Breaking barriers
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CHAPTER 5

Early events in the dengue virus life cycle - virus cell-binding and endocytosis in presence and absence of antibodies
Introduction

Dengue virus (DENV), of which four serotypes (DENV-1 to DENV-4) exist, causes the most common arthropod-borne viral infection in the world. DENV is transmitted to humans by mosquitoes of the *Aedes* genus, mainly *Aedes aegypti* and *Aedes albopictus*. Over the past 50 years, the incidence of DENV infections increased more than 30-fold. Currently, DENV is endemic in over 100 countries located mostly in the tropical and subtropical parts of the world. Annually, 390 million estimated DENV infections occur of which 96 million infections lead to clinical disease. Moreover, it was recently published that in 2013 an estimated 13,586 deaths could be attributed to DENV infections worldwide. Next to its major health burden, DENV also has a high economic burden, which reached close to 10 billion US dollars worldwide in 2013.

Whereas most DENV infections are asymptomatic or subclinical, the virus can cause a wide spectrum of clinical illness. The WHO classified dengue disease in three different categories: I) dengue fever without warning signs, II) dengue with warning signs and, III) severe dengue. Dengue fever without warning signs is a relatively mild flu-like disease which is characterized by high fever, pains and aches (including headache, pain behind the eyes and muscle and joint pains), rash, nausea and leucopenia. Although dengue without warning signs is mostly self-limiting, the disease can be highly debilitating. Dengue with warning signs is characterized by similar symptoms as general dengue fever with the addition of specific ‘warning signs’, like abdominal pain, fluid accumulation and mucosal bleeding. As the warning signs might forebode severe dengue, patients with dengue with warning signs should be under strict observation. Finally, severe dengue is characterized by plasma leakage with or without bleeding. Around the time fever drops, an increased capillary permeability accompanied by an increased hematocrit value can lead to hypovolemic shock. This reaction, also known as dengue shock syndrome (DSS), can result in organ impairment, metabolic acidosis, disseminated vascular coagulation and severe hemorrhage. If untreated, mortality can reach 20%, whereas appropriate symptom management, such as fluid replacement, reduces mortality to below 1%. Next to DSS, severe dengue also includes patients with fluid accumulation accompanied by respiratory distress, severe bleeding without shock, hepatitis, neurological disorders and myocarditis.

To date, no antivirals are available to treat DENV infections and treatment is solely symptomatic. Furthermore, the prospect of a safe and effective dengue vaccine recently got hampered, as the most advanced dengue vaccine did not meet expectations in a recent large clinical trial. The vaccine is now only licensed for individuals older than 9 years living in endemic countries.

Viral structure

DENV is a small icosahedral virus of 50 nm in size. It contains a single positive-stranded
11 kb RNA molecule which is complexed to multiple copies of the capsid (C) protein. The nucleocapsid is surrounded by the viral envelope, a host-derived membrane, which harbors two transmembrane proteins: envelope (E) and membrane (M). The viral surface of mature DENV virions consists of 90 homodimers of the E protein, which lie flat against the viral envelope. The 90 E-homodimers are arranged in 30 rafts of three homodimers, positioned head-to-tail in a herringbone-like pattern (Figure 1 and 10,11). DENV-E is anchored in the viral membrane via a stem anchor domain and two transmembrane domains. The DENV-E ectodomain consists of three structurally distinct domains, DI to DIII, connected by flexible hinges. Of these domains, DI is the linker region which connects DII and DIII via short flexible loops. DII is involved in the dimerization of the E protein. Furthermore, it contains a highly conserved hydrophobic fusion loop at its distal end. DIII, an immunoglobulin-like domain, forms small protrusions from the viral surface. Via these protrusions, this domain likely interacts with cellular attachment factors11–13. At 37°C, DENV virions are dynamic ‘breathing’ structures that occasionally expose their viral membrane and cryptic sites of E and M. However, at 28°C, the temperature DENV encounters when replicating inside a mosquito, the viral structure seems quite rigid14.

Figure 1. Structure of mature and immature DENV virions. (A) Outer structure of a mature DENV virion. The DENV-E proteins form homodimers and are arranged in 30 rafts of three homodimers. (B) Top (left) and side (right) view of the solubilized E-homodimer (sE) of mature DENV virions, depicting domain I (red), domain II (yellow), domain III (blue) and the fusion peptide (green). E-homodimers are formed by a head-to-tail arrangement of two E-proteins. (C) Cross-section of a mature and immature DENV virion. In the mature viral particle the DENV-E proteins lie flat on the viral membrane, anchored by a stem anchor and two transmembrane domains. In immature DENV virions, three prM/E-heterodimers arrange together to form spike-like structures. prM is present at the top of the spike and covers the fusion loop within E DII. (D) The outer structure of an immature DENV virion. Images were adapted from 18,19 with permission from from Elsevier.
In the infected cell, progeny DENV particles are assembled in an immature form. Immature DENV particles contain prM, the premature form of M, at their viral surface. prM interacts with the E protein to form heterodimers. Immature DENV particles are covered with a total of 60 spikes, each spike consisting of three prM/E heterodimers (Figure 1 and 15). Also partially immature virions exist; these particles have mosaic patches of both smooth mature and spiky immature structures 16,17.

