



REVIEW ARTICLE

Dengue virus and the complement alternative pathway

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Dengue disease is an inflammatory-driven pathology, and complement overactivation is linked to disease severity and vascular leakage. Additionally, dysregulation of complement alternative pathway (AP) components has been described, such as upregulation of complement factor D and downregulation of complement factor H (FH), which activate and inhibit the AP, respectively. Thus, the pathology of severe dengue could in part result from AP dysfunction, even though complement and AP activation usually provide protection against viral infections. In dengue virus-infected macrophages and endothelial cells (ECs), the site of replication and target for vascular pathology, respectively, the AP is activated. The AP activation, reduced FH and vascular leakage seen in dengue disease in part parallels other complement AP pathologies associated with FH deficiency, such as atypical haemolytic uraemic syndrome (aHUS). aHUS can be therapeutically targeted with inhibitors of complement terminal activity, raising the idea that strategies such as inhibition of complement or delivery of FH or other complement regulatory components to EC may be beneficial to combat the vascular leakage seen in severe dengue.

Keywords: alternative pathway; complement; complement factor B; complement factor H; dengue virus

Complement is a key part of the immune response, and complement activation can be mediated by (a) the classical pathway (CP), (b) lectin pathway (LP) and (c) the alternative pathway (AP) (Fig. 1) [1]. Complement activation generates products and protein complexes specialised in clearing pathogens. These may act *via* mechanisms such as lysis mediated by the membrane attack complex (MAC), the structural details of which

have been recently described [2], or *via* complement cleavage products, such as C3a, acting as modulators of inflammation that can increase the permeability of the endothelial cell (EC) barrier and recruit cells of the immune system [3,4]. Complement activation is potentially damaging, and it must be regulated to prevent collateral host-cell damage. In contrast to the CP and LP, which are triggered by specific pathogen

Abbreviations

ADE, antibody-dependent enhancement; aHUS, atypical haemolytic uraemic syndrome; AMD, age-related macular degeneration; AP, alternative pathway; C3G, C3 glomerulopathy; C3GN, C3 glomerulonephritis; CP, classical pathway; CRP, C-reactive protein; DDD, dense deposit disease; DENV, dengue virus; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; EC, endothelial cell; FB, factor B; FD, factor D; FH, factor H; FI, factor I; GBM, glomerular basement membrane; HCV, hepatitis C virus; hpi, hours postinfection; HSV, herpes simplex virus; HUVEC, human umbilical vein endothelial cells; IFN, interferon; LP, lectin pathway; MAC, membrane attack complex; MBL, mannose-binding lectin; NS1, nonstructural protein 1; PBMCs, peripheral blood mononuclear cells; RCA, regulators of complement activation; RRV, Ross River virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

