently unstable protease complexes. Other regulatory factors function downstream to inhibit the terminal portion of the complement cascade, blocking formation of the MAC.

5.1 Soluble regulatory factors

Complement factor H (CFH), the principal negative regulator of the AP, is a circulating plasma protein that recognizes and binds to host surfaces, protecting them from activation and amplification of the complement cascade. CFH is able to discriminate between healthy host cells and altered host cells or pathogens through binding of its C-terminal domains 19–20 to specific glycan markers [17]. Once bound to host surfaces, CFH exerts inhibitory effects on the complement system by causing dissociation of Bb from C3 convertases (termed its “decay accelerating activity” or DAA) and serving as a co-factor for CFI (also referred to as “cofactor activity” or CA). By blocking further activation and amplification of the AP, CFH also serves to block complement activation through the LP or CP. Deficiency or impaired function of CFH leaves healthy host cells and tissues highly vulnerable to complement-mediated attack.

Complement factor I (CFI), along with its cofactors (CFH, C4BP, CD46 or CR1), inhibits C3 convertase formation by cleaving and inactivating C3b (in the AP) and C4b (in the CP and LP) [18]. Near or complete deficiency of CFI leads to uncontrolled activation of the AP systemically due to inability to cleave C3b and C4b [19].

Complement component 1 inhibitor (C1INH) binds to the activated C1r and C1s subunits of the C1 complex in the CP and to MASPs in the LP, shutting off cleavage of C2 and C4, limiting further activation of the CP and LP, respectively.

MAPI (also known as Map44) is a soluble inhibitor of the LP, which prevents MASPs from binding to MBL/ficolins, and thus prevents cleavage of C2 and C4, and subsequently, formation of the C3 convertase [20].

5.2 Membrane-associated regulatory factors

In addition to the soluble regulatory factors described above, there are a number of membrane-associated complement regulators that are relevant to discussion of hematologic complement disorders. An important distinction between soluble and membrane-bound regulatory factors is that the membrane factors can only regulate complement activation on the cells which express them, while soluble factors can circulate and bind to a variety of cells and surfaces.

CD46, also known as membrane cofactor protein (MCP), is a transmembrane complement inhibitor that serves as a cofactor to CFI in the proteolytic cleavage and inactivation of membrane-bound C3b (in the AP) and C4b (in the LP and CP). CD46 is widely expressed, though notably absent from erythrocytes.

CD55, also referred to as decay accelerating factor (DAF), is a GPI-anchored protein that both inhibits formation of C3 convertases and promotes their decay [21].

CD59, another GPI-anchored protein, serves as the major inhibitor of the terminal complement pathway through binding to C8 and C9, blocking assembly of the MAC [22]. Both CD55 and CD59 are widely expressed and are present on normal erythrocytes.

Thrombomodulin is a transmembrane protein expressed in endothelial cells that binds thrombin and activates protein C, downregulating further thrombin generation. It also serves as another link between coagulation and complement in that it activates thrombin activatable fibrinolysis inhibitor (TAFI), which cleaves and inactivates the anaphylatoxins C3a and C5a. In addition, it may also function to inhibit activation through the LP and CP via its lectin-like domain and through the AP by enhancing the degradation of C3b by CFH and CFI [23].

Complement receptor 1 (CR1) is a membrane protein found on erythrocytes and leukocytes that serves as an immune adherence receptor and has a variety of roles in regulating complement, including accelerating decay of the C3 and C5 convertases and serving as a cofactor for CFI.

6. Definition of a complementopathy

For the purposes of this review, we will define complementopathy as a disorder in which: (1) activation of the complement system is a driving factor in the disease pathophysiology, and (2) there is evidence that inhibition of complement disrupts or halts the pathogenic process of the disorder.

7. Paroxysmal nocturnal hemoglobinuria (PNH)

PNH is an acquired clonal hematopoietic stem cell disorder in which uncontrolled complement activity leads to systemic complications, principally through intravascular hemolysis and thrombosis [24]. In virtually all cases, the disease arises from somatic mutation of *PIGA* (Table 3), an X-linked gene whose product is required for the first step in glycosylphosphatidylinositol (GPI) anchor biosynthesis; thus, the *PIGA* mutant PNH stem cell and all blood cells derived from this cell have a marked deficiency or absence of GPI anchored proteins. Those most relevant to hemolysis and thrombosis in PNH are the GPI-anchored, complement regulatory proteins CD55 and CD59. CD55 inhibits the C3 convertase in the AP and CD59 inhibits formation of the MAC (terminal complement). Hemolysis in PNH is chronic because of a continuous state of complement activation through the AP, but paroxysms resulting in brisk hemolysis coincide with increases in complement activation that may occur with inflammatory states, pregnancy, infections, or surgery. The most common clinical manifestations include fatigue, dyspnea, hemoglobinuria and abdominal pain [25], but thrombosis is the leading cause of death in PNH [26].