**Dengue virus infection & disease pathogenesis**

DENV infection is initiated through a bite of an infected mosquito. During feeding, the mosquito injects virus-containing saliva into the bloodstream with spillover into the epidermis and dermis. In the skin, keratinocytes and Langerhans cells are primarily infected. Infected Langerhans cells subsequently migrate to the lymph node and recruit monocytes and macrophages. These cells that are important target cells for DENV replication and dissemination 20,21. Next to the above mentioned cell types, DENV can also infect hepatocytes and cells residing in the bone marrow 20,22–24. Additionally, leukocytes were found to be targeted in experimentally infected nonhuman primate models 25. Finally, DENV can infect endothelial cells *in vitro*, however whether DENV also infects endothelial cells *in vivo* is still under debate 26–28.

DENV infections can lead to a wide spectrum of clinical manifestations. Disease becomes apparent 3-14 days after infection 1. Primary infections are often associated with relatively mild dengue fever. Upon recovery, lifelong immunity against re-infections with a similar DENV serotype is obtained. Cross-protection against heterotypic re-infections is also elicited, yet this cross-protection is transient 29. In fact, heterologous secondary DENV infections are correlated with severe disease 20,29–31. Tertiary and quaternary infections do not often trigger severe disease. Actually, a reduced risk of illness is observed in these patients 32,33.

The increased incidence of severe disease during secondary infection can be explained by the phenomenon called ‘original antigenic sin’. During a secondary DENV infection memory B- and T-cells are rapidly activated, which lead to a swift and elevated B- and T-cell response. However, this antibody and T-cell response is more directed to the primary infecting DENV serotype, the ‘original antigen’, than to the currently infecting DENV serotype. As a result, high numbers of cross-reactive antibodies and low-affinity T-cells, which do not efficiently clear the virus, are circulating 34–38. The aberrant immune response leads to high levels of proinflammatory cytokines. Proinflammatory cytokines can affect the permeability of the vascular endothelium and can consequently cause plasma leakage. The exact mechanism that leads to plasma leakage during severe dengue is poorly understood. Vascular leakage seems to be related to cytokines like TNFα, IL-6, IL-8, IL-10, IL-12, macrophage migration inhibitory factors, HMGB1, MCP-1 and matrix metalloproteases, rather than to infection of endothelial cells lining the blood vessels 34,39–41. As platelets closely interact with endothelial cells, also platelet
dysfunction might aid in the increased capillary fragility\textsuperscript{20,42}. Interestingly, Beatty \textit{et al.} recently described that DENV-NS1 triggers endothelial barrier dysfunction \textit{in vitro}. Furthermore, addition of DENV-NS1 could induce both vascular leakage and the induction of important inflammatory cytokines in mice\textsuperscript{43}.

Halstead \textit{et al.} observed that severe dengue peaked in two distinct populations of young children. The first peak was observed in infants of 6 to 9 months old, who were born to DENV-immune mothers. The second peak was seen in children who previously experienced an episode of DENV and were re-infected with another serotype. The fact that passive transfer of antibodies from mother to child could enhance dengue disease led to the hypothesis that antibodies exacerbate DENV infection, a phenomenon known as antibody-dependent enhancement (ADE) of infection\textsuperscript{44}. Multiple studies show that ADE can indeed occur. For example, \textit{in vitro} DENV infection can be enhanced when serum of patients with a secondary DENV infection is added\textsuperscript{45}. Moreover, DENV infection in the presence of anti-DENV antibodies can elicit lethal ADE in mouse models\textsuperscript{46}. These and other observations led to the general consensus that ADE is an important contributor to severe dengue disease.