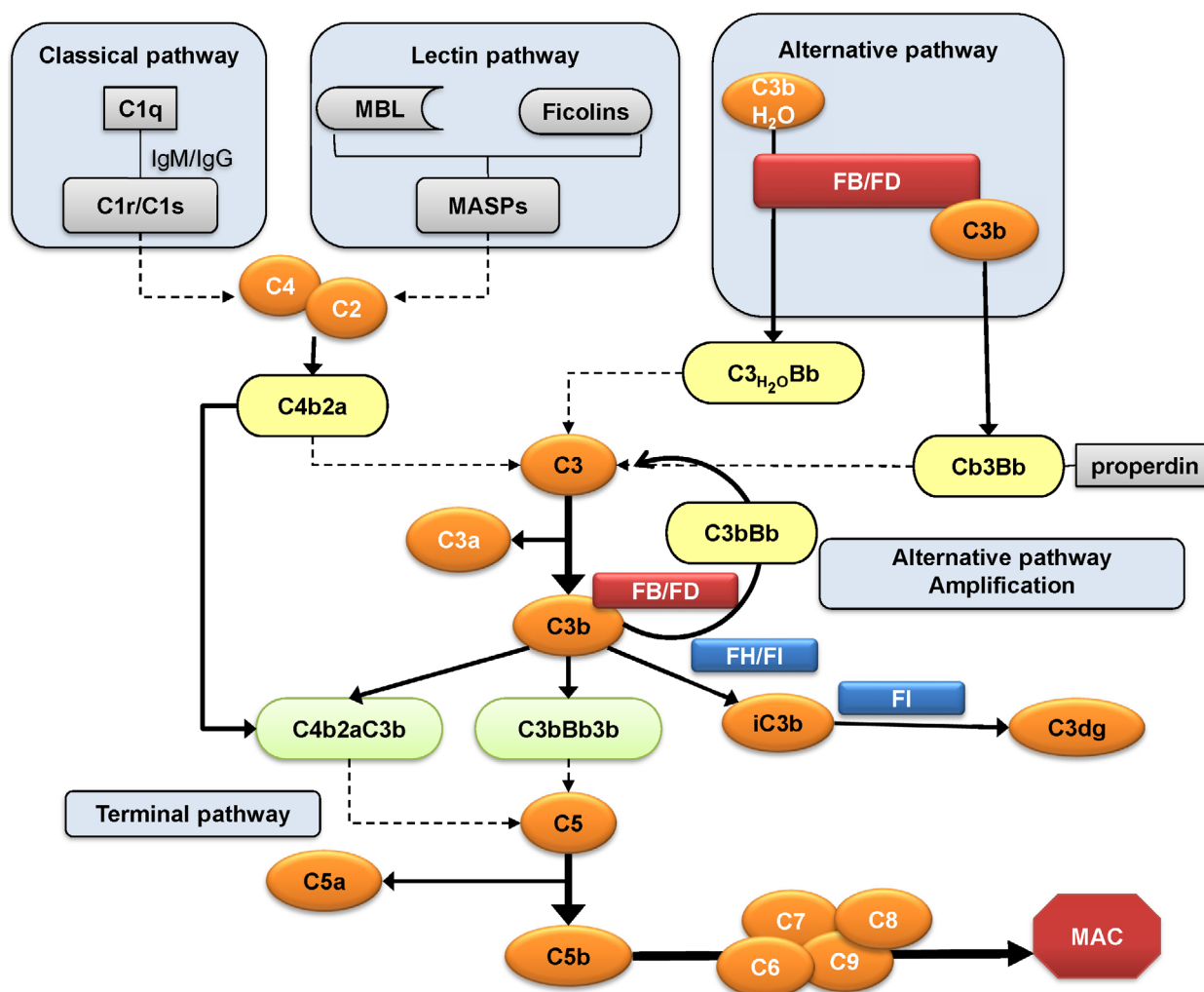


Fig. 1. Schematic illustration of the complement system. Complement is activated by three major pathways. The CP is activated when C1q interacts with IgM/IgG bound to antigen or targets such as CRP. C1q-associated C1s cleaves C4 and C2 to form the CP C3 convertase (C4b2a). The LP is initiated by carbohydrate pattern recognition receptors such as MBL and ficolins which form a complex with MBL-associated serine proteases (MASPs). MASP-2 activates the complement system by cleaving both C4 and C2 to form the C4b2a C3 convertase. The AP is activated by spontaneous hydrolysis of C3 (C3-H₂O) and also functions as an amplification loop for the cleavage of C3 triggered by CP or LP. Fluid-phase C3-H₂O or C3b in the fluid phase or surface-attached is bound by complement FB. Complement FD cleaves C3-H₂O or C3b-bound FB, resulting in the generation of Bb and formation of the AP C3 convertase (C3bBb). The resultant CP, LP and AP convertases function to cleave C3 to C3a, a potent anaphylatoxin, and C3b that can attach to target surfaces. In addition, C3b can bind to either the CP/LP or AP C3 convertases resulting in a change in the substrate specificity of the convertases from C3 to C5. These C5 convertases (C4b2aC3b and C3bBb3b) cleave C5 to C5a and C5b. Release of C5b promotes assembly of the C5b-C9 membrane attack complex (MAC) which can directly lyse pathogens or pathogen-infected cells. C3b is further cleaved by FI, in conjunction with a cofactor such as FH, to generate degradation products such as iC3b and C3dg. Complement immunomodulatory split products, C3a and C5a, are generated during complement activation.

recognition, the AP is generally considered to be constitutively active with ongoing ‘tick-over’, low-level spontaneous hydrolysis of C3 and an amplification loop to promote C3 cleavage [5,6] (Fig. 1). The AP amplification loop drives ~80–90% of total C5 cleavage

downstream of both CP activation and LP activation in humans [7,8]. Thus, the AP is a significant contributor to total complement activity and regulation to prevent overactivity of the AP in both the absence and presence of a pathogen is therefore critical.

