Blood Spotlight

HUS and the case for complement

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Hemolytic-uremic syndrome (HUS) is a thrombotic microangiopathy that is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. Excess complement activation underlies atypical HUS and is evident in Shiga toxin–induced HUS (STEC-HUS). This *Spotlight* focuses on new knowledge of the role of *Escherichia coli*–derived toxins and polyphosphate in modulating complement and coagulation, and how they affect disease progression and response to treatment. Such new insights may impact on current and future choices of therapies for STEC-HUS. (*Blood*. 2015;126(18):2085-2090)

Introduction

Hemolytic-uremic syndrome (HUS) is a thrombotic microangiopathy (TMA) with microvascular and arteriolar wall thickening, swollen endothelial cells, and fibrin- and platelet-rich thrombi which compromise blood supply to end organs, particularly the kidney.[1,2] Fragmentation of erythrocytes occurs from shear stress across partially obstructed vessels. Thrombocytopenia is caused by platelet consumption in clots and the reticuloendothelial system.

HUS is classified as either typical or atypical. Typical HUS is most often acquired from food contaminated with enterohemorrhagic *Escherichia coli* that produce Shiga-like toxins (Stx)[3] and is thus referred to as Shiga toxin–induced HUS (STEC-HUS). Other microorganisms have also been implicated (<5%-10%).[4] After exposure to STEC-HUS, an incubation period of ~3 days ensues, followed by 2 to 5 days of watery diarrhea, nausea, and fever (~30%). Gastroenteritis, with bloody diarrhea proceeds in ~50% to 70%, and is usually self-limited, but rarely is complicated with massive hemorrhage and/or bowel perforation.[5] A minority of STEC-exposed patients subsequently develop HUS (5%-25%), and this often has serious long-term sequelae.[6] Approximately 25% of STEC-HUS patients develop chronic renal insufficiency. Neurologic involvement (eg,, strokes, seizures) is evident in 10% to 20% of cases and these account for much of the 1% to 5% mortality. Other organs may also be involved.

The remaining noninfective cases of HUS are referred to as atypical (aHUS). aHUS is chronic and recurring with >50% developing end-stage renal failure, and has an early mortality of 10% to 25%. There is overwhelming evidence that aHUS is caused by excess complement activation.[7] Thus, most patients respond well to the complement inhibitor, eculizumab. In contrast, specific therapies for STEC-HUS are lacking.[4]

Recent advances in our understanding of complement and coagulation and the role of *E coli*–derived toxins (Stx and serine protease autotransporters of *Enterobacteriaceae* [SPATEs]) and polyphosphate in modulating these pathways, as discussed in this *Spotlight*, may help explain why STEC-HUS is less responsive to eculizumab, and hopefully aid in the rational design of STEC-HUS therapies.

The complement system

Complement comprises over 30 soluble and membrane-bound proteins, coordinated to eliminate pathogens and damaged cells.[8] Complement activation proceeds via 3 pathways: classical (CP), lectin (LP), and alternative (AP). The CP is triggered by antigen-antibody complexes recognized by C1q, and the LP by sugars recognized primarily by mannose-binding lectin. This causes activation of proteases that cleave C4 and C2 to form the C4b2a LP/CP C3 convertase which proteolyses C3 into C3b and C3a. The AP constitutively generates fluid-phase C3b-like $C3H_2O$. This binds to factor B (FB) that is cleaved by factor D (FD), yielding $C3(H_2O)Bb$. $C3(H_2O)Bb$ cleaves C3, yielding C3b that is necessary to form surface-bound C3 convertase, C3bBb. With more C3b, substrate specificity of the convertase shifts to C5, generating C5a and C5b. C5b triggers assembly of the C5b-9 membrane attack complex (MAC) that lyses target pathogens/cells.

Complement is tightly regulated. The major fluid-phase AP-negative regulator, factor H (FH), competes with FB binding to C3b, acts as a cofactor for factor I (FI) cleavage/inactivation of C3b, and accelerates convertase decay. FH also interacts with thrombomodulin and von Willebrand factor (VWF), augmenting FI inactivation of C3b.[9-11] Membrane glycoproteins, CD55 and CD46, also promote convertase decay.[12] Anaphylatoxins C3a and C5a are modulated by their receptors[13] and/or degraded by plasmin,[14] matrix metalloproteinases,[15] and activated thrombin-activatable fibrinolysis inhibitor (TAFIa).[16] MAC formation is suppressed by CD59, mortalin/GRP75,[17] clusterin, and vitronectin. Recently, we showed that polyphosphate also interferes with MAC assembly.[18]

aHUS: molecular defects causing excess complement activation

Mutations of genes that encode complement components, or antibodies that alter their function, account for ~60% to 70% of patients with inherited or sporadic aHUS. Affected genes and associated

