Mapping the Interactions of Dengue Virus NS1 Protein with Human Liver Proteins Using a Yeast Two-Hybrid System: Identification of C1q as an Interacting Partner

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Abstract

Dengue constitutes a global health concern. The clinical manifestation of this disease varies from mild febrile illness to severe hemorrhage and/or fatal hypovolemic shock. Flavivirus nonstructural protein 1 (NS1) is a secreted glycoprotein that is displayed on the surface of infected cells but is absent in viral particles. NS1 accumulates at high levels in the plasma of dengue virus (DENV)-infected patients, and previous reports highlight its involvement in immune evasion, dengue severity, liver dysfunction and pathogenesis. In the present study, we performed a yeast two-hybrid screen to search for DENV2 NS1-interacting partners using a human liver cDNA library. We identified fifty genes, including human complement component 1 (C1q), which was confirmed by coimmunoprecipitation, ELISA and immunofluorescence assays, revealing for the first time the direct binding of this protein to NS1. Furthermore, the majority of the identified genes encode proteins that are secreted into the plasma of patients, and most of these proteins are classified as acute-phase proteins (APPs), such as plasminogen, haptoglobin, hemopexin, α2-HS-glycoprotein, retinol binding protein 4, transferrin, and C4. The results presented here confirm the direct interaction of DENV NS1 with a key protein of the complement system and suggest a role for this complement protein in the pathogenesis of DENV infection.

Introduction

Dengue constitutes a major global health concern. It is estimated that nearly half of the worldwide population lives in risk areas and that fifty to one hundred million infections occur each year, including 500,000 hospitalizations of patients with severe dengue illness [1,2]. Dengue virus (DENV) is a member of the Flaviviridae family, and it cocirculates as four distinct antigenic serotypes (DENV1–4). Infection with DENV may induce a spectrum of symptoms varying from none to severe plasma leakage, hemorrhage and organ impairment [3]. The mechanism underlying endothelial cell dysfunction and vascular leakage is of primary importance; however, it is far from being understood. Several studies have been published attempting to elucidate the principal phenomenon that leads to severe disease. Indeed, it has been established that the risk of developing severe dengue may be associated with secondary heterologous infection, leading to the phenomenon of antibody-dependent enhancement (ADE) [4], in addition to high viral loads [5–7] and multiple host factors including age, gender, genotype and prior immunity, among others [8,9]. Disease severity can also be correlated to circulating levels of certain cytokines and chemokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interferon-gamma (IFN-γ), interleukin 8 (IL-8), macrophage inflammatory protein 1 (MIP-1) [10–18], and complement components (C3a, C5a, factor D and factor H) [19–22]. Despite the fact that several cell types and tissues have been described as potential sites for DENV replication and release of plasma immune mediators, the liver is one of the most important infection target organs [23,24].

The flavivirus nonstructural protein 1 (NS1) is a 50 kDa intracellular homodimeric glycoprotein that plays a pivotal role in DENV replication [25], and there is evidence that it also plays an important role in dengue severity and pathogenesis [6,26]. Although lacking a membrane-anchoring domain, the NS1 protein associates with organelle membranes and in particular with lipid-rafts, suggesting that it is involved in signal transduction pathways [27]. This association likely occurs via a GPI anchor [28]. The DENV NS1 protein is also secreted into the plasma as a lipid-associated barrel-shaped hexamer that is detectable in patient serum in the first few days after the onset of clinical symptoms in both primary and secondary infections [29,30]. Recent reports highlight the involvement of the NS1 protein in the modulation of the complement system and the vascular leakage process, which facilitate immune complex formation [20]. Moreover, the NS1 protein elicits autoantibodies that react with platelets and extracellular matrix proteins [31] or that interfere with endothelial
Dengue Virus NS1 Protein Interacts with C1q

Results

Identification of DENV NS1-interacting partners using the yeast two-hybrid system

Considering the importance of the NS1 protein during DENV infection and its possible involvement with liver dysfunction [33], we aimed to understand its role in DENV infection and pathogenesis. Therefore, we performed a yeast two-hybrid screening to detect novel putative NS1 interacting-partners using a liver cDNA library. We identified 50 different NS1-interacting partners, including the C1q protein. Coimmunoprecipitation, ligand binding ELISA, and immunofluorescence assays were also performed to confirm the direct binding of NS1 to human C1q. These results indicate the association of the DENV NS1 protein with another complement component, which suggests a role for this complement protein in DENV pathogenesis.