Complement AP-mediated diseases

Dysregulation of AP is associated with multiple noninfectious diseases, such as atypical haemolytic uraemic syndrome (aHUS) (Box 1). aHUS can be associated with gene mutations in complement factor H (FH), a negative regulator of AP activity, with these mutations leading to reduced targeting and activity of FH [9,10]. Approximately 50% of aHUS patients have a recognised gene variant affecting regulation of the AP [11,12]. Over 1000 different variants have been implicated, with loss-of-function mutations in exons encoding the host surface-binding C terminus of FH featuring most prominently. Loss-of-function mutations in genes encoding other AP-negative regulators, CD46 (membrane cofactor protein) and factor I (FI), and gain-of-function mutations in genes encoding key AP C3 convertase components C3 and factor B (FB) have been also reported [9]. Around 10% of presentations are acquired forms of aHUS with an inhibitory anti-FH antibody detected in serum [13–15]. Similarly, other diseases of AP dysregulation such as C3 glomerulopathies are associated with loss-of-function mutations in FH [16–18]. Furthermore, genome-wide association studies have linked FH polymorphisms with age-related macular degeneration (AMD) [19–21] in addition to genetic mutations in other complement

components [22]. The importance of AP regulation is further highlighted by the regulators of complement activation (RCA) gene cluster [23], comprised of genes encoding a number of proteins that modulate complement activity in the fluid phase and at the cell surface by mechanisms that dissociate, prevent the formation of or proteolyse active C3 convertases [24]. The actions of these negative regulators of complement are important in the determination of ‘self’ and ‘nonself’, where surface-bound RCA components, such as FH, identify a site as ‘self’ and protect it from complement actions. Further description of the RCA and its importance in controlling complement activity is highlighted in the accompanying review in this Special Issue (Sahu *et al.*, 2020).

Dengue: a complement AP-mediated disease?

In addition to noninfectious pathologies arising from AP dysregulation, failure to control the AP properly in response to a pathogen infection can also result in damage to the host, and immune-mediated disease. Dengue virus (DENV) infection is one such infection where the severe forms of disease, in particular, are driven by an immunopathogenesis with major roles for macrophages and EC and a proposed role for

Box 1. Uncontrolled AP activation in noninfectious human disease

Increased AP activity due to failure of its regulatory mechanisms can lead to a heterogeneous group of human inflammatory diseases characterised by microvascular abnormalities: aHUS, C3 glomerulonephritis (C3GN), dense deposit disease (DDD) and AMD.

aHUS, also known as complement-mediated HUS, is a rare, life-threatening disease defined clinically by nonimmune microangiopathic haemolysis, thrombocytopaenia and acute kidney injury. Unregulated AP activity leads to inappropriate, primarily local complement consumption, sometimes diagnostically reflected by reduced systemic C3 [25], damage to ECs and complement deposition throughout the microvasculature [26]. Kidney damage is often severe and irreversible.

C3 glomerulopathy (C3G) is an umbrella term for two histopathologically distinct kidney diseases, C3GN and DDD, both caused by AP dysregulation [27]. In common, C3GN and DDD feature deposition of complexes of complement products into the glomerular basement membrane (GBM), disrupting kidney function [16,17]. In contrast to the acute overactivation of the AP and systemic endothelial damage seen in aHUS, C3G results from a chronic systemic overactivity of the AP, and characteristically a reduction in circulating C3 [28], with slow aggregation of complement products only in susceptible areas such as the GBM [28].

In AMD patients, poorly controlled AP activation beneath the retina disrupts the vasculature and architecture of the retina and creates the typical central vision loss seen in AMD patients.

Thus, AP dysregulation, in acute or chronic settings, in the circulation or locally in tissues, can damage the vasculature and tissue function leading to severe human diseases.