The absence of CD59 is most responsible for the clinical manifestations in PNH. Accordingly, rare cases of inherited mutations in *CD59* leading to loss of CD59 on the cell surface have been well documented (Table 3) [27, 28]. The phenotype of these patients mimics PNH in that they manifest chronic intravascular hemolysis with paroxysmal flares of hemolysis and a propensity for thrombosis. Unlike PNH, pedigrees with inherited CD59 deficiency also present with relapsing immune-mediated peripheral neuropathy. In classical PNH, the CD59 deficiency is only found on the blood cells; in patients with germline *CD59* mutations, CD59 is deficient in all cells in the body. Thus, germline CD59 deficiency may be associated with demyelination via activation of terminal complement.

7.1 Treatment of PNH with eculizumab

Eculizumab is a humanized monoclonal antibody that blocks terminal complement by binding to C5 and sterically preventing C5 from binding to convertases [29]. This prevents cleavage of C5 into C5a and C5b. It is the only FDA approved therapy for PNH [30–33]. The drug blocks intravascular hemolysis, reduces the risk for thrombosis and improves quality of life. Eculizumab is also effective in patients with PNH resulting from germline *CD59* deficiency and rare cases of PNH caused by other genes involved with GPI anchor synthesis [34, 35]. It is administered intravenously every 7 days for the first 5 weeks and then every 14 $\pm$ 2 days thereafter. Eculizumab inhibits the formation of the MAC by blocking terminal complement at C5, and in doing so, compensates for the CD59 deficiency of PNH patients. It does not compensate for the loss of cell surface CD55; thus, eculizumab is highly effective in abrogating the intravascular hemolysis in PNH, but most PNH patients

on eculizumab continue to experience mild to moderate extravascular hemolysis due to C3 fragment deposition on the PNH red cells (Figure 2) [36]. Deposition of C3d can be detected in more than 50% of PNH patients treated with eculizumab with the direct anti-globulin test (C3 positive but IgG negative) in conjunction with a mild to moderate anemia and elevated reticulocyte count [37]. Terminal complement deficiency predisposes to *Neisserial* infections. Accordingly, all patients treated with eculizumab should be vaccinated against *Neisseria* and cautioned to seek immediate medical attention if they acquire a febrile illness. Despite adequate vaccination, the risk of acquiring a *Neisserial* infection is roughly 0.5% per year, so patients and physicians must remain vigilant.

The majority of classical PNH patients will respond to eculizumab; however, the hemoglobin response is highly variable and may depend on the degree of underlying bone marrow failure, concurrent inflammatory conditions, genetic factors and the size of the PNH red cell clone following therapy [38]. Up to 25% of PNH patients continue to require occasional red cell transfusions despite treatment with eculizumab. The most common reason for continued transfusions is extravascular hemolysis. An increase in the percentage of PNH red cells after eculizumab therapy correlates with response but also with extravascular hemolysis. The PNH red cells that are protected by eculizumab are CD55 deficient and become susceptible to opsonization by C3 fragments and premature removal in the spleen and liver (Figure 2) [36]. Pharmacogenetics may also influence response to therapy. Polymorphisms in the complement receptor 1 (*CR1*) gene are associated with response to eculizumab. CR1, through binding C3b and C4b, enhances the decay of the C3 and C5 convertases [39]. The density of CR1 on the surface of red cells modulates binding of C3 fragments to the GPI-negative red cells when C5 is inhibited. PNH patients with polymorphisms in *CR1* that lead to low CR1 levels (L/L genotype) are more likely to be suboptimal responders to eculizumab than patients with intermediate (H/L genotype) or high (H/H genotype) levels of CR1. Other genetic polymorphisms may also lead to unresponsiveness to eculizumab. A heterozygous missense mutation in *C5*, c.2654G→A, retains the functional properties of C5 but prevents binding and blockade by eculizumab [40]. The c.2654G→A polymorphism is present in 3.5% of the Japanese population and accounts for the lack of response to eculizumab in patients who carry the mutation.