DENV NS1 directly interacts with C1q

Because the interaction of flavivirus NS1 with complement system proteins and its regulators appears to play an important role in the immune evasion process [32,35,36], we focused our studies on the confirmation of the DENV NS1 and C1q interaction. First, we performed a coimmunoprecipitation assay in which the anti-NS1 polyclonal antibody was immobilized to the resin and subsequently incubated with NS1, which was purified in its hexameric form from the supernatants of DENV-infected BHK cells, and purified human C1q protein. Because C1q is able to bind to the Fc region of antibodies [37], we also assessed the interaction of purified C1q with the anti-NS1-coated resin (control). The elution fractions were then analyzed by Western blot using another specific anti-NS1 polyclonal antibody and an anti-C1q monoclonal antibody. Two bands of approximately 30 and 50 kDa corresponding to C1q and NS1, respectively, were obtained, indicating that C1q coimmunoprecipitated with the NS1 protein (Fig. 3A). As expected, a band of approximately 30 kDa was also observed in the control experiment (Fig. 3A). However, the band intensity for C1q in the presence of NS1 was more than four-fold higher than that in the absence of NS1 (control), and this difference is statistically significant \( p = 0.0177 \). These findings confirm the interaction between the NS1 and C1q proteins as determined by the yeast two-hybrid screening.

Previous studies have shown that anti-NS1 antibodies are able to cross-react with some human proteins, such as blood clotting factors and adhesion molecules [31,33]. Therefore, to verify whether the C1q precipitation in the control experiment was caused by its binding to the Fc region of the anti-NS1 antibody or its cross interaction, we performed an identical experiment as described above using a nonspecific IgG1-coated resin. We observed a sharp band of approximately 30 kDa in the elution fraction, indicating that the C1q protein was also immunoprecipitated by IgG1 at low levels (Fig. 3A). Analysis of the band intensity revealed that there is no significant difference between coimmunoprecipitation using IgG1 and the control experiments, but there is a significant difference between coimmunoprecipitation using IgG1 and coimmunoprecipitation in the presence of NS1 \( p = 0.0111 \), Fig. 3B). These results indicate that the anti-NS1 antibody does not cross-react with C1q.

To assess whether NS1 directly binds C1q, an ELISA assay was designed in which the purified C1q was immobilized onto the plate and incubated with increasing concentrations of NS1 purified from the supernatant of DENV-infected BHK cells. As a control, we used the supernatant of mock-infected BHK cells subjected to an identical protocol used in the NS1 purification. To detect the interaction, we used a specific conformational anti-NS1 monoclonal antibody. We observed a significant increase in the optical density (OD) values when both proteins were incubated together, whereas no difference in the OD value was observed in the control experiment (Fig. 4A). Statistical analysis using two-way ANOVA revealed a significant difference between the control and binding curves. This result clearly indicates that NS1 binds C1q directly in a dose-dependent manner.

antibody-dependent, complement-mediated cytolysis [10]. DENV NS1 also exhibits complement antagonism by binding directly to complement proteins, including C4 and C1s, which leads to the degradation of C4 in solution and, consequently, to the inhibition of complement activation [32]. Alcon-LePoder and coworkers (2005) demonstrated that the liver is the major site for NS1 protein accumulation and preincubation of hepatocytes with soluble NS1 enhances subsequent infection by a homologous strain of DENV [33]. However, the mechanism by which NS1 is involved in dengue pathogenesis remains unclear.

To understand the role of the NS1 protein in DENV infection and pathogenesis, a yeast two-hybrid system was used to screen for the interacting partners of the DENV NS1 protein using a human liver cDNA library. We identified 50 different NS1-interacting partners, including the C1q protein. Coimmunoprecipitation, ligand binding ELISA, and immunofluorescence assays were also performed to confirm the direct binding of NS1 to human C1q. These results indicate the association of the DENV NS1 protein with another complement component, which suggests a role for this complement protein in DENV pathogenesis.

Next, to select putative positive clones, we first grew the recombinant cells containing the bait and prey plasmids in triple drop-out media. This selection yielded 2,080 colonies. We identified the recombinant colonies containing a positive NS1 protein-interacting partner by HIS3, ADE2 and reporter gene activation, as visualized by cell growth on triple and quadruple drop-out media plates and by blue color staining following the colony-lift assays (Fig. 1). This analysis led to the identification of 50 different genes, including genes that encode the complement proteins C1q and C4 in addition to genes that encode several acute-phase proteins (APPs), such as plasminogen, haptoglobin, and hemopexin, among others (Table 1). Interestingly, the interaction of NS1 with the complement C4 protein has been described previously [32].