During re-infection, antibodies bind to the virus and direct the virus towards cells that express the Fc-receptor (FcR). These FcR-bearing cells include among others monocytes, macrophages and dendritic cells, the natural target cells of DENV. Whether an antibody neutralizes or enhances infection depends on multiple factors. Based on studies on WNV, Pierson \textit{et al.} proposed that antibody-mediated neutralization of flaviviruses can be described by a ‘multiple hit’ model\textsuperscript{47}. In this model, a certain number of available epitopes must be occupied by antibodies to evoke neutralization. Both antibody affinity and the accessibility of the epitope contribute to the neutralization potency of an antibody. Consequently, the exact number of bound epitopes required for neutralization differs per antibody. When the number of bound antibodies does not exceed the neutralization threshold, ADE might be evoked. At sub-neutralizing concentrations, anti-DENV antibodies still target the virus towards FcRs and trigger internalization\textsuperscript{48–51}. Yet, once inside the cell the antibodies fail to neutralize the virus. It is speculated that upon internalization the acidic endosomal pH cause low affinity antibodies to dissociate from their epitopes, after which the virus is ‘free’ to fuse\textsuperscript{52}. High affinity antibodies, however, likely do not dissociate from their epitope at low pH and therefore only at low occupancy membrane fusion can be observed. To date, all anti-E antibodies tested elicit ADE when the antibody concentrations fall below the threshold for neutralization\textsuperscript{53,54}. Besides efficiently directing DENV towards its target cells, antibody-mediated DENV cell entry is believed to suppress the antiviral immune response, thereby promoting viral replication and virus particle production. This phenomenon is called ‘intrinsic’ ADE\textsuperscript{55}. To date, the mechanism behind ‘intrinsic’ ADE has not been elucidated. Hence, antibodies direct DENV particles to target cells and antibody-mediated cell entry of DENV increases virus particle production per infected
cell. Indeed, individuals that develop severe disease often have a high viral load early in infection\textsuperscript{56–58}.

The E glycoprotein is the major antigenic structure of DENV. Neutralizing antibodies were found to recognize all three domains of E\textsuperscript{59}. Whereas in mouse the antibodies against surface exposed epitopes in DIII appear to be the most potent in neutralizing infection, for humans antibodies that bind quaternary epitopes on the complete virion are the most potent. Binding to quaternary epitopes likely cross-links multiple E-proteins and thereby blocks the conformational changes needed for fusion\textsuperscript{59–63}. In human serum potently neutralizing anti-DENV antibodies are rare\textsuperscript{59,64}, which indicates that only a small fraction of the complete antibody arsenal is responsible for virus neutralization. Most human DENV-specific antibodies are cross-reactive and have a low epitope-affinity\textsuperscript{35} and thus potentially evoke ADE. In addition, a high proportion of human anti-DENV antibodies target prM and thus (partially) immature DENV. Most anti-prM antibodies belong to the category of cross-reactive, but poorly neutralizing antibodies\textsuperscript{35,49,64,65}. As a consequence also these antibodies readily promote ADE both \textit{in vivo} and \textit{in vitro}. Importantly, anti-prM antibodies render completely non-infectious immature DENV particles infectious in cells expressing the FcR\textsuperscript{48}. Therefore, these particles are considered important contributors to severe disease.

Notably, only 2\% of the individuals experiencing a heterologous DENV infection develops severe disease\textsuperscript{20}. This observation indicates that ‘original antigenic sin’ of both B- and T-cells and ADE are not the sole determining factors for severe disease. Also, the genetic background of both the virus and the host influence the severity of infection. It is reported that some DENV strains are more pathogenic than others; whereas Asian DENV-2 genotypes are often linked to severe disease, American DENV-2 genotypes elicit milder outcomes\textsuperscript{66}. Furthermore, the sequence of infection also influences the outcome of secondary infections. A secondary infection with DENV-2 is 5 to 7 times more often associated with severe dengue than secondary infections with either DENV-1 and DENV-3\textsuperscript{31}. Host factors which influence the incidence of severe disease include among others HLA-type, gene variations of certain host proteins (FcγRII, the Vitamin D receptor, TNFα, TGFβ and CTLA-4) and activation of the complement system\textsuperscript{20,29,31}. To conclude, the development of severe dengue is multifactorial and still relatively poorly defined.

**Virus life cycle**

**Virus replication & assembly**

DENV infection is initiated when the viral genome is released into the cell cytosol. After release, the viral genome is transported to the endoplasmatic reticulum (ER) where translation of viral RNA is initiated. The DENV genome acts as a single mRNA encoding seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5)
and three structural proteins (C, prM and E), which are cleaved into single proteins by autoproteases. The ectodomains of prM, E, NS1 and parts of NS4A and NS4B are oriented to the inside of the ER lumen, whereas the other proteins are oriented towards the cytosolic side of the ER. The seven non-structural proteins act together to initiate RNA replication. Replication occurs inside membrane invaginations of the ER, whose formation is likely triggered by DENV-NS4A. The membrane invaginations have an open connection to the cytosol, presumably to allow exit of newly formed viral RNA. Virus assembly occurs close to the site of replication. The positively charged region of the cytoplasmic C interacts with the viral RNA. Subsequently, an amorphous nucleocapsid buds into the ER lumen, thereby acquiring a lipid envelope containing prM and E. The newly assembled virions are immature and have a ‘spiky’ appearance.