7.2 Indications for eculizumab

Eculizumab is expensive and must be administered indefinitely; thus, in patients with mild or no symptoms a watchful waiting approach is reasonable, regardless of the of the PNH clone size. Severe anemia, thrombosis, frequent pain paroxysms, debilitating fatigue, worsening renal insufficiency, or dyspnea are indications to initiate therapy [41]. Thrombosis is the most urgent indication to start eculizumab. It is also important to note that terminal complement inhibition is the most effective way to prevent subsequent thrombosis in PNH [42]. Moreover, many PNH patients may be able to discontinue anticoagulation if their PNH is controlled with eculizumab, highlighting close interaction between complement activation and thrombosis. The decision to start eculizumab should not be based solely on the PNH clone size; however, patients with a large clone (>50% PNH granulocytes and >10% PNH red cells) coupled with a markedly elevated LDH (indicator of intravascular hemolysis) and a robust reticulocyte count (indicator of adequate bone marrow reserve) are

most likely to benefit. Eculizumab does not treat underlying bone marrow failure. Patients with severe bone marrow failure from aplastic anemia (hypocellular bone marrow, severe thrombocytopenia, and a low reticulocyte count) are unlikely to derive benefit from eculizumab unless they are having thrombotic complications. In these patients (aplastic anemia with a small PNH clone) it is best to direct initial therapy toward the bone marrow failure.

7.3 Monitoring the PNH patient on eculizumab

A complete blood count, reticulocyte count, LDH, and biochemical profile weekly for the first 4 weeks, and then monthly thereafter, should be used to monitor PNH patients starting eculizumab. A direct anti-globulin test should be obtained in patients with evidence of persistent hemolysis and PNH flow cytometry should be obtained yearly since the PNH clone size may fluctuate over time. The LDH usually returns to normal or near normal within days to weeks after starting eculizumab; however, the reticulocyte count usually remains elevated and the hemoglobin response is highly variable. The reticulocyte count often remains elevated because most PNH patients on eculizumab continue to have extravascular hemolysis due to opsonisation of C3dg coated red cells (Figure 2).

In classical PNH patients who are transfusion dependent, a marked decrease in red cell transfusions is usually observed, with over 70% achieving transfusion independence [43]. Breakthrough intravascular hemolysis and a return of PNH symptoms occurs in less than 5% of PNH patients treated with eculizumab. This typically occurs 1 or 2 days before the next scheduled dose, and is accompanied by a spike in LDH. If this occurs regularly, the interval between dosing may be shortened to 12 days, or the dose of eculizumab may be increased to 1200mg, every 14 +/- 2 days. Increased complement activation may accompany infections (upper respiratory viruses, influenza, viral gastroenteritis, etc.) or trauma and may result in transient breakthrough hemolysis. These single episodes of breakthrough hemolysis do not require a change in dosing since patients usually return to their baseline hemoglobin once the inflammation has resolved.

8. Atypical hemolytic uremic syndrome (aHUS)

aHUS is a thrombotic microangiopathy (TMA) characterized by hemolytic anemia, thrombocytopenia and renal failure [44]. Historically, hemolytic uremic syndrome (HUS) has been divided into typical HUS (primarily STEC-HUS due to Shiga toxin-producing *Escherichia coli*) and atypical HUS, referring to non-STEC mediated HUS. Newer classification systems have been proposed, including a division of HUS into primary causes due to mutations in complement genes or autoantibodies against complement regulatory proteins (aHUS), or secondary causes, including infection, drug toxicity or autoimmune disorders. Other systems have proposed naming of TMAs based on cause, for example, utilizing “complement-mediated TMA” to refer to aHUS in order to ameliorate confusion when discussing TMAs [45], however, there is no universally accepted naming convention at this time. For purposes of this review, we will use the term aHUS to refer to hereditary or sporadic/acquired forms that are complement-mediated.

The majority of aHUS cases are thought to be related to underlying genetic abnormalities (Table 3) in complement proteins and complement regulatory factors or development of autoantibodies against complement factors[46]. In most instances, aHUS appears to be a “two-hit” disease, in which genetic alterations predispose to the disorder, but a second hit is required to manifest the disease clinically. Presumably, conditions that trigger inflammation (surgery, pregnancy, infection, etc.), lead to increased complement activation, and susceptible individuals have dysfunctional regulation that ultimately leads to uncontrolled complement activity and damage to host cells and tissues.