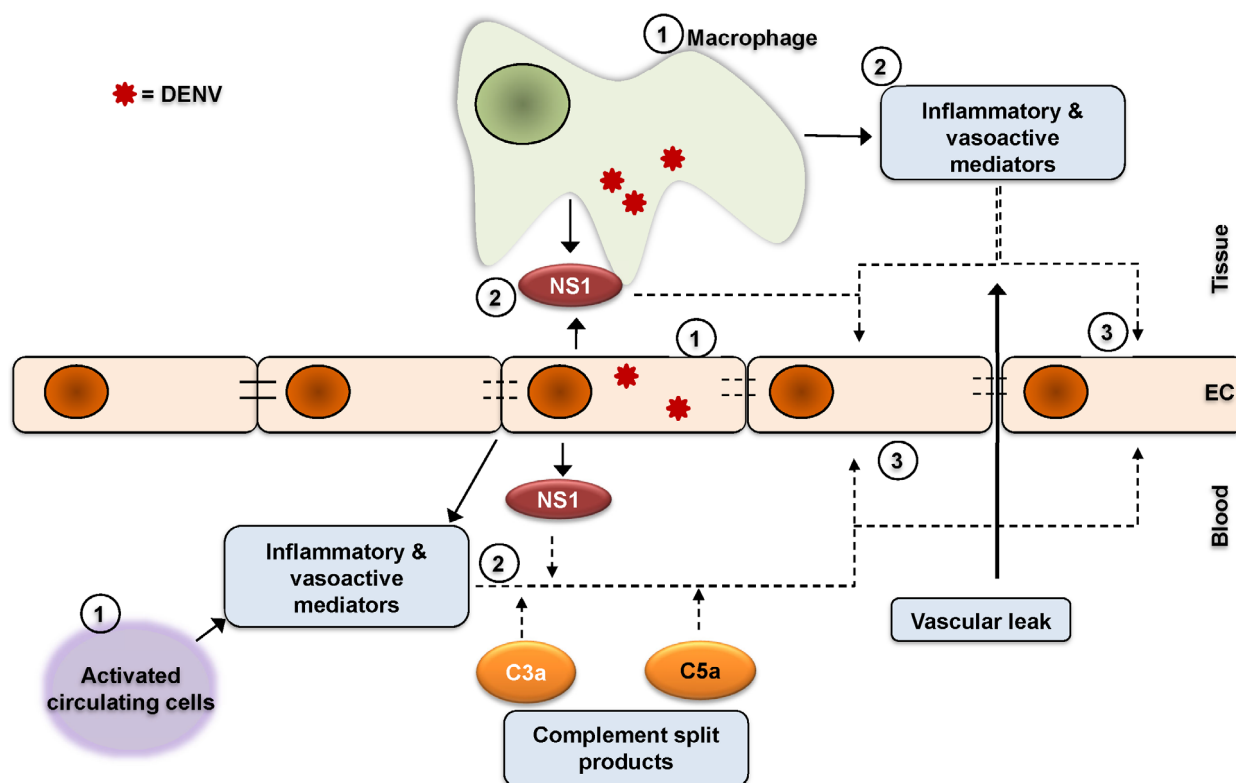


Fig. 2. Proposed pathogenesis of dengue vascular leak. DENV infects macrophages and ECs and causes activation of other circulating immune cells ① that leads to production of pro-inflammatory and vasoactive factors in the tissues and circulation, including complement split products and the viral NS1 protein ②. These multiple pathogenic tissues and circulating signals converge on the endothelium, leading to increased EC permeability and vascular leakage ③.

Box 2. Fundamental facts about dengue

Vector: *Aedes aegypti* mosquitoes

Endemic countries: over 100, with a focus on the Americas, South-East Asia and Western Pacific

Number of WHO estimated cases: 50–100 million pa

Number of cases determined by mathematical modelling: 390 million in 2010 [41]

Treatments: none

Therapies: supportive

Commercial vaccine: DengVaxia (Sanofi Pasteur)

complement products in the pathogenesis of dengue vascular leak syndrome (Fig. 2) [29–31].

Dengue virus is the most prevalent mosquito-borne viral infection for which there are no specific treatments (Box 2) [29,30]. There is a controversial licensed DENV

vaccine only appropriate for use in people with prior dengue immunity [29,30,32] with newer vaccine prospects on the horizon [33]. DENV infection ranges from an asymptomatic or mild, febrile illness (dengue), to a concerning febrile illness with markers such as mild vascular leak and thrombocytopaenia (dengue with warning signs) and potentially fatal 'severe dengue' with vascular leak syndrome and vascular haemorrhage [dengue haemorrhagic fever (DHF)] which may progress to hypovolaemic shock [dengue shock syndrome (DSS)] [32]. The mediators of DENV-induced vascular leak syndrome include a multifactorial array of inflammatory and vasoactive cytokines and chemokines, with a likely key role for $\text{TNF-}\alpha$ [29,34,35]. Other mediators include actions of the viral protein nonstructural protein 1 (NS1), including effects mediated *via* toll-like receptor 4 [36,37] or the glycocalyx [38–40] (Fig. 2). DENV-induced vascular leakage is also associated with elevated levels of complement cleavage products (Fig. 2) [31], suggesting excessive complement activation. Although the activation of complement is clearly vital for the host to control infection, the role of dysregulated complement AP in contributing to disease is the focus of this review.

