Chapter 2

Dengue Virus Life Cycle and Pathogenesis
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Introduction

Dengue virus (DENV) – mainly transmitted by the Aedes aegypti mosquito – is the most common mosquito-borne viral infection worldwide. DENV causes an estimated 390 million infections per year, of which about 100 million are symptomatic [1]. Each of the four serotypes of DENV (designated DENV 1-4) can cause disease with symptoms including fever, rash, headache, and musculoskeletal pain [2]. Approximately 500,000 to 1 million individuals however develop more severe disease including abdominal pain, bleedings, organ impairment, hematological aberrations, and plasma leakage. Severe dengue can be fatal if not properly treated, and each year about 25,000 patients die from dengue infection [3,4].

Severe dengue is almost exclusively seen during secondary infection with a heterotypic DENV serotype and during primary infection of infants with declining levels of maternal antibodies [5-7]. This observation points towards a pathogenic role of the immune system in severe disease development. In contrast, upon homotypic re-infection, the newly infecting DENV is effectively neutralized and individuals do not develop disease [8]. Also, third and tertiary infections usually do not lead to disease symptoms [9]. In this chapter, the viral structure and life cycle of DENV will be described, followed by a review of the host cell tropism and the mechanisms controlling disease outcome.

Viral structure & life cycle

Dengue virus (DENV) belongs to the flavivirus genus within the Flaviviridae family. Other flaviviruses are for example West Nile virus (WNV), Yellow Fever virus (YFV), Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV), and the emerging Zika virus (ZIKV) [10,11]. DENV virions are small spherical particles with a diameter of approximately 50 nm. Each virion contains a single-stranded, positive-sensed RNA genome of 10.8kB in length, which is packaged by the capsid (C) protein to form the nucleocapsid. The nucleocapsid is surrounded by a host-cell derived lipid bilayer in which 180 copies of the membrane protein (M) and envelope protein (E) are anchored. The E ectodomain consists of three structural domains (DI, DII and DIII). The E proteins are arranged as homodimers in a head-to-tail orientation. The homodimers are lying tangential to the viral membrane in a herringbone-like pattern, thereby giving the particle a “smooth” surface (figure 1 a,b) [12-15].

The first step in the viral life cycle (figure 2) involves binding of the E glycoprotein to a receptor or attachment factor on the host target cell. The putative
receptor binding domain has been allocated to E DIII [16-19]. Upon binding, DENV particles enter the host cell via clathrin-mediated endocytosis. The internalized virions are delivered to Rab5-positive early endosomes, which further mature into Rab7-positive late endosomes [20,21]. Upon exposure of the virus to the acidic lumen of the endosome, the E protein homodimers dissociate, which results in the exposure of the fusion loop situated on DII (Figure 1, green). The hydrophobic fusion peptide subsequently inserts into the endosomal membrane and E homotrimeric are formed. Then, DIII folds back towards the fusion loop, which forces the opposing outer leaflets of the membranes together. The outer membranes subsequently merge, which is referred to as hemifusion. This short-lived intermediate is followed by complete fusion and expansion of the fusion pore. Finally, the nucleocapsid is released to the cytosol [12,22-24]. It has been suggested that fusion mainly occurs from within Rab7-positive late endosomes [20], although contrasting reports have been published considering the location of membrane fusion [25]. In addition to low pH, negatively charged lipids were shown to support membrane fusion of DENV. This observation strongly points towards fusion from within late endosomal compartments as negatively charged lipids are abundantly present within these compartments [26].

Upon the release of the nucleocapsid to the cytosol, the capsid proteins dissociate from the viral genome. The viral genome is translated at the rough endoplasmic reticulum (ER) into a single polyprotein and processed into 3 structural proteins (prM, E and C) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The ectodomains of prM and E, NS1 and parts of NS4A and NS4B are facing the ER lumen whereas the other proteins are present at the cytosolic side of the ER. NS4A, presumably together with other viral and host proteins, subsequently triggers membrane rearrangements and vesicle formation by ER membrane

Figure 1 DENV envelope proteins on the mature virion
The E protein consists of three functional domains (DI, DII & DIII). Receptor-binding is presumably mediated by DIII (blue). The fusion loop (green) is located at the tip of DII (yellow). DI is depicted in red. On the mature virions, E homodimers lie flat on the membrane in a head-to-tail orientation, giving the particle a smooth appearance. Adapted from [39] and reprinted with permission from Elsevier.
invagination. These vesicles were found to contain NS proteins, viral RNA, and host cell factors and serve as sites for viral RNA replication. Newly synthesized viral RNA is released to the cytosol and associates with C proteins to form the nucleocapsid. The nucleocapsid subsequently buds into the ER lumen, thereby acquiring a lipid membrane in which prM and E proteins are anchored. The newly assembled virus particles have a “spiky” appearance with prM and E heterodimers arranged as 60 trimeric spikes on the viral surface [27-29] (figure 3).