8.1 Diagnosis of aHUS

Diagnosis of aHUS is a challenge, as it remains a clinical diagnosis that must be distinguished from other TMAs. Although aHUS classically presents with renal involvement, it may be accompanied by a wide range of end-organ damage. Patients with aHUS often present with abnormalities of multiple organ systems, including the CNS (seizures, stroke, encephalopathy), GI tract (gastroenteritis, pancreatitis, transaminitis, colitis) and cardiovascular system (myocardial infarction, severe hypertension). Clinical manifestations are therefore not reliable for diagnosis, and as a result, establishment of a diagnosis and initiation of appropriate treatment are frequently delayed for aHUS, and TMAs as a whole. Diagnostic evaluation for a patient presenting with suspected TMA should include a peripheral blood smear to evaluate for evidence of microangiopathic hemolytic anemia (presence of schistocytes and thrombocytopenia, Figure 3), LDH and reticulocyte count. It is important to screen for Shiga toxin to evaluate for STEC-HUS and obtain an ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif 13) level to evaluate for TTP. A diagnosis of TTP is confirmed if the ADAMTS13 activity is <10%; however, there is no confirmatory test for the diagnosis of aHUS. If the ADAMTS13 activity is >10% and the Shiga toxin assay is negative, aHUS remains a strong possibility in the differential diagnosis [47]. Often, patients presenting with TMAs are started on treatment with plasma exchange while awaiting these results because of the difficulty in making a definitive diagnosis. In addition, considering the high cost of treatment with eculizumab and lack of an objective test to diagnose aHUS, therapy is frequently delayed while awaiting more certainty in the diagnosis, not started until patients fail to respond to plasma exchange, or not administered at all. Given these challenges, there has been interest in development of diagnostic tests to help confirm a diagnosis of aHUS so that the appropriate therapy can be started in a timely manner. The ability to distinguish between TTP and aHUS using markers of terminal complement activation has previously been assessed, and while the median and mean levels of C5a and C5b-9 differed among a cohort of patients, there was considerable overlap between the two groups, making this unreliable for differentiating between TTP and aHUS on an individual case basis. The modified (or “indirect”) Ham test, a functional assay of AP activation, has shown promise in being able to reliably distinguish between TTP and aHUS on the basis of degree of AP activation; however, this assay awaits further validation with larger cohorts of patients and further development for use as a clinical assay [48]. The principal of the modified Ham test is similar to the acidified serum (Ham) test that was used to diagnose PNH before the routine use of flow cytometry. In PNH, there is a genetic mutation in *PIGA* that causes a loss of complement regulatory proteins (CD55 and CD59) on red cells. Acidifying normal human serum activates AP and leads to specific lysis of

PNH erythrocytes because they are unable to protect themselves from complement in acidified serum. In most aHUS cases there are genetic mutations or antibodies that lead to activation AP in the patient's serum. Thus, when PNH-like reagent cells (*PIGA* null TF-1 cells) are incubated with aHUS serum, they rapidly accumulate C5b-9 and undergo cell death within 30 minutes in a WST-1 viability assay. The modified Ham test is positive during the acute phase of the disease and in remission, in agreement with aHUS being a two-hit disease. Interestingly, the modified Ham test is also positive in STEC-associated HUS in the acute phase of the disease but not after patients achieve remission, likely due to direct, transient activation of the AP by Shiga toxin binding to and inactivating CFH [49, 50].

8.2 Genetics of aHUS

Approximately 60% of cases of aHUS are thought to have an underlying genetic component that typically involves genes that regulate the AP [51]. In general, there are two mechanisms by which mutations in complement components can lead to inappropriate activation of the AP: (1) loss-of-function mutations in complement inhibitors that normally protect healthy host cells and tissues, and (2) gain-of-function mutations in complement activators, which can overwhelm the normal regulatory system (Figure 4). However, the majority of aHUS cases are sporadic, occurring in the absence of any prior family history. Furthermore, even in familial forms of aHUS, penetrance is incomplete [52–54]. Taken together, these findings support the model of aHUS as a two-hit disease, in which genetic alterations confer susceptibility, but additional genetic or environmental risk factors influence the risk for development of disease in carriers. This is supported by the fact that ~20% of patients with aHUS are found to have mutations in more than one gene [55], and some patients with autoantibodies against CFH also have mutations in *CFH* and other complement genes [56]. Another common theme is that even different mutations in the same complement gene can lead to a very pleiotropic range of disorders.