Thus, to identify the corresponding proteins of these identified genes, we subjected their sequences to BLASTX analysis (available at the NCBI website). Next, we arranged them according to their primary cellular localization, as shown in Figure 2. We determined that NS1-interacting partners belonged to a wide range of protein classes including extracellular milieu-released proteins (35% of all proteins screened by the yeast-two hybrid system) that includes, for example, the apolipoprotein A2 and H, C1q, C4, haptoglobin, hemopexin, plasminogen, and transferrin; cytoplasmic-resident proteins (23% of all proteins) such as alcohol dehydrogenase 1B, aldehyde dehydrogenase 1 and 7, and eukaryotic translation elongation factor 1; mitochondrial-resident proteins (11% of all proteins), such as, for example, 3-hydroxybutyrate dehydrogenase, monoamine oxidase B and phosphoenolpyruvate carboxykinase 2; plasma membrane proteins (11% of all proteins) such as CD14, endoplasmic reticulum-resident proteins (4% of all proteins) such as carboxylesterase 1; and lysosomal proteins (4% of all proteins) such as prosaposin. These results indicate the ability of the NS1 protein to interact with different classes of host proteins, primarily those localized in the extracellular region.
The NS1 protein is glycosylated in at least two sites [39] and coexists as different oligomeric states (dimers and/or hexamers). However, there is considerable discussion concerning which oligomeric state of NS1 interacts with other proteins and whether these interactions occur through its carbohydrate moiety. Previous studies from our group demonstrated that bacterial recombinant NS1 protein is able to form dimers in a conformation similar to those produced in insect cells depending on the refolding protocol used [34]. To evaluate whether recombinant NS1 purified from bacteria was able to bind C1q, we performed an identical experiment as described above for NS1 purified from BHK cells. As a control, we used purified bovine serum albumin (BSA). We observed that recombinant NS1 was also able to bind C1q directly in a dose-dependent manner similar to what was observed for NS1 purified from BHK cells. Note that the peak of the OD value measured for each NS1 protein was similar. We also performed an identical experiment but in the opposite order, i.e., immobilizing the NS1 protein (either from DENV-infected BHK or from recombinant bacterial cells) onto the plate followed by incubation with increasing concentrations of purified human C1q. These data were similar to those shown in Figure 4 (data not shown).

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| Abbreviation | Gene name | NCBI ID  | No. of clones detected |
|--------------|-----------|----------|------------------------|
| ACTG1        | actin, gamma 1 | NG_011433.1 | 2                      |
| ADH1B        | alcohol dehydrogenase 1B (class I), beta polypeptide | NG_011435.1 | 4                      |
| AHSG         | alpha-2-HS-glycoprotein | NG_011436.1 | 2                      |
| ALDH1L1      | aldehyde dehydrogenase 1 family, member L1 | NG_012260.1 | 2                      |
| ALDH7A1      | aldehyde dehydrogenase 7 family, member A1 | NG_008600.2 | 5                      |
| ALDOB        | aldolase B, fructose-bisphosphate | NG_012387.1 | 13                     |
| AMBP         | alpha-1-microglobulin/bikunin precursor | NM_001633.3 | 5                      |
| APEH         | N-acylaminoacyl-peptide hydrolase | NM_001640.3 | 2                      |
| APOA2        | apolipoprotein A-II | NM_001643.1 | 1                      |
| APOH         | apolipoprotein H (beta-2-glycoprotein) i | NG_012045.1 | 5                      |
| AZG1         | alpha-2-glycoprotein 1, zinc-binding | NM_001185.3 | 1                      |
| BCKDHA       | branched chain keto acid dehydrogenase E1, alpha polypeptide | NM_00709.3 | 1                      |
| BDH1         | 3-hydroxybutyrate dehydrogenase, type I | NM_004051.4 | 1                      |
| C1QA         | complement component 1, q subcomponent, A chain | NG_007282.1 | 2                      |
| C4A          | complement component 4A | NM_007293.2 | 2                      |
| C7orf10      | C7orf10 chromosome 7 open reading frame 10 | NM_001193311.1 | 1                  |
| CC5          | copper chaperone for superoxide dismutase | NM_005125.1 | 1                      |
| CD14         | CD14 molecule | NG_023178.1 | 2                      |
| CES1         | carboxylesterase 1 | NG_012057.1 | 1                      |
| COL18A1      | collagen, type XVIII, alpha 1 | NM_030582.3 | 1                      |
| CT5B         | cathepsin B | NG_009217.1 | 1                      |
| CUTA         | cutA divalent cation tolerance homolog (E. coli) | NM_001014840.1 | 1                  |
| ECHDC1       | enoyl CoA hydratase domain containing 1 | NM_00100230.1 | 2                  |
| EEF1A1       | eukaryotic translation elongation factor 1 alpha 1 | NM_001402.5 | 3                      |
| EMR2         | egf-like module containing, mucin-like, hormone receptor-like 2 | NM_152916.1 | 1                      |
| ENTP5        | ectonucleoside triphosphate diphosphohydrolase 5 | NM_001249.2 | 1                      |
| FAH          | fumarylacetoacetate hydrolase (fumarylacetoacetase) | NG_012833.1 | 1                      |
| FH           | fumarate hydratase | NG_012338.1 | 1                      |
| GPX2         | glutathione peroxidase 2 | NM_002083.2 | 1                      |
| HP           | haptoglobin | NG_012651.1 | 1                      |
| HPN          | hepsin | NM_002151.2 | 1                      |
| HPX          | hemopexin | NM_000613.2 | 1                      |
| LSR          | lipolysis stimulated lipoprotein receptor | NM_205834.2 | 1                      |
| MAOB         | monoamine oxidase B | NG_008723.1 | 1                      |
| MPND         | MPN domain containing | NM_00159846.1 | 1                  |
| MT2A         | metallothionein 2A | NM_005935.3 | 13                     |
| MTERFD2      | MTERF domain containing 2 | NM_182501.3 | 1                      |
| MTHFD1       | methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methylenetetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase | NG_012450.1 | 1                      |
| PARP12       | poly (ADP-ribose) polymerase family, member 12 | NM_022750.2 | 2                      |
| PCK2         | phosphoenolpyruvate carboxykinase 2 (mitochondrial) | NG_008162.1 | 2                      |
| PKD1         | polycystic kidney disease 1 | NG_008171.7 | 1                      |
| PLG          | plasminogen | NG_016200.1 | 2                      |
| PSAP         | prosaposin | NM_00142465.1 | 2                  |
| RBP4         | retinol binding protein 4, plasma | NG_009104.1 | 2                      |
| RELN         | reelin | NG_011877.1 | 4                      |
| SAT1         | spermidine/spermine N1-acetyltransferase 1 | NG_012929.1 | 1                      |
| SEPP1        | selenoprotein P, plasma, 1 | NM_0085486.1 | 1                      |
| SIVA1        | SIVA1, apoptosis-inducing factor | NM_006247.3 | 7                      |
| TF           | transferrin | NM_001063.3 | 1                      |
identification of C1q as an NS1-interacting partner but also reveal that this interaction occurs directly. Moreover, the finding that bacterial recombinant NS1 protein is also able to bind C1q indicates that this interaction is independent of posttranslational modification.