Complement and viral infections: focus on DENV and the AP

Protective and pathogenic effects of complement during viral infections

As expected, antibody opsonisation and activation of the CP are key components of protection against many important human viral infections, such as the respiratory RNA viruses influenza A virus [42], respiratory syncytial virus [43] and the DNA herpesvirus family, herpes simplex virus (HSV) [44], varicella zoster virus [45] and Epstein–Barr virus [46]. More recently, the importance of the CP in control of a novel human neurotropic virus, Chandipura virus, mediated by C1q neutralisation and aggregation, has been demonstrated [47]. Additionally, the LP is protective in diverse viral infections such as HIV [48] and Ebola virus infections [49]. In many instances, however, complement activity has been linked to viral pathogenesis [50,51], for example hepatitis C virus (HCV), where C1q receptor (C1qR) on activated T cells binds to the HCV core protein to inhibit T-cell proliferation and drive hepatic inflammation [52]. A number of studies with Ross River virus (RRV) have found a link between complement activation and joint pathology, where C3a is found in the synovial fluid of joints associated with inflammatory disease [53], and RRV infection in C3 and C receptor 3 (lacking CD11b)-deficient mice show decreased joint pathology [53,54]. Additionally, mannose-binding lectin (MBL) levels are increased in the serum and synovial fluid of RRV patients and disease is reduced during RRV infection in MBL-deficient mice, suggesting an association of MBL with increased RRV pathogenesis [55]. The pathogenic effect of MBL was shown to be dependent on the glycans of the RRV envelope protein increasing MBL deposition on RRV-infected cells [56].

Additionally, viruses have mechanisms to evade complement [57]; for example, the HSV1 glycoprotein C binds C3b and prevents cell lysis [58,59]. As described by Sahu *et al.*, in this issue, a number of viruses have strategies to influence components of the RCA gene cluster, for instance using molecular mimicry in variola or vaccinia virus to generate virally encoded RCA mimics [60,61]. HIV, mumps virus and human cytomegalovirus can incorporate RCA components, such as DAF/CD55 and CD59 into the virus envelope to negatively regulate complement activity at the virion surface [62–65].

The *flavivirus* genus comprises globally and medically relevant viruses such as yellow fever virus (YFV), West Nile virus (WNV), DENV and Zika virus (ZIKV) where similarly their interaction with complement has

been studied [66]. Antibody-mediated activation of the CP is protective in WNV, Kunjin (a nonpathogenic subtype of WNV) and YFV infection [67–69]. The LP and MBL are protective against DENV and WNV infection [69,70], through recognition and binding of glycans on the surface of WNV and DENV virions [69]. C1q also binds DENV E protein and virions to decrease infectivity [71]. Further, the disruption of MBL and C1q binding by specific isotypes of nonneutralising antibody has been associated with antibody-dependent enhancement (ADE) of DENV infection – a well-known correlate of severe dengue disease [72]. In contrast, C1q antibody binding facilitates antibody neutralisation and restricts the potential for ADE in WNV [73]. Several different flavivirus components such as the NS1 protein, with intracellular roles in viral replication [74] and secretion at high levels extracellularly and in the circulation of infected patients [75], also modulate complement activation by direct binding. For instance, *in vitro* studies with recombinant proteins have demonstrated that DENV, WNV and YFV NS1 bind C1s and C4 to promote C4b production which potentially reduces the availability of C4 to target the virion and evades CP and LP activity [76]. DENV, ZIKV and WNV NS1 can bind to vitronectin, a terminal complement regulator leading to decreased C9 polymerisation and MAC formation [77]. DENV NS1 can also interact with clusterin and inhibit MAC formation [78]. In a mechanism similar to that described for bacteria such as *Neisseria spp* [79,80] and *Streptococcus pneumoniae* [81,82], WNV NS1 protein binds and recruits FH to the virion which results in increased FI-mediated cleavage of C3b to form (AP inactive) iC3b and protect the virion from complement-mediated lysis [83]. Interestingly, mosquitoes may also utilise FH captured during a blood meal to protect the midgut epithelium from complement-mediated damage [84]. In contrast, DENV NS1 is not reported to have the same capacity to bind FH [83,85]. Recent data, however, do suggest that DENV NS1 can regulate the AP and modulate pathogenicity. A T164S mutation in DENV NS1 is associated with increased disease in AG129 mice. This mutant virus also induces upregulation of the transcriptional profile for complement components including the AP positive mediator, factor D (FD) in DENV-infected human peripheral blood mononuclear cells (PBMC) [86].

Complement, the AP and FH during DENV infection

Early studies have described excessive complement activation associated with increased dengue disease

severity, with lower circulating levels of C3, C4 and FB, indicative of complement consumption [87,88]. Additionally, lower C3 and increased C3 cleavage is seen in patients in the acute stage of DHF compared with DF and higher FD and lower FH are seen in DHF/DSS compared with DF patients [89]. These changes are restored during convalescence [89]. Histopathological studies from 13 fatal DENV cases demonstrated hepatic deposition of C9, in some instances in conjunction with C1q and C3b, as well as C9, C1q and C3b deposition in the spleen, with associated tissue damage [90]. Additionally, reduced CH₅₀ (indicating overall consumption and resultant reduction in complement function) and higher C3a and C5a – complement split products resulting from complement activation, have been detected in the sera of DHF and DSS patients [87,88,91,92]. Importantly, C3a and C5a have vasoactive properties that may be linked to the vascular leak syndrome seen in DENV-infected patients. Circulating changes in complement components during DENV infection are summarised in Fig. 3.