A genetic link between mutations in *CFH* and familial aHUS was first established in 1998 [57] and *CFH* remains the most frequently mutated complement gene in aHUS. The majority of *CFH* mutations cluster in the C-terminal domains 19–20, which are important for binding to C3b and to host cells [17, 58, 59]. There are unique determinants for CFH self-recognition/binding among different cell and tissue types, and this feature may partially account for the tissue-specificity of the various diseases associated with mutations in *CFH* [60, 61].

The *CFH* gene resides as part of a gene cluster on chromosome 1q32 that arose via large genomic duplications, termed the regulators of complement activation (RCA) cluster, which also includes five CFH-related genes (*CFHR1-CFHR5*) [64]. Some *CFH* mutations have been traced to gene conversion between *CFH* and *CFHR1*, and non-allelic gene rearrangements at the RCA cluster have also occurred, resulting in *CFH-CFHR* gene hybrids that have been found in patients with aHUS [63–66].

CFI mutations are found in both sporadic and familial aHUS [67]. Many of the *CFI* mutations found in aHUS lead to impaired secretion or function [68, 69]. Recently, larger deletions containing all or part of *CFI* have also been described [70, 71].

CD46 mutations were first described in patients with familial aHUS in 2003 [72, 73], and since that time, many additional mutations have been identified, many of which lie within the extracellular complement control protein (CCP) domains [74].

Although C4BP is primarily a negative regulator of the CP, it can potentially regulate the AP through acting as a cofactor for CFI to mediate inactivation of C3b. Given this role, it has been proposed that *C4BP* mutations found in association with aHUS may have a pathogenic role, or at least influence the risk of developing the disease [75].

While mutations in *CFH*, *CFI*, *CD46* and *C4BP* represent loss-of-function mutations in complement regulatory factors, gain-of-function mutations have also been identified in components that normally serve as complement activators, including *C3* and *CFB* [76].

Mutations in *THBD* have also been identified in patients with aHUS; however, as with many complement gene variants that have been described, they are present in the heterozygous state and their impact from a causative perspective remains unclear [23]. Mutations in diacylglycerol kinase- ϵ (*DGKE*) were found to cause a rare form of congenital aHUS with recessive pattern of inheritance and early onset in infancy; however, *DGKE* is an intracellular protein and the mechanism by which it causes aHUS has not been fully elucidated [77, 78].

8.3 Autoantibodies in aHUS

While many patients with aHUS will have identifiable genetic susceptibility factors, approximately 10 percent of patients have detectable autoantibodies against CFH or other complement components [79]. In some cases, these may represent acquired autoimmune forms of aHUS, but in other cases, autoantibodies have been found in conjunction with alterations in complement genes. In particular, autoantibodies often occur in the setting of gene rearrangements among *CFH* and the *CFH-related* genes in the RCA gene cluster, such as those causing homozygous deficiency of *CFHR1* [80]. Similar to clustering of *CFH* mutations in domains 19–20, the majority of CFH autoantibodies found in aHUS are directed against the same C-terminal region of CFH [81], which is critical for its binding to host cells and C3b.

8.4 Treatment of aHUS

Due to the challenges in establishing a diagnosis of aHUS, as discussed above, initial therapy with plasma exchange is often started after sending plasma for ADAMTS13 levels and stool cultures to rule out Shiga toxin. While a proportion of patients may respond at the beginning, and show improvements in LDH and thrombocytopenia, the extent of improvement is often limited. In TTP, plasma exchange helps to correct the underlying ADAMTS13 deficiency, whereas in aHUS, in most cases, plasma exchange does not halt excessive complement activation and complement-mediated end-organ damage continues. Many patients progress to end-stage renal failure or death despite plasma exchange or infusion [46, 82]. Even aHUS patients who respond favorably to plasma exchange initially can become dependent on or resistant to this treatment. Plasma-based therapy remained the only treatment for aHUS until eculizumab was FDA-approved for use in 2011 based on the results from two open-label prospective phase 2 clinical trials [83]. Patients treated with