Immature DENV particles mature by passing through the Golgi and Trans Golgi Network (TGN). Trafficking occurs in an Arf4-, Arf5- and possibly microtubule-dependent manner. The mildly acidic pH in the Golgi leads to a global conformational change of the viral proteins. The E/prM heterodimers dissociate and 90 E-homodimers are formed. These 90 homodimers lie flat on the surface of the virus particle, while prM is still attached to the fusion loop of DENV-E. Due to the conformational change, the prM cleavage site becomes exposed. This cleavage site is a substrate of the cellular serine protease furin, which cuts prM into a pr-peptide and M. During exocytosis the pr-peptide stays attached to E and prevents premature fusion events. Upon release into the pH-neutral extracellular milieu the pr-peptide dissociates from the virus. A relatively smooth spherical mature virus particle, which is fully infectious, remains.

Furin processing during viral egress is rather inefficient. Next to mature virions, DENV infected cells secrete a high percentage partially mature and immature particles. Although the actual percentage differs per cell type, the percentage of shed immature virions can reach up to 45%\(^6\). Additionally, the majority of virions released from DENV-infected cells can be immunoprecipitated with antibodies against prM, indicating that most viral particles have some degree of immaturity\(^6,74\). Upon egress, uncleaved prM reorganizes back to prM/E heterodimers, leading to a spiky instead of smooth viral surface\(^75\). Fully immature DENV particles are essentially non-infectious\(^48,75\), indicating that prM cleavage is important for DENV infectivity. Partially immature virions can still be infectious\(^76\), yet the required threshold for prM cleavage in DENV infectivity is unknown.

**DENV cell binding, entry and fusion during primary DENV infection**

**Cell binding**

DENV recognizes its target cells by binding of the E glycoprotein to a receptor molecule\(^8\). In the field of virus entry, a difference is made between attachment factors and *bona fide* virus receptors. Binding to attachment factors is rather non-specific and serves to concentrate the virus at the cell membrane, whereas interaction with a virus receptor is
Figure 2. DENV life cycle. DENV enters the cell via receptor-mediated endocytosis. Upon entry, the virus is delivered to early endosomes and transported towards the perinuclear region. At low pH, DENV fuses from within the late endosomal compartment, thereby releasing the viral nucleocapsid. After uncoating, the genome is transported to the ER where replication and protein translation take place. Virus assembly starts by the budding of a newly formed nucleocapsid into the ER lumen, thereby acquiring E and prM. The virus is transported through the Golgi and TGN towards the cell surface. In the TGN, the host protease furin cleaves the prM protein. The pr-peptide remains associated with the virus particle until its release into the extracellular environment. Reprinted from 18 with permission from Elsevier.

specific and required for entry77. To date, no bona fide DENV receptor has been identified. Combined with the notion that DENV infects several tissues in both mosquitoes and humans, it can be hypothesized that attachment to the target cell is sufficient for DENV infection. In this section, I will describe the different attachment factors that DENV uses to bind its target cells.

Glycosaminoglycans
Glycosaminoglycans (GAGs) are expressed on nearly all animal cell types78 and serve as important attachment factors for DENV. Indeed, pre-incubation of DENV with heparin
or highly sulfated heparin sulfate, which bind to GAG-binding sites on DENV, prevent DENV binding to cell surface GAGs. Furthermore, pretreatment of the cells with heparin, heparin sulfate or heparinase III impaired DENV infection. Interaction of DENV with GAGs is thought to occur via two lysines (K291 and K295) located in E-DIII. Indeed, heparin treatment completely abolished binding of DENV-E-DIII to BHK21 cells. In correspondence with these results, DENV-E-DIII was found to bind only weakly to mutant cells lacking heparin sulfate.

**Glycosphingolipids**

In both mammalian and mosquito cells the carbohydrate moieties of neutral-charged glycosphingolipids are suggested to be attachment factors for DENV. Glycosphingolipids are found in all vertebrate and arthropod tissues, with sub-families expressed in a tissue-dependent manner. In the mammalian cells lines K562 and LLC-MK2, human lymphoblast and rhesus macaque endothelial kidney cell lines respectively, DENV-2 recognized nLc4Cer (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Cer). In the mosquito cell line AP-61, DENV-2 was found to bind the glycosphingolipid L-3 (GlcNAcβ1-3Manβ1-4Glcβ1-1 Cer).

**C-type lectins: DC-SIGN and L-SIGN**

In dendritic cells, a positive correlation between DENV infection and the expression of the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) was found. Next to DCs, DC-SIGN is expressed in macrophage subpopulations in the dermis, mucosal surfaces, lymph nodes and peripheral tissues. Similar to DC-SIGN, the expression level of L-SIGN, a DC-SIGN homologue, correlated with DENV infection. L-SIGN is expressed on liver endothelial cells, in the lymph nodes, gastrointestinal tract and the placenta.