For the AP, lower FB, higher FD and lower FH have been reported in DHF/DSS compared with DF patients [89,91]. This low FH is reminiscent of other noninfectious AP-mediated pathologies (Box 1) that as described above can result from underlying genetic changes in FH. There are contradicting reports of the association of FH polymorphisms with dengue severity. One study in a Thai population found no association of FH or FB polymorphisms with the severity of

secondary DENV, although it was acknowledged that the study was underpowered [85]. A second study found a significant association of a FH promoter variant in the NFκB binding site with high FH levels and reduced dengue disease severity, suggesting that the ability of FH to respond to inflammatory signals can influence disease [93]. Overall, the literature suggests that low circulating FH protein is associated with more severe dengue disease outcomes (Fig. 3).

In terms of transcriptional changes, recent data described above have shown that *in vitro* infection of human PBMC with DENV is associated with transcriptional changes in a number of components of the complement cascade including the AP [86]. Infection with wild-type (WT) or a DENV NS1 mutant virus associated with increased disease severity in mice resulted in an early (6 hours postinfection [hpi]) decrease in C3 mRNA followed by an increase at 24 hpi. Both WT and NS1-mutant viruses increased mRNA for FB and decreased mRNA for FD. A greater decline in FD mRNA was observed in the NS1 mutant compared with WT virus, and when cells were infected in the presence of an antibody, both these strategies reflect increased disease severity [86]. Analysis of publicly available transcriptional profiling data *via* NCBI GEO database [94] has shown that in whole blood during acute DENV infection [95], where the expected induction of antiviral responses such as the interferon (IFN)-stimulated gene viperin is evident, the mRNA for both FH and FB is increased (Fig. 4A). In contrast, mRNA for other complement components

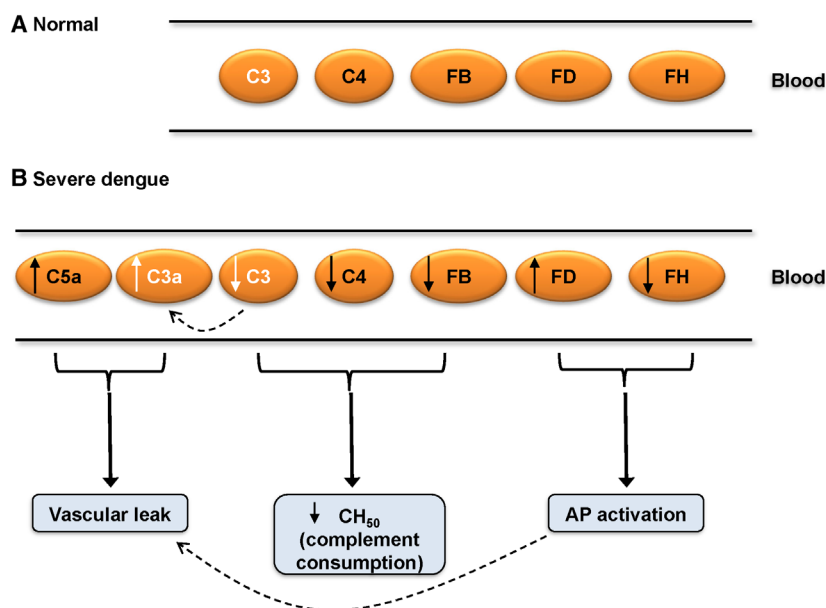


Fig. 3. Schematic of the described changes in circulating complement proteins during DENV infection. (A) normal: intact C3, C4 and FB are present in the circulation in addition to complement AP effector; FD and negative regulator; FH; (B) severe dengue: C3, C4 and FB are reduced, consistent with an observed reduction in CH₅₀. Reduced C3 is associated with an increase in the C3 cleavage product C3a, as well as C5a, which are known vasoactive factors and proposed to promote vascular leak. Additionally, FD is increased and FH decreased consistent with activation and increased AP activity that is proposed to further promote vascular leakage.