Colocalization of the NS1 protein with endogenous C1q

To evaluate whether these proteins colocalize during DENV infection in vivo, we performed an immunofluorescence assay of mock- and DENV-infected human acute monocytic leukemia (THP-1) cells using both anti-NS1 and anti-C1q antibodies. Although the liver is the principal production site of complement components, hepatocytes do not express the C1q protein [40]. In fact, it is the liver-resident macrophages (Kupffer cells) that are responsible for the production of C1q [41]. Alternatively, we used a mononuclear phagocytic cell line (THP-1) that has been shown to express C1q [42]. We observed that infected cells were labeled for both C1q (green) and NS1 (red) proteins (Fig. 5). When the images were merged, distinct yellow regions were observed, indicating the colocalization of NS1 with C1q in these areas (Fig. 5, detail). The C1q labeling was also observed in mock-infected cells, and it appeared at an identical position as that observed in DENV-infected cells in which no NS1 was detected. These results clearly indicate the colocalization of DENV NS1 with C1q in cell culture.

Discussion

In the present study, we identified 50 putative interacting partners of DENV2 NS1 by screening a human liver cDNA library using a yeast two-hybrid system. In this screening, we detected the NS1 interaction with a key protein of the complement system, C1q. According to recent reports, the flavivirus NS1 seems to interact with complement system proteins and its regulators exhibiting immune-evasion functions, which contributes somehow to disease pathogenesis [32,35,36]. Based on this information, we focused our efforts on the confirmation of the DENV NS1 and C1q interaction by coimmunoprecipitation, ELISA and immunofluorescence assays. The complement system consists of more than 30 proteins that are present as either soluble blood-borne or membrane-associated proteins. This collection of proteins organizes into a hierarchy of proteolytic cascades beginning with the identification of pathogenic surfaces and leading to i) the generation of potent proinflammatory mediators (anaphylatoxins); ii) the opsonization (coating) of the pathogenic surface using various complement opsonins (e.g., C3b); and iii) targeted lysis of the pathogenic surface by assembling membrane-penetrating pores known as the membrane attack complex (MAC) [43,44]. Viruses have developed strategies to limit or retard their recognition by the complement cascade and inhibit the immune response. These strategies include i) secretion of molecules that