Antibodies against DC-SIGN can block DENV infection. Moreover, expression of DC-SIGN in cells that are naturally negative for this molecule, made these cells susceptible to DENV infection. Furthermore, polymorphisms in the promoter of DC-SIGN is associated with an increased risk of severe disease, further supporting the role of DC-SIGN in DENV infection. Binding of DENV to DC-SIGN occurs via the interaction of the DC-SIGN carbohydrate recognition domain and sugar groups on E-N67 or E-N153. Interestingly, immature DENV particles can also bind DC-SIGN. This interaction might either be facilitated by a sugar group on prM-N69 or by sugar groups on E-N67 and E-N153. As glycosylation patterns differ between mammalian and insect cells and can also differ between cell types, virus origin may impact the ability of virions to infect cells via DC-SIGN. Indeed, Dejnirattisai et al. described that DENV virions produced on DCs lack mannose-rich glycosylation and thus lose their affinity to DC-SIGN and DCs in general.
C-type Lectins: Mannose receptor

In macrophages, the mannose receptor (MR) was identified as a DENV attachment factor\textsuperscript{100}. Next to expression in macrophages, the MR is expressed in selected populations of dendritic cells and nonvascular endothelium\textsuperscript{101}. Comparable to DC-SIGN, anti-MR antibodies were able to block infection, whereas overexpression of the MR led to increased binding. The carbohydrate recognition domain of MR recognized all DENV serotypes and purified DENV-E, but does not bind deglycosylated DENV-E\textsuperscript{100}. These results implicate that the N-linked glycans on E-N67 and E-N153 likely recognize the MR.

CD14, Heat Shock protein 70 and Heat shock Protein 90

CD14, a LPS-receptor, was implicated in DENV cell binding of monocytes and macrophages\textsuperscript{102}. Although CD14 is best known for its expression on monocytes and macrophages, low levels are also expressed in different endothelial and epithelial cells\textsuperscript{103}. Addition of LPS prior to DENV infection inhibited DENV infection and this inhibition was independent of the inflammatory response induced by LPS. Although antibodies against CD14 could not directly block DENV infection, binding of LPS to CD14 was shown to be critical for the LPS-induced reduction of DENV infection. Consequently, it was proposed that DENV does not directly bind CD14, but requires a CD14-associated cell surface molecule for infection\textsuperscript{102}.

The observations of Chen et al. may be explained by the finding that heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90), two ubiquitously expressed LPS receptors, are DENV ligands\textsuperscript{104}. Although under normal circumstances HSP70 and HSP90 were described as CD14-independent LPS cell surface receptors\textsuperscript{105}, they were found to cluster around CD14 when incubated with LPS prior to DENV infection. The clustering of the heat shock proteins and CD14 possibly prevents binding of DENV to HSP70 and HSP90\textsuperscript{104}.

GRP78/BiP

In HepG2 cells, a hepatocellular carcinoma cell line, GRP78 was found to bind DENV-2. GRP78 is chaperone protein, which normally resides in the ER, but can also be localized to the plasma membrane. Antibodies against GRP78 affected DENV binding and infection in HepG2 cells, indicating GRP78 is a DENV attachment factor\textsuperscript{106–108}. Yet, GRP78 does not seem to mediate binding of all DENV serotypes to HepG2 cells, as DENV-1 was found to bind the high affinity laminin receptor in this cell line\textsuperscript{109}.

αvβ3 integrins

In HMEC-1, human dermal microvascular endothelial cells, αvβ3 integrins were found to mediate DENV infection. Integrins are ubiquitously expressed in adult tissues, yet αvβ3 integrins are most prominently expressed on vascular endothelial cells and in pathological tissues\textsuperscript{110}. Pre-incubation of DENV-2 with soluble αvβ3 integrins strongly
inhibited infection. Moreover, siRNA-mediated knockdown of αvβ3 integrins reduced entry by 90%. Interestingly, DENV infection can substantially enhance the expression levels of αvβ3 integrins\textsuperscript{111}.

**PhosphatidylSerine receptors: TIM and TAM families and CD300a**

The TIM and TAM receptor families – which are mainly expressed on cells of the immune system, but are also expressed in the reproductive, hematopoietic, vascular, and nervous systems\textsuperscript{112,113} – have been found to enhance binding and entry of many different viruses. TIM and TAM receptors normally mediate the phagocytic removal of apoptotic cell by binding to phosphatidylserine (PS). It is suggested that these receptors also recognize PS present in the viral envelope and can thereby concentrate virus at the cell surface. Indeed, poorly susceptible cells can be successfully infected with DENV when the TIM and TAM receptors are expressed on the plasma membrane of these cells. Furthermore, DENV entry is inhibited by antibodies against TIM and TAM and RNA silencing of these respective proteins\textsuperscript{114}.