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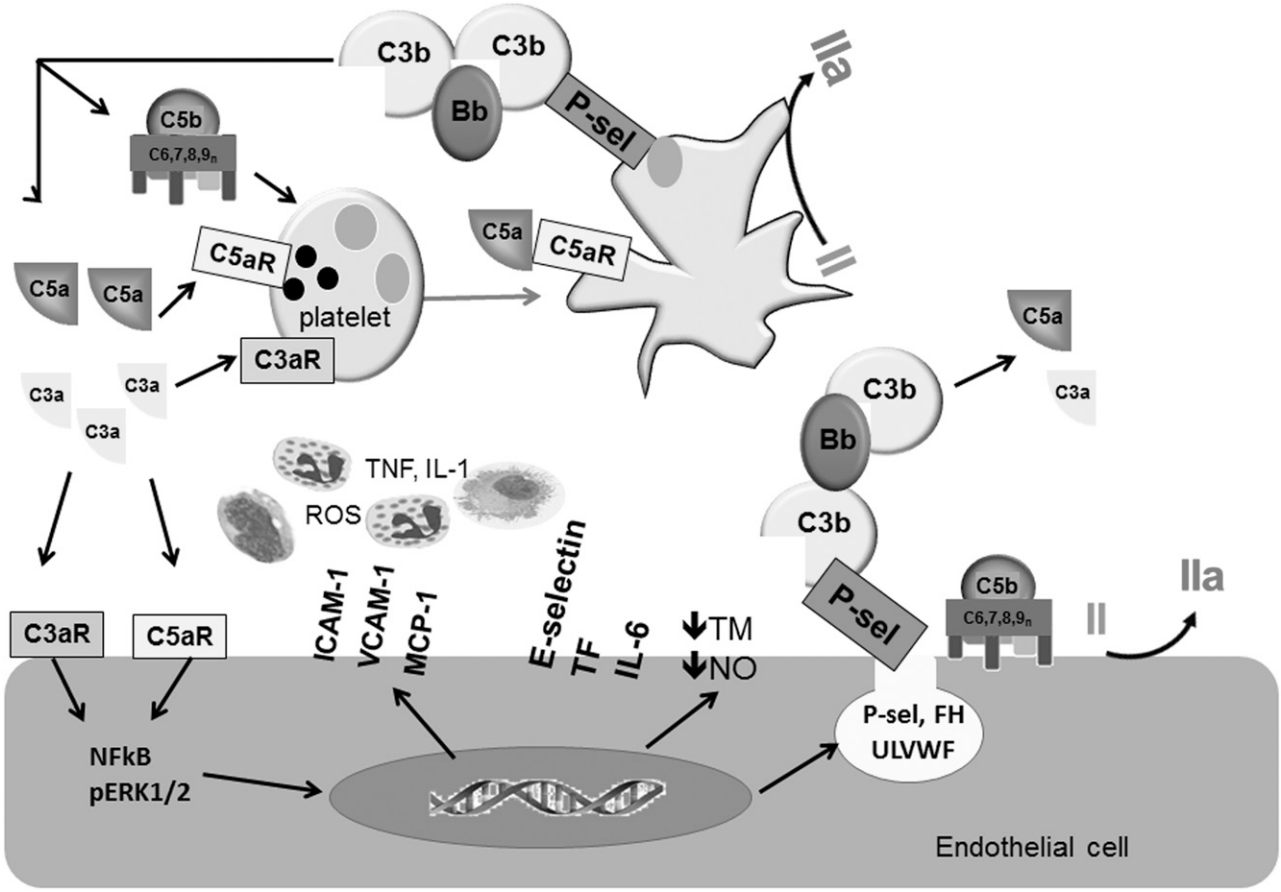