The virions are then transported via the Golgi and Trans Golgi Network (TGN) to the plasma membrane of the cell. During exocytosis the virus particles further mature. The acidic lumen of the TGN triggers a global conformational re-arrangement of the viral spike proteins leading to dissociation of the prM-E heterodimers and formation of E homodimers which are capped by prM. This

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**Figure 2 DENV life cycle**

DENV enters the cell via receptor-mediated endocytosis followed by fusion from within acidic endosomes and release of the nucleocapsid. Viral replication takes place in endoplasmic reticulum (ER) membrane invaginations. Virus assembly occurs by budding of a newly formed nucleocapsid into the ER lumen. The newly assembled immature particles are transported through the Golgi and Trans Golgi Network (TGN) to the surface of the cell. Within the TGN, enzymatic cleavage of the prM protein occurs by the host protease furin. The pr peptide remains associated with the virion till its release to the extracellular milieu. Reprinted from [39] with permission from Elsevier.
rearrangement allows proteolytic cleavage of prM by the host protease furin, which separates the pr peptide from the M protein (figure 2). The pr peptide functions to stabilize the E protein while transiting acid compartments during virus egress. It stays associated with M at low pH, and is released after secretion of the particle in the pH-neutral extracellular space [30-33]. DENV-infected cells have been described to secrete virions with varying maturation degree [34]. Indeed, electron micrographs revealed the presence of virions with a spiky surface (immature), smooth surface (mature), and surfaces with both spiky and smooth characteristics in varying distributions (partially immature). At least 40% of all particles are partially immature and 3% is fully immature when produced in mosquito cells [35,36]. Comparable levels of prM were seen in viruses produced in mammalian endothelial and human embryonic kidney cells, whereas dendritic cells appear to produce more mature virus [37]. Fully immature DENV virions were found to be essentially non-infectious, as the pr peptide is capping the E protein thereby preventing virus-cell binding and membrane fusion [32,38]. It is not known to which degree prM cleavage must have taken place to enable viral infectivity, yet it is clear that not all prM proteins have to be cleaved [39].

**Figure 3 DENV envelope proteins on the immature virion**
DENV virions are assembled as immature virions (left side). On these particles, the fusion loop of the E protein is covered by the prM protein (purple). Color coding of EDI, DII, DIII is same as in figure 1. Three E-prM heterodimers form one spike on the immature particles, with the prM protein at the tip of the spike (right side). Adapted from [39] and reprinted with permission from Elsevier.

**Primary dengue virus infection**

Dengue is transmitted to humans via mosquito bites. The mosquito usually probes the skin several times before a blood vessel is reached. Most DENV particles are inoculated in the skin and a minor fraction of particles is directly delivered to the blood. DENV particles can infect cells resident in the skin, including keratinocytes, Langerhans cells and dendritic cells (DCs) [40-43]. Infected Langerhans cells and DCs migrate to the lymph nodes, where other DENV target cells are infected,
including monocytes (which can also be directly infected by virus injected in the blood vessels) and macrophages [40,44,45]. Moreover, monocytes are recruited to the dermis where they differentiate into DCs that can be infected as well [46]. The virus then spreads to other parts of the body and becomes a systemic infection [2]. Although cells derived from the mononuclear lineage are the main target cells of DENV, also a variety of other cell types is permissive to infection, like for example cells of hepatic and endothelial origin or fibroblasts [47,48]. Such a wide cell tropism suggests that DENV uses several receptors or attachment factors. Indeed, molecules that were described to interact with DENV are (amongst others) heat shock protein 70, heat shock protein 90, GRP78/BiP, a 37/67 kDa high-affinity laminin receptor, heparin sulphate, CD14, and C-type lectin receptors [45,49-58]. C-type lectin receptors are expressed on cells of the myeloid lineage. For example, Dendritic Cell Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN/CD209) is abundantly expressed on immature dendritic cells (imDC) and on certain types of macrophages [59]; the mannose receptor is mainly expressed on imDCs and macrophages, but also on dermal fibroblasts and keratinocytes [60]; and C-type lectin domain family 5, member A (CLEC5A/MDL-1) is expressed on monocytes and macrophages [58]. The infected cells produce large amounts of progeny virus, and approximately three days after the mosquito bite, viremia can be detected in the blood of infected patients. Peak titers are usually measured three days after the appearance of fever. Viral load can reach 10^6-10^8 viral copies/ml, though in patients with severe disease, higher titers are observed as well. DENV viremia is cleared on average 5 days after onset of fever [61,62].