eculizumab had significant improvements in thrombocytopenia and renal function. Dialysis was able to be discontinued in 4 out of 5 patients who were initially on dialysis in the first trial, and in both trials, at least 80% of patients achieved TMA event-free status by week 26 of treatment. Earlier intervention was also associated with greater improvement in renal function, supporting recommendation to begin treatment with eculizumab while awaiting results of testing for autoantibodies or complement gene mutations. While patients with PNH typically remain on eculizumab indefinitely, the optimal duration for treatment with eculizumab in aHUS has not been defined. Returning to the idea of aHUS being a two-hit disease, once the trigger for an acute episode has resolved, it may be safe to discontinue eculizumab treatment in selective patients [48, 84, 85]; however, they remain at risk for relapse/recurrence. As with PNH patients on eculizumab, aHUS patients treated with eculizumab are at increased risk of *Neisseria* infections and should be vaccinated and educated accordingly.

9. HELLP syndrome (HELLP)

HELLP syndrome, characterized by hemolysis, elevated liver enzymes and low platelets, is a severe form of pre-eclampsia that poses risk of serious morbidity and mortality to both the mother and fetus during pregnancy [86]. Patients with HELLP have clinical manifestations similar to aHUS, including thrombotic microangiopathy, renal dysfunction, hypertension, seizures and altered mental status, and indeed, dysregulation of complement has been implicated in its pathogenesis [87, 88]. Mutations in complement regulatory genes, similar to those found in patients with aHUS, including *CFH*, *CFI* and *CD46*, have also been reported in women with pre-eclampsia [89], HELLP [90] and pregnancy-associated HUS [91] (Table 3). In addition, a recent study evaluating a cohort of women with classical and atypical HELLP syndrome, nearly 80% of these patients had detectable systemic activation of the AP by the modified Ham test; addition of eculizumab to serum of HELLP patients also blocked complement mediated killing *in vitro* [88]. This finding, along with the prevalence of complement gene mutations in women with pre-eclampsia, strongly supports the idea that a proportion of cases of HELLP syndrome likely represent a form of pregnancy-triggered aHUS, in which dysregulation of complement is a major driver of disease pathophysiology.

9.1 Treatment of HELLP

Delivery of the fetus is the treatment of choice for HELLP syndrome (again, consistent with the two-hit model of disease), but delivery before 34 weeks is associated with excess fetal morbidity and mortality. Prior to delivery, treatment is largely supportive with steroids to increase fetal lung maturity, magnesium sulfate for maternal seizure prophylaxis, and anti-hypertensives [92, 93]. Interestingly, there are now case reports showing that eculizumab may be effecting in treating HELLP syndrome, but this must be considered an off-label use of the drug [94].

10. Cold agglutinin disease (CAD)

CAD is an antibody and complement-mediated hemolytic anemia that leads to extravascular and intravascular hemolysis with a propensity for thrombosis. CAD is classified as primary or secondary. Primary CAD is a clonal B cell lymphoproliferative disorder that is now

referred to as “primary cold agglutinin-associated lymphoproliferative disease”; it is distinct from lymphoplasmacytic lymphoma (MYD88 L265P negative), marginal zone lymphoma, and other low grade lymphoproliferative diseases [95]. Immunophenotyping often reveals marrow infiltration with a population of monoclonal B cells which are CD20(+), IgMs(+), IgDs(+), CD27(+), CD5(+/-), CD11c(-), CD23(-) and CD38(-). Somatic mutated monoclonal IGHV4-34 gene rearrangement is found in most cases. Secondary CAD is a syndrome associated with a variety of infectious (*Mycoplasma pneumoniae*) and neoplastic disorders (aggressive lymphomas, Hodgkin’s lymphoma, carcinomas, etc.). Cold agglutinins are autoantibodies (typically IgM) that agglutinate RBCs at 4°C, but may also act at warmer temperatures. Most pathogenic cold-agglutinins are of the IgM class, have a thermal amplitude that exceeds 28–30°C and are present at a titer of 1:256 or greater. In contrast, non-pathogenic cold-agglutinins are low titer and have low thermal amplitude. The peripheral blood smear of patients with CAD shows red cell agglutination (Figure 5), in contrast to patients with warm autoimmune hemolytic anemia, whose blood smear often reveals microspherocytes. The red cell agglutination of CAD is often associated with severe artefactual elevation of the MCV.