Figure 1. Complement activation and TMA. The scheme highlights some of the mechanisms by which unregulated complement activation provokes the development of microvascular thrombi via unrelenting activation/damage to the endothelium and platelet. With excess complement activation, the abundant anaphylatoxins C3a and C5a bind to their widely expressed cognate receptors. Endothelial cells and platelets can thus be activated, whereupon intracellular proinflammatory and procoagulant signaling cascades are recruited (eg, nuclear factor κB [NFκB], phospho-ERK 1/2 [pERK1/2]). In endothelial cells, these upregulate expression of adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule [VCAM-1], E-selectin), the release of proinflammatory cytokines (eg, monocyte chemotactic protein 1 [MCP-1], interleukin-6 [IL-6]), exposure of TF, suppressed expression of the anticoagulant/anticomplement thrombomodulin (TM), and reduced release of nitric oxide (NO). P-selectin, FH, and ULVWF multimers are secreted from endothelial cell Weibel-Palade bodies, and they are released from α-granules and/or the cytoplasm (for FH) of platelets. P-selectin and ULVWF facilitate platelet adhesion/aggregation, but also are receptors for C3b binding, allowing assembly of the AP C3 and C5 convertases (C3bBb and C3bBbC3b, respectively). These further amplify complement activation, with release of more C3a and C5a. Downstream, complement activation yields terminal pathway complexes (C5b-7, sC5b-9) which induce TF exposure, endothelial membrane "flipping" to support prothrombinase assembly, and the transformation of prothrombin (II) to the procoagulant, platelet activating and proinflammatory protease, thrombin (IIa). Thrombin and other procoagulant enzymes (eg, factor Xa) also feed back to cleave C5, fueling further activation of complement. C3a, C5a, and cell-released chemokines also recruit inflammatory cells, which in turn exacerbate endothelial damage via the release of reactive oxygen species and cytokines (eg, tumor necrosis factor α [TNFα], high-mobility group box 1 [HMGB1]). Activated platelets also release their granule contents which are primarily procoagulant and proinflammatory. Platelet and endothelial microparticles, released in response to exposure to C3a and C5a, carry complement factors and express TF to further promote coagulation and complement. ULVWF multimers support platelet adhesion/aggregation and the formation of thrombi, as well as further activation of complement. In aHUS, eculizumab interferes with cleavage of C5 to C5b and C5a, and effectively reverses the unrelenting, self-propelling activation of the cascades that otherwise result in the TMA.

frequencies include FH (20%-30%), FI (5%-10%), CD46 (10%-15%), thrombomodulin (3%-4%), C3 (2%-10%), and FB (1%-4%).[19-21] Hybrid forms of FH and FH-related proteins (3%-5%) have reduced activity,[22] and autoantibodies against FH (5%-10%) reduce its binding to endothelium or C3b.[23] A mutation in diacylglycerol kinase-ε is a rare cause of aHUS, but its link to complement is not well established.[24,25]

Spiraling activation of complement and coagulation

Complement overactivation, as occurs in aHUS, undermines vasculoprotective properties via several mechanisms[26] (Figure 1). C3a/C5a stimulate secretion of cytokines, promote leukocyte adhesion[27] and tissue factor (TF) expression, suppress thrombomodulin, and induce P-selectin and VWF release. C3a/C5a also activate platelets,[28] causing granule secretion, exposure of P-selectin, and release of procoagulant microparticles. P-selectin recruits leukocytes and platelets and is a C3b receptor[29] for AP convertase assembly, amplifying complement activation. C5b-7 activates TF on monocytes,[30] and sC5b-9 activates platelets and endothelial cells, induces VWF and cytokine release, and promotes prothrombinase assembly and release of TF-expressing microparticles.[31] Thrombin loops back to liberate C5a and generate a more damaging MAC.[32,33]

Defects in processing VWF are linked to another TMA, thrombotic thrombocytopenia purpura.[34] But VWF also contributes to complement activation, and likely participates in HUS-associated thrombosis. When secreted, VWF is anchored to endothelium as ultra-large (ULVWF) multimeric strings.[35] These are normally cleaved by a disintegrin and metalloproteinase with a thrombospondin

type 1 motif, member 13 (ADAMTS13) into smaller, less procoagulant forms. FH binds to VWF and also reduces multimers,[36,37] limiting complement and platelet activation/aggregation. Loss-of-function FH mutations that cause aHUS would facilitate formation of platelet-rich thrombi, and allow unregulated complement activation with generation of procoagulant products.

Overall, it is not surprising that a drug that restrains complement is effective for aHUS. Eculizumab interferes with C5 cleavage to C5a and C5b-9[38]; its efficacy in aHUS is >80%.[39]

STEC-HUS and complement activation

Does complement activation occur in STEC-HUS? If so, does it explain the TMA? Most patients infected with STEC exhibit heightened complement activation,[40] with increased generation of C3a, Bb, and sC5b-9, and C3 and C9 deposits on platelet-leukocyte aggregates and microvesicles.[41-43] Complement activation therefore likely contributes to endothelial damage and thrombosis in STEC-HUS. Clinical validation is, however, lacking, as there have not been controlled studies of eculizumab for STEC-HUS. In 2 small reports of affected children with neurologic impairment, benefit for some appeared to be derived from eculizumab.[44,45] A similar result was reported for a small number of French adults.[46] However, review of its use in the large European outbreak did not reveal any benefit.[47] Controlled, prospective studies are needed. Nonetheless, the stunning success of eculizumab in aHUS has not been seen with STEC-HUS, suggesting that modulating complement, by itself, is inadequate.