The TIM and TAM families are, however, not the only PS-receptor which can bind DENV and initiate viral entry. It was recently published that human CD300a, a novel phospholipid receptor which is expressed on myeloid cells, can also facilitate entry of all four DENV serotypes in a PS-dependent manner\textsuperscript{115}.

**Claudin-1**

Although E-DIII is considered the main receptor binding domain of DENV, binding of prM to claudin-1, a component of the tight-junctions between epithelial cells or endothelial cells\textsuperscript{116,117}, can also initiate DENV entry. In Huh cells, a human hepatocarcinoma cell line, knockdown of claudin-1 significantly attenuated DENV production. DENV-prM was found to bind the extracellular loop of claudin-1. Moreover, the specific point mutations I32M, C54A and C64A in the claudin-1 extracellular loop abolished the interactions between prM and claudin-1. Interestingly, claudin-1 expression was found to be upregulated in the early phase of DENV infection, whereas it was down-regulated at the later stages of infection. Claudin-1 down-regulation appeared to be mediated by the DENV C protein\textsuperscript{118,119}.

**Other (possible) DENV attachment factors**

To date, several other DENV attachment factors of were identified without further characterization. These proteins include a 65 kDa membrane protein in the human neuroblastoma cell line SK-NSH which bound to DENV-2\textsuperscript{120}; a 74 kDa and a 44 kDa protein in the Green Monkey Kidney cell line Vero which interacted with DENV-4\textsuperscript{121}; 29, 34 and 43 kDa proteins from the human umbilical vein endothelial cell line ECV304 with bound to DENV-2\textsuperscript{122}; and diverse proteins from HepG2 membrane preparations which bound all four DENV serotypes\textsuperscript{123}.
Furthermore, in mosquito cells, prohibitin, HSP90, a high affinity laminin receptor and 67 kDa protein, have among others been identified as DENV attachment factors\textsuperscript{124}. Of these proteins, prohibitin is of special interest as this molecule has also been identified as a chikungunya virus (CHIKV) attachment factor in mammalian cells\textsuperscript{125}.

**DENV cell entry and fusion**

In most cell types studied DENV enters via endocytosis, yet one group reported that DENV directly penetrates the plasma membrane\textsuperscript{126}. The main cell entry route of DENV in HeLa\textsuperscript{127,128}, A549\textsuperscript{129}, Huh7\textsuperscript{130}, HepG2\textsuperscript{24,131}, BS-C-1\textsuperscript{128} and C6/36\textsuperscript{132–134} was found to be clathrin-mediated endocytosis (CME). Van der Schaar \textit{et al.} visualized the DENV-2 entry process in BS-C-1 cells, a Green Monkey Kidney epithelial cell line\textsuperscript{128}. In these cells, DENV particles diffuse along the plasma membrane towards pre-existing clathrin-coated pits (CCP). Next, DENV colocalizes with clathrin for approximately 80 seconds. During this time the DENV particle is likely trapped into the CCP, after which the pit evolves and scissions from the plasma membrane in a dynamin-dependent manner. After scission from the plasma membrane, the clathrin-coated vesicle is transported inside the cell after which the vesicle loses its clathrin coat and delivers the virus to the early endosome. DENV internalization was found to be a rapid process; a large proportion of virus particles was observed in early endosomes within 5 minutes post-infection\textsuperscript{128}. Subsequently, the virus-containing early endosome matures into a late endosome. In BS-C-1 cells, DENV primarily fused from within Rab7-positive late endosomes after residing approximately 5 minutes in this endosomal compartment. On average, fusion occurred 10-13 minutes post infection\textsuperscript{135}. During DENV endocytosis, the virus is transported towards the perinuclear region in a microtubule-dependent manner. Microtubule-dependent transport was found to be important but not essential for infection, as disruption of microtubules inhibited DENV infectivity by 30%\textsuperscript{135}.

Although DENV-2 entry in BS-C-1 cells was predominantly mediated by clathrin, serotype- and cell type-specific differences were reported. For example, DENV-2 was found to enter Vero cells via a clathrin-, caveolin- and lipid raft-independent endocytic pathway, whereas DENV-1 was found to enter Vero cells via CME\textsuperscript{129}. Moreover, in HepG2 cells, DENV was described to hijack both CME and macropinocytosis for entry\textsuperscript{24}. In addition to the serotype and cell type, also the cell line on which the virus was produced has been reported to influence the route of DENV cell entry. Mosquito-cell C6/36 derived DENV entered Vero cells via clathrin-independent endocytosis\textsuperscript{129}, whereas Vero-derived DENV followed CME for entry\textsuperscript{136}. This finding is probably a consequence of a change in receptor binding, as DENV propagated on Vero was found to have a reduced affinity towards heparin sulfate\textsuperscript{136}.