10.1 Mechanism of hemolysis in CAD

Cold agglutinins with a thermal amplitude of greater than room temperature bind to red cells in acral parts of the circulation (fingers, toes, ears, etc.) and are commonly directed against the I antigen. Binding of IgM activates the classical complement pathway (CP) (Figure 1). C1 esterase activates C4 and C2, ultimately generating the C3 convertase which cleaves C3 to C3a and C3b. Upon return to central portions of the circulation (~37°C) the IgM-CA dissociates from the cell surface but C3b remains bound to the cell. The C3b coated red cells bind to complement receptors on macrophages of the reticuloendothelial system, predominantly in the liver (extravascular hemolysis). C3b of the surviving red cells is eventually cleaved, leaving a high number of circulating red cells with C3d on the surface. Thus CAD patients have a direct anti-globulin test that is strongly positive for C3d but negative for IgG and IgM. The CP activity does not proceed to activate the terminal pathway in most cases, due to the expression of CD55 and CD59 on the red cell membrane; however, in more severe cases, usually in association with an inflammatory state (infection, surgery etc.) intravascular hemolysis resulting from terminal activation may occur.

10.2 Treatment of CAD

Patients with primary CAD should be instructed to avoid cold temperatures if possible and especially to keep their hands, feet, and ears protected in cold environments; however, symptomatic patients will often need pharmacologic therapy. Rituximab is the most common first-line therapy [96]. It leads to remission (median duration of 1 year) in roughly 50% of patients. Relapsed patients may respond to subsequent rituximab therapy. Fludarabine plus rituximab has a 75% response rate (median duration over 5 years), but significantly more toxicity [97]. Rituximab and bendamustine combination therapy and bortezomib-based therapy also has activity in primary cold agglutinin disease. There are case reports of eculizumab therapy in the occasional cold agglutinin disease patient with intravascular hemolysis and there is *in-vitro* evidence that a monoclonal antibody that targets C1s can help to control extravascular hemolysis [98]. However, complement inhibitors are

not yet FDA approved for use in CAD. In patients with secondary CAD, treatment should target the underlying cause.

Conclusions

Proper activation of the complement system is crucial to its homeostatic and innate immune functions, yet excessive or inappropriate activation can lead to a variety of disease states. Complementopathies are disorders in which impaired regulation of complement is a driving factor in disease pathogenesis and therapy directed at complement inhibition has the potential to improve the disease course. A number of hematologic disorders can be classified as complementopathies, including PNH, aHUS, HELLP and CAD. We expect this list will continue to grow as further research is conducted on the role of the complement system in a variety of human disease states – research that will be crucial to the development of novel diagnostic assays and therapies for complementopathies.

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Practice points

- The complement system plays important roles host defense, immune surveillance and homeostasis
- Inappropriate activation or impaired regulation of complement can lead to damage to healthy host cells and tissues
- Dysregulation of complement is a driver in a number of human diseases, including hematologic disorders – PNH, aHUS, HELLP and CAD – which can be termed complementopathies
- A complementopathy is a disorder in which (1) the pathophysiology is driven primarily by complement, and (2) there is evidence that inhibition of the complement cascade ameliorates the disease manifestations

Research agenda

- Development of rapid diagnostic assays for complementopathies
- Development of novel complement inhibitors for use as alternative or adjunctive therapies in combination with eculizumab
- Clinical trials to determine whether patients with aHUS can safely discontinue eculizumab
- Clinical trials to determine whether complement inhibition with eculizumab or other novel agents can block hemolysis in CAD

Figure 1. Schematic of the complement cascade

The three primary routes for activation of complement are: (1) the lectin pathway (LP), (2) the classical pathway (CP), and (3) the alternative pathway (AP). The LP and CP are activated when specific triggers are recognized by host pattern-recognition receptors (PRRs). The AP is constitutively active. Initial activation through the LP or CP generates a shared C3 convertase (C4b•C2a). In the AP, C3b pairs with factor B (FB) to form the AP proconvertase (C3b•B), which is processed by factor D (FD) to form the AP C3 convertase (C3b•Bb). Both types of C3 convertases cleave C3 to generate C3a and C3b. C3a is an anaphylatoxin, a substance that promotes an inflammatory response. C3b that lands on the surface of a healthy host cell is quickly inactivated; C3b that attaches to the surface of a pathogen or altered host cell triggers a rapid amplification loop to generate more C3b, resulting in opsonization. C3b also complexes with the C3 convertases to form the C5 convertases (C4b•C2a•C3b and C3b•Bb•C3b). In the terminal complement cascade, C5 convertases cleave C5 into C5a (an anaphylatoxin) and C5b. C5b combines with C6–9 to form the membrane attack complex (MAC), also referred to as the terminal complement complex (TCC). Regulatory factors (Table 2) act at various stages of the cascade to control complement activation via their decay accelerating activity and/or cofactor activity. Additional abbreviations: PAMPs = pathogen-associated molecular patterns; MBL = mannose-binding lectin; MASP_s = mannose-binding lectin-associated serine proteases.