Shiga toxin: direct effects on multiple pathways

The most frequent cause of STEC-HUS is *E coli* strain 0157:H7, but many others have been identified.[48,49] Stx is the key virulence factor.[50-52] Upon ingestion, STEC colonize the gut, adhere to epithelial cells, destroy the brush border villi, and cause diarrhea.[52] Stx is secreted through the epithelium where it can contact blood, but only trace amounts of circulating free toxin are found. Rather, Stx preferentially binds to platelets, neutrophils, monocytes, and possibly erythrocytes.[53,54] Transfer of toxin to cells in target tissues is achieved via binding to globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4), expressed by endothelial cells of the intestine, brain and kidney, podocytes, mesangial cells, and renal tubular epithelial cells.[55] An intriguing alternative virulence mechanism has been described in which Stx is internalized by circulating blood cells, and then released in microvesicles, which in turn are transferred into glomerular and peritubular capillary endothelial cells via Gb3-independent pathways for uptake by renal cells.[56] Whatever the mechanism, intracellular Stx interferes with ribosomal apparatus and blocks protein synthesis.[57] Stx also induces release of P-selectin and VWF from platelets and endothelial cells, which further activates platelets and neutrophils, induces neutrophil extracellular trap formation,[58] and amplifies the AP[29,59] (Table 1). It interferes with FH, rendering the endothelium more vulnerable to complement.[60] Stx also stimulates release of C3- and C9-bearing microparticles from platelets and monocytes[41,42] and in animal models, causes AP-dependent microvascular thrombosis, apoptosis of renal tubular cells, and podocyte loss and dysfunction.[61,62]

Thus, at some stage(s) of the syndrome, Stx directly or indirectly activates complement and, in turn, coagulation. However, Stx has many complement-independent effects (Table 1). Upon internalization, Stx triggers proinflammatory signals,[55] promotes chemokine release, upregulates leukocyte adhesion molecules and TF, suppresses endothelial TF pathway inhibitor and thrombomodulin, induces platelet-neutrophil interactions,[41] promotes release of TF-bearing microparticles,[63] and induces neutrophil production of reactive oxygen species.[64] Stx also interferes with ULVWF cleavage by ADAMTS13[65] and FH,[66] enhancing platelet adhesion/aggregation. Overall, Stx uses multiple means to promote thrombosis, not all of which are sensitive to anticomplement interventions.

SPATES: aiding Stx in crime

In addition to Stx, >25 SPATEs are produced by pathogenic strains of *E coli*[67] and their activities may further explain the variable response of STEC-HUS to eculizumab. SPATE proteases cleave/inactivate chemokines and adhesion molecules, disrupt leukocyte chemotaxis, transmigration, and activation, and dampen inflammatory and prothrombotic responses.[68] Several SPATEs have recently been shown to modulate complement (Table 1). For example, the serine protease Pic is secreted by strains of HUS-inducing STEC, including 0104:H4. Pic suppresses complement activation by proteolysing C2, C3/C3b, and C4/C4b.[68,69] Pic also synergizes with FI/FH to inactivate C3b, and des-arginates C3a, rendering it less active. EspP is a serine protease strongly associated with *E coli* 0157:H7.[70] EspP dampens complement activation by proteolysing C3/C3b and C5.[71] Interestingly, EspP also inactivates coagulation factor V and α2-antiplasmin, contributing to mucosal bleeding and facilitating bacterial invasion. StcE is a metalloprotease that is also secreted by *E coli* 0157:H7.[72,73] Correlated with high virulence,[74] StcE cleaves C1-esterase inhibitor (C1-INH), but enhances its capacity to neutralize C1s and MASPs. StcE also binds to host cell surfaces (eg, platelets, endothelial cells) and tethers C1-INH so that it can protect the pathogen and host cell by subverting complement activation.

By circumventing immune destruction, several SPATE proteases confer a survival advantage to the STEC, favoring persistence, invasion, and migration. Therapeutic interventions that suppress complement might therefore not be of benefit.