Early in DENV infection the innate immune response is activated, which is important for restricting the infection and viral clearance [63,64]. The expression of IFNs and other pro-inflammatory cytokines is triggered by activation of pattern recognition receptors, including Toll-like receptor (TLR) 3, TLR 7 and TLR 8; but also the C-type lectins DC-SIGN, CLEC-5, and MR [58,65-68]. Subsequent binding of IFNs to infected and neighboring cells leads to activation of the JAK/STAT pathway and induces an antiviral state of the cell. Moreover, activation of natural killer (NK) cells leads to secretion of type-II IFNγ, which is likewise important in the antiviral defense [63,69]. DENV can however counteract the antiviral effects of IFNα/β binding by downregulating the JAK/STAT pathway [70]. Therefore, activation of the adaptive immune system is essential for complete clearance of infection.

IFN production not only induces an antiviral state, it also stimulates the adaptive immune system by inducing imDC maturation and activation of lymphocytes [71]. Approximately 6 days after infection, DENV specific B and T cells appear. The majority of antibodies against DENV are generated against E and prM, which suggests that the secreted virus particles indeed consist of a mixture of virions with varying maturation state [72-74]. Serotype-specific, strongly neutralizing antibodies are often targeting the E protein and can efficiently prevent infection [75]. NS1 antibodies are produced as the NS1 protein is expressed on the surface of infected
cells and secreted in the extracellular space [76,77]. NS1 antibodies were shown to mediate complement-mediated lysis of DENV infected cells [78,79]. Activated CD4\(^+\) and CD8\(^+\) T cells also induce lysis of DENV-infected target cells and produce a range of antiviral cytokines. Thus, infection is cleared through natural killer cells and macrophages, assisted by the presence of neutralizing antibodies and activated T cells [8,80].

**Re-infection: Neutralization versus enhancement of infection**

Upon re-infection with DENV, the adaptive immune response is rapidly activated and anti-DENV antibodies are secreted. In case of a homologous re-infection, the pre-existing and newly produced antibodies efficiently prevent infection and together with a rapid activation of memory T-cells, the individual is protected from disease [81]. If heterotypic re-infection occurs shortly after primary infection (estimations range from six months up three years), cross-reactive antibodies are present in high titers thereby protecting the individual from disease [82,83]. However, if re-infection occurs after a longer time-span, the pre-existing cross-reactive antibodies are no longer protective against heterologous infection and may even enhance the severity of disease. Indeed, severe disease development is strongly associated with a heterologous secondary infection [62,84]. In contrast, third and tertiary DENV infections only rarely lead to disease as cross-immunity is then sufficient to prevent infection [9].

**Role of antibodies in homologous re-infection**

During homologous re-infection, antibodies play an important role in neutralization. NS1 antibodies have been described to induce complement-dependent lysis of infected cells [76] and lysis of antibody-opsonized viruses [85]. Antibodies directed against the viral spike proteins can directly neutralize viral infectivity. The dogma is that antibody-bound virions are taken up by Fc-receptor expressing cells and are delivered to the endocytic pathway for degradation. Importantly, Fc-receptors are expressed on myeloid cells, which are the main natural target cells of DENV. Therefore, in case of dengue, neutralization has to occur at a post-attachment step [86,87]. Indeed, multiple high-affinity antibodies were identified that neutralized infection by prevention of membrane fusion [88-90]. Most neutralizing antibodies were found to target the E protein. In murine studies, antibodies binding to the DIII domain were most potent, whereas in humans, antibodies targeting quaternary structures and cross-linking multiple E proteins have been described as strongly neutralizing. These antibodies likely act by preventing the essential conformational changes required for membrane fusion [37,91-95]. Neutralization of DENV can be described as a “multiple hit” phenomenon, in which a certain amount of available epitopes must have bound to prevent infection. For strongly neutralizing antibodies, only a fraction of epitopes has
to be covered to efficiently prevent infection, whereas weakly neutralizing antibodies have to bind more available sites to mediate neutralization [96]. Thus, in homotypic re-infection, type-specific antibodies displaying high affinity to the virus are present in high titers and prevent infection.