The majority of studies report DENV fusion from within Rab7-positive late endosomes\textsuperscript{128,130,134,137}, yet also for endocytic trafficking strain-specific differences have been reported. For example, DENV-2 NGC only requires functional Rab5 for infection\textsuperscript{127}, which is suggestive for fusion from within early endosomes. DENV-2 PR159 S1 on the other
hand requires both functional Rab5 and Rab7 for infection\textsuperscript{128}. Additionally, DENV-2 strain 16681 requires functional Rab7 for infection of Vero cells, suggestive for fusion from within late endosomes. Yet, in the same cell line DENV-2 NGC does not require functional Rab7, but is affected by a DMN of Rab22\textsuperscript{137}. Based on the dependence on Rab22, DENV-2 NGC was argued to fuse from perinuclear recycling endosomes in this cell type\textsuperscript{137}. However, the evidence for fusion from within recycling endosomes is rather frail, as Rab22 primarily aids trafficking to the TGN instead of trafficking to perinuclear recycling endosomes\textsuperscript{138}. To our knowledge, direct evidence for fusion from within recycling endosomes has not been reported to date. To summarize, in our view the strongest evidence points towards DENV fusion from within Rab7-positive late endosomes. This view is further supported by the notion that negatively charged lipids, which are specifically enriched in late endosomes, were found important in DENV membrane fusion\textsuperscript{139}.

**DENV cell binding, entry and fusion during secondary infection**

*Cell binding*

Upon secondary infection, antibodies bind to the virus and direct the immunocomplex to immune cells expressing FcγRs. Three classes of FcγRs exist: FcγRI, II and III. Of these classes, FcγRII and III consist of multiple subtypes. The FcγRI, IIA, IIB and IIIA are expressed on myeloid cells and are therefore important for either neutralization or ADE of DENV particles\textsuperscript{140}. Furthermore, during secondary DENV infection, individuals usually have high IgG antibody titers, which consist mainly of IgG1 and IgG3\textsuperscript{59}. IgG1 efficiently binds to all FcγR subtypes, whereas IgG3 mainly interacts with FcγRIIA and IIB\textsuperscript{140}.

The role of the different FcRs in neutralization and enhancement of DENV infection has been poorly investigated. FcγRIIB is likely involved in DENV neutralization. At high antibody concentrations, DENV-antibody-complexes aggregated and this was observed to crosslink FcγRIIB, thereby preventing FcR-mediated uptake of virus particles\textsuperscript{141}. Additionally, Chawla \textit{et al.} showed that FcγRI and FcγRIIA neutralize DENV infectivity\textsuperscript{142}. Yet, using the identical monoclonal IgG1 antibody, FcγRIIA-mediated neutralization of DENV infection required significantly higher antibody concentrations than FcγRI-mediated DENV neutralization\textsuperscript{142}. In line with this, the antibody-virus-complex preferentially bound FcγRI over FcγRIIA\textsuperscript{142}. Both FcγRI and FcγRIIA were also described to facilitate ADE\textsuperscript{50,142,143}, whereas the role of FcγRIIA in DENV-ADE seems to be limited\textsuperscript{144}. At enhancing antibody concentrations, DENV entry mediated through interactions with FcγRIIA led to the highest levels of produced virus progeny\textsuperscript{50,142}. Moreover, Rodenhuis-Zybert \textit{et al.} showed that under ADE conditions immature DENV bound to K562 cells via interactions with FcγRI\textsuperscript{48}. Taken together, these data indicate that although FcγRI and FcγRIIA can both induce neutralization and enhancement of DENV infection, FcγRIIA is primarily disposed to ADE of DENV infection, while FcγRI is more prone to DENV neutralization. In addition, the phagocytosis-inhibiting Fcγ-receptor FcγRIIB primarily mediates DENV neutralization.
**Endocytosis and fusion**

All virus-antibody-complexes are targeted to FcR-expressing cells for degradation. Under neutralization conditions, antibodies likely prevent the virus from undergoing the conformational changes that are required for fusion. Indeed, several antibodies have been identified to “freeze” the particle in a certain conformation\(^61,62\). Alternatively, high concentrations of antibody may completely prevent DENV uptake into FcR-bearing cells by evoking aggregation of the DENV-antibody-complexes\(^141\). ADE of DENV infection is only observed when the antibody titer falls below the threshold required for neutralization. Low affinity antibodies likely dissociate from the particle upon delivery to acidic organelles thereby allowing membrane fusion and infection.