**Table 1.** Cont.

| Abbreviation | Gene name       | NCBI ID    | No. of clones detected |
|--------------|-----------------|------------|------------------------|
| THNSL1       | threonine synthase-like 1 | NM_024838.4 | 1                      |

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It is known that C1q binds to the Fc region of the antibody complement regulatory proteins on the surface of infected cells, mimicking or recruiting host complement regulators and expression of surface proteins that interact with the antibody Fc region, thereby preventing C1q-dependent action; ii) incorporation of complement components on the virion; and iii) upregulation of C3 convertase promotes C3b-mediated opsonization and assembly of C5b-C9 MAC [37,45,46]. This pathway is also activated in the absence of antibodies due to the direct interaction of C1q with viral surface proteins, as observed for the p15E protein of oncornavirus [47] and gp41 and gp120 of the human immunodeficiency virus (HIV-1) [48,49].

The flavivirus NS1 protein has been described as an immune evading protein [43] that binds complement regulators such as human clusterin, which inhibits MAC formation [50], and C4b-binding protein (C4BP), which regulates complement activation by interacting with C4b [36]. In addition, the NS1 protein binds complement proteins such as C4, also identified in this work, forming a complex with proC1s/C1s and leading to C4 degradation [32]. These interactions may help the virus to reduce complement activation via the classical and lectin pathways [32,36]. Moreover, several studies have indicated that the complement system can restrict ADE. ADE is a phenomenon associated with dengue severity that occurs when non-neutralizing antiviral antibodies enhance viral entry into host cells, leading to increased efficiency of infection [4].

Experiments with mouse sera lacking individual complement components have indicated that C1q is sufficient to restrict ADE in West Nile virus (WNV) infection [51], and this has also been demonstrated using human sera in DENV infection [52]. C1q can also increase the potency of antibodies targeted against WNV by modulating the stoichiometric requirements for virus neutralization [33]. Our results indicated that DENV2 NS1 can directly bind the complement protein C1q, and this interaction is not dependent on the glycosylated form of DENV NS1. Nevertheless, further experiments are required to ascertain whether this interaction will modulate complement activation.

Interestingly, most of the identified NS1-interacting partners are proteins that are secreted to the extracellular milieu, such as plasminogen, haptoglobin, hemopexin, α2-HS-glycoprotein, retinol binding protein 4, transferrin, and C4 and are classified as APPs. They actively participate in the acute-phase response (APR), and their plasma concentration varies during the inflammatory process. The APR is an important pathophysiological phenomenon that supersedes the normal homeostatic mechanisms during infection. The APR is an essential component of the innate immune system and plays an important role in limiting hepatic tissue injury. Several APPs can initiate, amplify or sustain inflammation, whereas others can attenuate it [54,55]. Increasing the circulating levels of proinflammatory cytokines, chemokines, and immune effectors, for example, can lead to initiation of the inflammatory process. APR proteins crosstalk with proteins involved in the coagulation process, as observed during septic shock. This crosstalk is of great importance in the induction of episodes of DHF/DSS [56,57]. Therefore, the NS1-plasminogen interaction would be a promising start point to study the abnormal activation of the fibrinolytic system associated with DHF/DSS events. There has been considerable discussion on the origin and maintenance of signals/effectors that trigger the vascular leakage phenomenon in DENV infection. However, the hypothesis that several factors, such as host genetic background, virus characteristics and immune response, contribute to dengue severity is commonly accepted [38]. During the course of DENV infection, increased levels of proinflammatory cytokines, chemokines and immune effectors, for example, can lead to initiation of the inflammatory process. APR proteins crosstalk with proteins involved in the coagulation process, as observed during septic shock. This crosstalk is of great importance in the induction of episodes of DHF/DSS [56,57]. Therefore, the NS1-plasminogen interaction would be a promising start point to study the abnormal activation of the fibrinolytic system associated with DHF/DSS events. There has been considerable discussion on the origin and maintenance of signals/effectors that trigger the vascular leakage phenomenon in DENV infection. However, the hypothesis that several factors, such as host genetic background, virus characteristics and immune response, contribute to dengue severity is commonly accepted [38]. During the course of DENV infection, increased levels of proinflammatory cytokines, chemokines and immune effectors, for example, can lead to initiation of the inflammatory process. APR proteins crosstalk with proteins involved in the coagulation process, as observed during septic shock. This crosstalk is of great importance in the induction of episodes of DHF/DSS [56,57]. Therefore, the NS1-plasminogen interaction would be a promising start point to study the abnormal activation of the fibrinolytic system associated with DHF/DSS events. There has been considerable discussion on the origin and maintenance of signals/effectors that trigger the vascular leakage phenomenon in DENV infection. However, the hypothesis that several factors, such as host genetic background, virus characteristics and immune response, contribute to dengue severity is commonly accepted [38]. During the course of DENV infection, increased levels of proinflammatory cytokines, chemokines and immune effectors, for example, can lead to initiation of the inflammatory process.
specific C1q-NS1 interaction may enable the virus to avoid complement activation through the classical pathway by possibly preventing antibody interaction and/or enabling C1-complex assembly. This hypothesis could be confirmed with in vivo studies by assessing complement activation in C1q, C1s or C1r-deficient mice challenged with DENV and/or inoculated with NS1 protein. Moreover, yeast two-hybrid screening revealed that DENV NS1 interacts with a wide range of proteins including some APPs that are important effectors of APR, which is an orchestrated mechanism focused on the reestablishment of normal liver and plasma homeostasis. Binding to and altering the properties of APPs may provide the molecular basis to link dengue infection and hemostatic abnormalities such as vascular leakage, thrombocytopenia and hemorrhage. However, further studies are necessary to confirm the role of NS1 interaction with C1q and APPs in the pathogenesis of DENV infection.