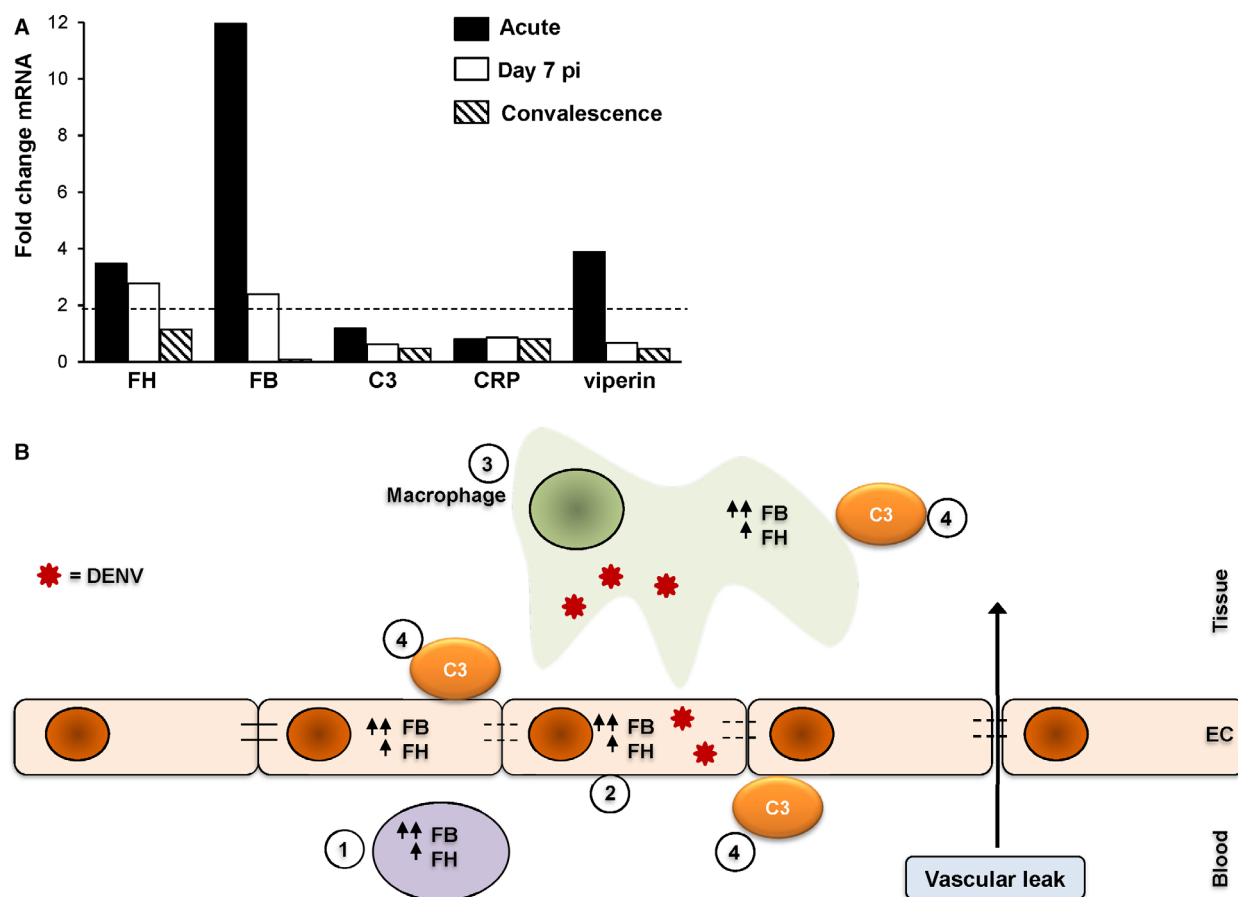


Fig. 4. Cellular changes in FB and FH mRNA during DENV infection. (A) Gene expression array data from whole blood from DENV patients ($n = 31$) during acute infection, < 72 h, days 4–7 pi and during convalescence (3–4 weeks) [95] were analysed by NCBI GEO database, accession no. [GSE28405](#) [94]. Data represent significant changes (adjusted $P < 0.05$) and are expressed as fold change relative to healthy controls ($n = 20$); (B) mRNA levels for FB are increased > FH in whole blood from DENV-infected patients, as in A ①, primary ECs [101,102] ② and primary macrophages ③ [102]. C3 protein deposition is increased on DENV-infected primary macrophages and EC ④ [102] reflecting increased AP activity and associated with increased EC permeability, indicated by the arrow.

such as C3 or the classical acute-phase reactant, C-reactive protein (CRP), is not increased (Fig. 4A). This profile of increased FB and FH mRNA resolves as infection progresses and the patient convalesces (Fig. 4A), which is in accordance with reported circulating protein changes [89]. Interestingly, poor induction of CRP during DENV infection has been reported in other studies [89,96,97]. CRP is known to bind to FH and recruit FH to sites of tissue damage with resultant downregulation of complement activity [98], and thus, low CRP may further hamper FH regulatory actions during DENV infection.