The cell entry pathway of DENV-antibody-complexes is poorly defined. As disruption of the FcR activation motifs was found to abolish DENV-ADE, DENV opsonized with enhancing concentrations of antibodies was suggested to be internalized via FcR-mediated phagocytosis\(^145\). Two additional studies described a relationship between phagocytic activity of the cell and infectivity of DENV-immune complexes\(^50,51\). Furthermore, antibody-bound WNV, a closely related flavivirus, was described to enter cells via CME and phagocytosis, depending on the size of the virus-antibody-complex\(^146\).

Antibodies were found to render immature virions infectious\(^48\). The infectivity of antibody-opsonized immature DENV was dependent on FcγRII, likely FcγRIIA, and intracellular furin\(^48\). To date, no detailed study on the cell entry mechanism of antibody-bound immature DENV has been performed, yet the following steps are expected to occur. Upon delivery of antibody-opsonized immature DENV to acidic endosomes, the virus undergoes a global conformational change allowing the host protease furin to cleave prM. Then, the pr-peptide dissociates from M and membrane fusion is triggered. The release of the pr-peptide is likely triggered by the more acidic pH inside endosomes compared to that of the pH in the secretory pathway\(^18,147\). Antibodies attached to the virion may dissociate from the virion once delivered to the acidic endosome or are released together with the pr-peptide upon low-pH induced pr-peptide dissociation.

**Molecular mechanisms involved in membrane fusion**

Membrane fusion is triggered by the acidic pH inside endosomes. At low pH, a series of conformational changes occur within the DENV surface proteins that lead to fusion of the viral membrane with the endosomal membrane and subsequent nucleocapsid release\(^8,75,148,149\). First, protonation of pH-sensing histidine residues in E\(^148,149\) and M\(^150\) promote the dissociation of the E homodimers at the viral surface. This leads to the outward projection of E-DII and consequently exposure of the fusion loop. Next, the hydrophobic fusion loop is inserted into the target membrane. Then, the three E monomers are thought to interact to form a trimer, which is stabilized through interactions between the DI domains. Next, DII is believed to fold back against the trimer, forming a hairpin-like conformation. The energy released by this step induces hemifusion. Thereafter, a fusion pore is formed and the viral nucleocapsid is released.
into the cytosol. Nour et al. showed that hemifusion occurs several minutes before the delivery of the viral genome to the cell cytosol, implying that genome delivery is a distinct step. Noteworthy, the group of Freire et al. recently showed that the supercharged C protein has the ability to transport nucleic acids across lipid membranes. Consequently, these authors speculated that C might play a role in DENV RNA delivery.

Next to low pH, the composition of the target membrane is important for DENV fusion. Cholesterol was found to promote membrane fusion activity of flaviviruses. At low pH, cholesterol appears to stimulate the interaction of the E protein and the target membrane. Yet, E does not directly interact with cholesterol. Therefore, these effects are likely due to a change of fluidity or the physical or chemical properties of the target membrane. Indeed, in mammalian cells depletion of cholesterol is associated with a decrease in membrane curvature, and this membrane curvature might be important during the DENV fusion process. In addition, anionic lipids promote DENV fusion, but likely downstream of hemifusion. It has been observed that DENV can fuse with the plasma membrane of insect cells upon and artificial pH drop, whereas no fusion is seen with the plasma membrane of mammalian cells under similar experimental conditions. One major difference between the plasma membrane of insect and mammalian cells is the high concentration of anionic lipids, such as bis(monoacylglycero)phosphate (BMP) and PS, in the plasma membrane of insect cells. Addition of these lipids to the plasma membrane of mammalian cells facilitated the low pH-induced fusion of DENV. Interestingly, anionic lipids are enriched in late endosomes of the endolysosomal pathway and might aid in efficient fusion from within these organelles.

**Concluding remarks**

In conclusion, dengue is a complex disease, of which the disease outcome depends on multiple, sometimes poorly defined, factors. The immune system and specifically the anti-DENV antibody response play a major role in the development of severe dengue. Therefore, a better understanding how antibodies control and enhance DENV infection is important.

At the start of this thesis the cell entry pathway and the subsequent intracellular trafficking of mature and immature DENV opsonized with enhancing concentrations of antibody were still elusive. Furthermore, the exact contributions of both ‘extrinsic’ (the increased binding and entry of FcR-bearing cells) and ‘intrinsic’ ADE to severe dengue were still unknown. Both topics are of importance to this thesis. Studying the DENV cell entry pathway and the intracellular antiviral response under ADE conditions will improve our understanding of DENV pathogenesis. Furthermore, it will also guide the rational development of a safe and universal DENV vaccine.
References