Materials and Methods

Cloning the ns1 gene into the pGBKT7 plasmid
The ns1 gene, from the cDNA of the DENV2 New Guinea C (NGC) strain, was amplified by PCR using the forward primer 5'-AATAAGGATCCATGATAGTGGTTGCGTTGTGAGC-3' containing the BamHI restriction site (underlined bases) and the reverse primer 5'-TATTGCCGCGCTTAGGCTGTGAC-CAAGGAGTTGAC-3' containing the NotI restriction site (underlined bases) and the TAA stop codon. The PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 1 min and 68°C for 2 min followed by a final extension at 68°C for 7 min. The amplified gene was cloned in frame with the GAL4 DNA-binding domain (BD) of the yeast expression vector pGBK7T (Clontech, CA, USA) to construct the bait plasmid (pGBK7T-NS1). The yeast strain AH109 was transformed with the pGBK7T-NS1 plasmid using the lithium acetate method and then grown in SD (synthetic defined) medium lacking tryptophan (SD–Trp). Autoactivation of the HIS3 reporter was confirmed by the growth of clones in SD medium lacking histidine, leucine and tryptophan (SD–His–Leu–Trp). The transformed cultures were then plated onto SD–His–Leu–Trp and SD–Ade–His–Leu–Trp media to select putative positives clones. The activity of the lacZ reporter gene was evaluated by the β-galactosidase assay (colony-lift filter) using the substrate X-gal on nitrocellulose membranes. To eliminate false positives, a plasmid linkage assay was also performed. The positive plasmids were sequenced, and their gene sequences were analyzed using the BLASTN and BLASTX software available at NCBI (www.ncbi.nlm.nih.gov).

Cell culture and infection
Baby hamster kidney fibroblast (BHK; ATCC, USA) and human acute monocytic leukemia (THP-1; ATCC, USA) cell lines were cultured in α-MEM Medium (Gibco) and RPMI Medium 1640 (Gibco, NY, USA), respectively, supplemented with 10% fetal bovine serum (Invitrogen, NY, USA), 0.22% sodium bicarbonate and 0.2% HEPES, pH 7.4, in a humid CO₂ incubation chamber at 37°C. After 2 days, BHK and THP-1 cells were mock-infected or infected with DENV2 strain 16681 at a multiplicity of infection of 2. All experiments were performed at 48 h post infection.

Purified NS1
DENV2 NS1 was produced and isolated from the supernatant of BHK cells that were infected with the DENV2 strain 16681. The supernatant was harvested, and NS1 was purified as described previously [62]. DENV NS1 from E. coli was expressed and purified as described previously [34].

Coimmunoprecipitation and Western blot
The coimmunoprecipitation was performed using a kit (Pierce, IL, USA). Purified anti-NS1 polyclonal antibody or IgG control antibody was attached to an N-terminal-binding resin. Purified

Figure 5. Colocalization of NS1 and C1q proteins by confocal microscopy. DENV2-infected THP-1 cells were labeled by incubation with polyclonal anti-NS1 (red stained) or monoclonal anti-C1q (green stained) antibodies. NS1 and C1q proteins were localized in vesicle-like structures in the cytoplasm, which is characteristic of secretory proteins. When the images were merged, distinct yellow regions were revealed, indicating colocalization of NS1 with C1q in these areas (detail). The subcellular localization of C1q was also analyzed in mock-infected cells, and it appeared at an identical position as that observed in DENV-infected cells, whereas no NS1 protein was detected in these cells. The cells were also incubated with DAPI for nuclear staining.