Polyphosphate

Beyond SPATEs, *E coli* and other bacteria rely on multiple mechanisms to evade complement.[75,76] Polyphosphate (polyP) is an anionic, linear polymer of phosphate that is found in all cells.[77-79] Identified first in cytoplasmic granules, it is localized in several cell compartments, and, in *E coli*, is prominent in the membrane.[80] In prokaryotes, polyP exhibits prosurvival properties as an energy source, a metal ion chelator, and a molecular chaperone, and is essential for microorganism pathogenicity.[81,82] In mammals, polyP promotes coagulation at several steps in the cascade.[83,84] We recently determined that polyP also dampens complement activation (Table 1), a finding in line with reports that a mutant form of *Neisseria meningitidis* with excess polyP, is protected against complement-mediated killing.[85]

PolyP destabilizes C5b,6, and reduces binding of the resultant C5b-7 and C5b-8 to the target membrane.[18] Thus, polyP in the

Table 1. Summary of diverse biological effects of Stx, SPATEs, and polyphosphate that modulate activation of complement and coagulation during STEC-HUS

Summary of diverse biological effects of Stx, SPATEs, and polyphosphate	
Stx	
Direct effects that promote complement activation	• Induces release and cell surface expression of P-selectin (receptor for C3b) • Induces release of VWF (site for AP convertase assembly) • Interferes with functions of FH • Induces release of C3- and C9-bearing microparticles from platelets and monocytes
Direct effects that induce endothelial damage and activation of coagulation	• Activates endothelial cells, platelets, monocytes, and neutrophils • Induces release of reactive oxygen species from neutrophils • Induces expression of TF • Suppresses expression of TM and TF pathway inhibitor • Induces endothelial expression of leukocyte adhesion molecules (e.g., ICAM-1, VCAM-1) • Induces platelet-neutrophil interactions • Interferes with cleavage of ULVWF by ADAMTS13 and FH • Promotes release of TF-bearing microparticles • Induces proinflammatory cytokine (e.g., TNF, IL-8) release by endothelial cells and monocytes
SPATEs	
Pic	• Proteolyses and inactivates C2, C3/C3b, C4/C4b • Des-arginates C3a
EspP	• Proteolyses and inactivates C3/C3b, C5 • Inactivates coagulation factor V and α2-antiplasmin
StcE	• Cleaves C1-INH and increases its neutralization of C1s and MASP • Binds to host/pathogen surfaces and tethers C1-INH
PolyP	• Promotes coagulation activation at several steps in cascade • Destabilizes C5b,6 and interferes with MAC assembly on host and pathogen • Binds to C1-INH and increases its neutralization of C1s and MASP • Binds to FH (function unknown)

ICAM, intercellular adhesion molecule; IL, interleukin; TM, thrombomodulin; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

membrane of STEC likely provides a barrier against MAC assembly.[80] PolyP binds to C1-INH[18] and enhances its activity, suppressing the CP and LP.[86] PolyP also binds to FH,[18] although the functional consequences are unknown. Interestingly, like polyP, C1-INH and FH are released from activated platelets, where they may coat and protect host cells[87] from complement activation, convertase assembly, and MAC binding/integration. Similar to C1-INH, FH may also be recruited to pathogen surfaces for immune evasion.[76] We speculate that released polyP binds to FH, C1-INH, and/or other cationic proteins on the surface of host cells and pathogenic *E coli*, providing an additional barrier against complement-mediated damage. Once again, this may help explain why a complement inhibitor alone may not be sufficient to resolve the manifestations of STEC-HUS.

Conclusions

For aHUS, where disturbances in complement regulation define a "starting point" for amplification of cascades that lead to TMA, intervening with eculizumab is effective and currently the favored first-line treatment. The situation is more complex for STEC-HUS. The virulence factor Stx(s), which is requisite for development of STEC-HUS, has complement-activating properties, but also triggers endothelial injury, podocyte and renal tubular damage, platelet activation and thrombosis, via pathways that likely vary during the course of the syndrome. It follows that preventing C5 cleavage with eculizumab may not be uniformly effective in abrogating the associated TMA. Furthermore, pathogenic *E coli* that cause HUS also use multiple means to prolong survival and enhance virulence, partly by encapsulating its toxin in microvesicles, synthesizing polyP, and secreting SPATEs along with Stx(s). These either evade or dampen complement-dependent immune-mediated killing, and again may help explain the suboptimal response to eculizumab. They also point to potential shortcomings of using HUS models that rely solely on Stx induction,[50,51] and underline the importance of seeking alternative therapeutic approaches. In that respect, efforts are under way to delineate the pathways by which the toxins traffick to target organs, and to characterize factors synthesized and/or secreted by enterohemorrhagic *E coli*. These are uncovering potential strain-specific targets to reduce pathogen persistence, replication and adhesion, biofilm formation, and Stx invasion.[88] Closer to the clinic, vaccines and neutralizing anti-Stx antibodies are in development,[89-91] and these will hopefully reduce the incidence and severity of STEC-HUS.

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