While the data above support transcriptional changes in AP components in circulating cells, changes in expression profiles and local AP regulation at the cell surface [99] such as the vascular endothelium [100] – the site of DENV-mediated vascular leakage, or the

macrophage – the primary target for viral replication *in vivo*, may be of particular importance to DENV-associated responses. Microarray analysis has previously described a 34-fold increase in FB mRNA in DENV-infected human umbilical vein endothelial cells (HUVECs) at 48 hpi in an *in vitro* model [101]. Studies from our laboratory using *in vitro* models of DENV infection in primary HUVEC and macrophages have demonstrated increased FB and FH mRNA with greater induction in FB mRNA and protein relative to FH, at 24–48 hpi [102]. Additionally, these changes in FB and FH are associated with increased C3 protein deposition on cells, and supernatant from infected cells can promote AP activity *in vitro*, together suggesting increased AP activity in the local environment of these important cell types [102]. These described changes in complement AP components within cells and at the

cell surface are summarised in Fig. 4B. Additionally, we have noted that in DENV-infected cells, FH mRNA is induced, but this does not result in a comparable increase in FH protein release into the milieu [102], suggesting that changes in complement components at the mRNA level are only one facet of the responses that occur during infection. This phenomenon where FH mRNA, but not protein, is induced remains to be investigated further, including definition of the specificity of this response to DENV, flaviviruses or viral infections in general.

Conclusions and Perspectives

There are important protective roles of complement, mechanisms for evasion of complement and clear pathogenic potential for dysregulated complement activity during viral infection. The ways that different viruses induce, evade and interact with complement proteins may impact final disease outcome. There are commonalities amongst pathogens in the mechanisms used to evade complement; for instance, pathogen recruitment or binding of FH is seen broadly in bacteria, viruses and even mosquitoes. DENV infection, where the disease is largely an immunopathogenesis, interacts with multiple complement pathways to gain transcriptional and post-transcriptional control of the AP. Moving forward from descriptive responses of the AP to DENV infection is needed to relate changes in complement activity to the specific vascular leak pathology of dengue disease. One key aspect to assist in these studies would be a manipulatable *in vivo* model, such as a mouse, but for DENV infection mouse models are troublesome. DENV does not replicate well in immunocompetent mice, due to an inability of DENV to evade components of the mouse pathogen recognition pathways and IFN responses [103,104]. An often-used mouse model to study DENV infection and DENV-induced vascular leak is the IFN receptor-deficient mouse (e.g. AG129) [35,105]. The AG129 mouse, however, may be problematic for studies of complement proteins, where expression is influenced by IFN [106].

The potential for complement-targeted therapeutics for dengue disease

Excitingly, the main implication of complement dysregulation and overactivity being an important contributor to severe DENV disease is that complement regulators could be useful for treating DENV-induced vascular leakage. Drug development against complement components is intense, and there are many

agents in development to control complement-mediated pathologies [107]. One clinically successful inhibitor of the complement terminal pathway is eculizumab, an anti-C5 monoclonal antibody, that is approved for the treatment of aHUS [108,109]. aHUS is a good clinical example of vascular-mediated pathology driven by the complement system [110] and DENV shows some similarities with these AP-mediated pathologies (Box 1), for example reduced FH, thrombocytopenia and a vascular leak pathology, suggesting that eculizumab may also be of use in dengue treatment. The reality, however, is that eculizumab is a very costly drug, and treating dengue with eculizumab would offer a proof of principle for the applicability of complement inhibitors but would not be a financially viable option in resource-poor settings where dengue is prevalent. Targeting the terminal complex of the complement pathway as eculizumab does, where all three pathways converge, including the protective response of the CP and LP, theoretically would require careful planning to avoid increasing viral replication. Fortunately, DENV viraemia declines during the critical phase of disease where vascular leakage can ensue [32] and the use of a complement inhibitor targeting the terminal complex at this stage of disease may not be problematic. Importantly, however, eculizumab does not affect C3a levels, and if this contributes to DENV vascular leakage [31], then eculizumab would not be beneficial. Hence, we need to understand more about the specific roles of complement components in DENV pathogenesis.

The development of other complement regulators, for example FH/complement receptor 2 fusion proteins, such as TT30 that target FH AP regulatory function [111], 'minifactor H', a variety of drugs targeting generation of C3 [107,112], agents targeting FD as trialled for AMD [113] or glycocalyx-modifying agents [110], are exciting ideas to promote AP regulatory activity at the site of DENV pathology. For a disease such as dengue vascular leak syndrome, where the pathogenesis is likely to involve multiple mediators, targeting the complement AP may offer a multi-pronged opportunity to modulate vasoactive actions and negate the recruitment of other immune cells and hence the activity of other pathogenic factors. Thus, the complement AP may be the perfect target to halt the vicious cycle of inflammation during DENV infection.

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