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NS1 from BHK supernatants and purified human C1q (Sigma-Aldrich, USA) were mixed and added to the anti-NS1-coated resin. C1q protein was also added to the anti-NS1- and IgG1-coated resin to serve as controls and to assess nonspecific binding. The samples obtained from the CoIP assay were separated by 12% SDS-PAGE and transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare, Sweden). The membrane was then blocked with 5% BSA in TBST (0.1% Tween 20 in TBS [25 mM Tris-HCl, pH 7.6, 3 mM KCl, and 140 mM NaCl]) for 2 h followed by overnight incubation with a mouse polyclonal anti-NS1 or mouse monoclonal anti-C1q antibody (Abcam, USA) in blocking solution. The membrane was then washed three times with TBST and incubated with anti-mouse IgG conjugated to horseradish peroxidase (Promega, USA) in blocking solution for 2 h. The membrane was washed again, developed with a Supersignal West Pico kit (Pierce, IL, USA) and exposed to Kodak MXG/PLUS film. The band intensity was measured by Scion Image software.

DENV2 NS1 and C1q ligand binding ELISA

Purified human C1q (Sigma-Aldrich, USA) at a concentration of 10 μg/mL was adsorbed to wells in MaxiSorp microtiter plates (Nunc) at 4°C overnight. After five washes with phosphate-buffered saline (PBS), nonspecific binding sites were blocked with 200 μL of 1% BSA in PBS containing 0.05% Tween 20 (PBST) for 1.5 h at 37°C followed by five washes with PBS. Purified NS1 from BHK or E. coli at specific concentrations were added to each well and incubated for 2 h at 37°C. Plates were then washed five times with PBST followed by a 1-h incubation with a DENV2 NS1-specific monoclonal (Abcam) or polyclonal antibody (1:1000 dilution). After five washes with PBS, anti-mouse IgG conjugated to horseradish peroxidase (Promega, USA) was added sequentially for 1 h at 37°C. After five final washes with PBST, the signal was developed by adding 100 μL of 0.4 mg/mL o-phenylenediamine (OPD) and 50 μL of 9 N H2SO4 stop solution to each well. The OD at 490 nm was determined by a 96-well plate reader.

Statistical analysis

Datasets were compared by a two-tailed, unpaired Student t test and statistical significance was achieved when p values were <0.05. Multiple comparisons were performed using two-way ANOVA (Bonferroni post-test), and asterisks indicate significant difference from the control (*p<0.05, **p<0.01, ***p<0.001).

Immunofluorescence confocal microscopy

THP-1 cells were washed in PBS, pH 7.2, and fixed for 30 min with 4% freshly prepared formaldehyde diluted in PBS. Cells were deposited on poly-L-lysine-treated microscope slides and permeabilized with 1.5% Triton X-100 in PBS, pH 7.2, for 25 min. The slides were incubated in blocking solution containing 1.5% BSA in PBS, pH 7.2, and then with purified anti-NS1 polyclonal and anti-C1q monoclonal antibodies diluted 1:100 in blocking solution for 1 h. The cells were washed and incubated for 45 min with Alexa 488-conjugated goat anti-mouse IgG antibody (Invitrogen, USA) or with Alexa 568-conjugated goat anti-rabbit IgG antibody (Invitrogen, USA) diluted 1:400 in blocking solution. The cells were subsequently incubated with 5 μM 4′,6-diamidino-2-phenylindole (DAPI, Sigma, USA) for 20 min at room temperature. The slides were mounted in N-propyl gallate and observed using a Leica TCS SP5 confocal microscope. All images were collected with LAS AF Lite 2.6 software (Leica Microsystems).