Figure 2. Eculizumab and residual extravascular hemolysis in PNH

PNH erythrocytes lack the GPI-anchored complement regulatory proteins CD55 and CD59. CD55 inhibits the AP C3 convertase and CD59 inhibits formation of the membrane attack complex (MAC), also known as the terminal complement complex (TCC). Patients with PNH primarily manifest with chronic intravascular hemolysis mediated by terminal complement. Eculizumab, a humanized monoclonal antibody, inhibits terminal complement by binding to C5. This effectively corrects for the absence of CD59 and blocks intravascular hemolysis. However, up to 25% of patients PNH patients on eculizumab require continued red cell transfusions, likely due to the fact that PNH erythrocytes protected from intravascular hemolysis by eculizumab, are subject to opsonization by C3b (C3b is shown, but other C3 fragments can also bind to the red cells) and extravascular hemolysis due to absence of CD55.

Figure 3. Peripheral blood smear in aHUS

Characteristic peripheral blood smear in aHUS showing schistocytes (fragmented erythrocytes) and thrombocytopenia, typical of microangiopathic hemolytic anemia.

Figure 4. Mechanisms of alternative pathway of complement dysregulation in aHUS

(A) The AP is constitutively active. If C3b lands on the surface of a pathogen or damaged/ altered host cell, the cascade proceeds with rapid amplification, resulting in opsonization by C3b, MAC assembly and generation of other inflammatory mediators. (B) If C3b lands on a healthy host cell, it is normally immediately inactivated by endogenous complement inhibitors. This includes CFH, which binds to the surface of host cells, displaces C3b and prevents formation of the C3 convertase (C3b•Bb). CFI, along with its cofactors, cleaves and inactivates C3b, preventing further downstream activation of the complement cascade and amplification loop. THBD enhances inactivation of C3b by CFH and CFI. CD46 indirectly inhibits assembly of C3 and C5 convertases by cleavage/inactivation of C3b and also serves as a cofactor for CFI. (C) In aHUS, mutations that cause loss-of-function of complement inhibitors (CFH, CFI, CD46 or THBD) or gain-of-function in complement activators (C3 or FB), lead to uncontrolled activation of complement on host surfaces, leading to damage to healthy cells and tissues.

Figure 5. Peripheral blood smear in CAD

Characteristic peripheral blood smear in CAD showing red blood cell agglutination.

Table 1

Complement pathways and activators

Pathway	Activator(s)
Lectin pathway (LP)	MBL, ficolins (via binding to carbohydrates on pathogens)
Classical pathway (CP)	IgG and IgM immune complexes
Alternative pathway (AP)	Slow spontaneous hydrolysis of C3

Table 2

Complement regulators

Soluble	Pathway(s)	Function(s)
CFH	AP, CP, LP	Prevents AP C3 convertase formation, CFI cofactor
CFI	AP, CP, LP	Inactivates C3b and C4b
C1INH	CP, LP	Inhibits C1r/C1s and MASP1/MASP2
MAP1	LP	Blocks MASPs from binding to MBL/ficolins
Membrane		
CD55	AP, CP	Inhibits formation/accelerates decay of C3 convertases
CD59	TP	Binds to C8 and C9 to block MAC assembly
CD46	AP, CP, LP	CFI cofactor, inactivates C3b and C4b
THBD	AP, CP, LP	Enhances C3b inactivation by CFH/CFI
CR1	AP, CP, LP	CFI cofactor, accelerates decay of C3/C5 convertases

Table 3

Genetic alterations in complementopathies

Disorder	Gene(s)
PNH	<i>PIGA</i>
CD59 deficiency	<i>CD59</i>
aHUS	<i>C3, CFB, CFH</i> (and <i>CFHR</i> gene rearrangements), <i>CFI, CD46, THBD, DGKE</i>
HELLP	<i>C3, CFH, CFI, CD46</i>
CAD	None known