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Author Contributions

Conceived and designed the experiments: EMS, JNC, RMB. Performed the experiments: EMS, JNC DA. Analyzed the data: EMS, JNC DA RMB. Contributed reagents/materials/analysis tools: MLN RMB. Wrote the paper: EMS, JNC DA RMB.
22. Nascimento EJ, Silva AM, Cordeiro MT, Brito CA, Gil LH, et al. (2009) Alternative complement pathway deregulation is correlated with dengue severity. PLoS One 4: e6762.
23. Rosen L, Drouet MT, Deubel V (1999) Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Am J Trop Med Hyg 61: 720–24.
24. Paes MV, Lenzi HL, Nogueira AC, Nuovo GJ, Pinho AT, et al. (2009) Hepatic damage associated with dengue-2 virus replication in liver cells of BALB/c mice. J Virol Methods 164: 102–108.
25. Mackenzie JM, Jones MK, Young PR (1996) Immunolocalization of the dengue virus nonstructural-1 protein (NS1) in the sera of infected patients. J Clin Microbiol 38: 1053–1057.
26. Young PR, Hilditch PA, Bletchly C, Halloran W (2000) An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of dengue patients. J Gen Virol 89: 2492–2500.
27. Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM, Young PR (2000) Dengue virus protein NS1 is expressed in a glycosylphosphatidylinositol-linked form that is capable of signal transduction. FASEB J 14: 1603–1610.
28. Alcon S, Talainin A, Debruyne M, Falconar A, Deubel V, et al. (2002) Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40: 576–381.
29. Gutsche I, Coulibaly F, Voss JE, Salmon J, d’Alayer J, et al. (2011) Secreted dengue virus nonstructural protein NS1 with lipid raft. J Gen Virol 92: 624–635.
30. Gutsche I, Coulibaly F, Voss JE, Salmon J, d’Alayer J, et al. (2011) Secreted dengue virus nonstructural protein NS1 with lipid raft. J Gen Virol 92: 624–635.
31. Falconar AK (1997) The dengue virus nonstructural-1 protein (NS1) generates antibody to common epitopes on human blood clotting, integrin/adhesion proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142: 897–916.
32. Avirutnan P, Klinosuda P, Yonan S, et al. (2010) Antagonism of the complement component C4 by flavivirus nonstructural protein 1. J Exp Med 207: 793–806.
33. con-LePoder S, Drouet MT, Roux P, Frenkiel MP, Arborio M, et al. (2005) The dengue virus nonstructural protein 1 NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A 102: 6003–6008.
34. Falconer AJ (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesion proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142: 897–916.
35. Falconer AJ (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesion proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142: 897–916.
36. Avirutnan P, Hauhart RE, Somnuke P, Yonan S, et al. (2010) Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. J Exp Med 207: 793–806.
37. Duncan AR, Winter G (1988) The binding site for C1q on IgG. Nature 332: 19111–19116.
38. Mehlhop E, nsarah-Sobrinho C, Johnson S, Engle M, Fremont DH, et al. (2007) Complement protein C1q inhibits antibody-dependent enhancement of flavivirus infection in an IgG subclass-specific manner. Cell Host Microbe 2: 417–426.
39. Rosen L, Drouet MT, Deubel V, Debruyne M, Falconar A, Deubel V, et al. (2002) Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 40: 576–381.
40. Idusogie EE, Presta LG, Gazzano-Santoro H, Topal K, Wong PY, et al. (2000) Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol 164: 4178–4184.
41. Vranakis JE (2005) The role of complement in innate and adaptive immunity. Curr Top Microbiol Immunol 266: 41–56.
42. Sals C, Kirschnik M, Kröpelin M, Daniel V, Oppeck C (1994) Complement activation by recombinant HIV-1 glycoprotein gp120. J Immunol 152: 6028–6034.
43. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
44. Mehlhop E, Nelson S, Just CA, Gorlato S, Johnson S, et al. (2009) Complement protein C1q reduces the stoichiometric threshold for antibody-mediated neutralization of West Nile virus. Cell Host Microbe 6: 383–391.
45. Gahay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340: 448–454.
46. Black PH (2003) The inflammatory response is an integral part of the stress response: Implications for atherosclerosis, insulin resistance, type II diabetes and metabolic syndrome X. Brain Behav Immun 17: 350–364.
47. Schouten M, Wiersinga WJ, Levi M, van der PT (2008) Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol 83: 586–545.
48. Armbrust T, Nordmann B, Kreissig M, Ramadori G (1997) C1q synthesis by tissue mononuclear phagocytes from normal and from damaged rat liver: up-regulation by dexamethasone, down-regulation by interferon gamma, and lipopolysaccharide. Hepatology 26: 90–106.
49. Chang NS, Immieri C, Martin J, Armand G (1994) Synthetic polysulfated hyaluronic acid is a potent inhibitor for tumor necrosis factor production. J Leukoc Biol 55: 778–784.
50. Avirutnan P, Mehlhop E, Diamond MS (2000) Complement and its role in protection and pathogenesis of flavivirus infections. Vaccine 18 Suppl 4: 1100–1107.
51. Lambiris JD, Ricklin D, Gruberbuch B (2008) Complement evasion by human pathogens. Nat Rev Microbiol 6: 132–142.
52. Yamanaka A, Drouet MT, Roux P, Frenkiel MP, Arborio M, et al. (2005) The dengue virus nonstructural protein 1 NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A 102: 6003–6008.
53. Mackenzie JM, Jones MK, Young PR (1996) Immunolocalization of the dengue virus nonstructural-1 protein (NS1) in the sera of infected patients. J Clin Microbiol 38: 1053–1057.
54. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
55. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
56. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
57. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
58. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
59. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.
60. Yamanaka A, Konishi K (2008) Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. J Virol 82: 927–937.