**Enhanced disease during heterologous secondary infection**

Approximately 2.5% of the individuals experiencing a secondary heterologous re-infection develop severe disease [2,3]. It is generally believed that antibodies play an important role in the development of severe disease as infants with declining levels of maternal antibodies were found to have an increased risk for severe disease during primary infection [7,84,97]. During a secondary heterologous re-infection, pre-existing cross-reactive antibodies can recognize newly infecting virus serotype. Functional studies revealed that cross-reactive antibodies generally have weakly neutralizing properties [72,73,98,99]. The presence of weakly-neutralizing cross-reactive antibodies is further increased through original antigenic sin, a phenomenon in which cross-reactive memory B-cells are expanded, but the number of immune cells against the currently remaining serotype remains low [8,73,98,100].

The cross-reactive antibodies bind to the virions, which are then internalized by Fc-receptor bearing DENV target cells. Intriguingly, these particles escape from degradation and fuse from within acidic endosomes [87]. This phenomenon is called antibody-dependent enhancement of infection (ADE). *In vitro*, all antibodies that can neutralize flavivirus infectivity also show enhancement of infection when antibody concentrations are low enough. Important factors in ADE are: antibody affinity, epitope accessibility, and antibody concentration. High affinity antibodies that bind to accessible epitopes are more prone to neutralization than low affinity antibodies that bind to cryptic sites. ADE occurs when the occupancy of viral particle by a neutralizing antibody is below the threshold of neutralization [39,74,96,101].

At ADE conditions, antibody-opsonized virions not only escape from degradation, these particles also have a higher chance to induce membrane fusion and productive infection [102]. This might be related to the alternative entry route by which antibody-opsonized DENV is taken up into the cell [103]. In addition, we and others showed that virtually non-infectious fully immature particles are rendered infectious by antibodies [104-106]. Upon entry of antibody-opsonized immature particles in Fc-receptor bearing target cells, furin cleavage and maturation occurs within the endosome, followed by infection of the cell [105,107]. ADE of infection of both mature and immature DENV particles leads to an enhanced infected cell mass and viral load early in infection. Epidemiological studies showed that an increased viral load is associated with a higher risk of developing severe dengue [61].

Furthermore, not only memory B cells but also memory T cells specific for the primary infecting serotype are preferentially expanded. Original antigenic sin of T cells leads to the generation of low avidity T cells that are less efficient in clearing
the infection. This results in the secretion of a vast amount of pro-inflammatory cytokine and modulators which can cause capillary leakage as seen in severe dengue [2,8,73,98,108,109]. Though these and other aspects like virulence of the infecting strain and host genetic factors are clearly associated with severe disease, the complete picture is not understood given the small percentage of individuals with a heterologous secondary infection that develop severe disease [2,3].

**Concluding remarks**

DENV has a tremendous clinical impact, yet no antiviral treatment or vaccine has been approved. The development of an efficient and – most importantly – safe vaccine is challenging, as it is of upmost importance to prevent vaccine enhanced disease through ADE and original antigenic sin [110]. Therefore, an optimal vaccine has to neutralize all four DENV serotypes and elicit a long-term antibody response. The most promising candidate vaccine so far is from Sanofi Pasteur. Their tetravalent chimeric DENV/Yellow Fever (YF) vaccine (prM and E proteins of DENV and the replicative backbone of the YF vaccine 17D) reduced hospitalization by 67%-80% and dengue hemorrhagic fever by 80%-90% [111,112].

The efficacy to protect against natural DENV infection was surprisingly low (30%-60%, depending on the study population) [111-114]. The low protection against disease was rather unexpected, as antibody titers towards all DENV serotypes were found using the in vitro plaque reduction neutralization test (PRNT) [114,115].

The “golden standard” PRNT involves cells of endothelial origin [116]. Infection of these cells by DENV can be prevented by neutralizing antibodies. However, as endothelial cells do not express Fc-receptors, the enhancing properties of antibodies by Fc-receptor mediated entry cannot be assessed. Therefore, neutralization in DENV target cells might require higher titers than the ones found in standard PRNTs. Indeed, several groups have shown that Fc-receptor bearing target cells often require higher antibody titers for neutralization, especially when cross-reactive antibodies are tested [117-119]. For this, assays employing Fc-receptor bearing cells might represent a better predicting neutralization test for DENV, yet this approach is challenging in an industrial setting. Alternatively, the definition of the correlate of protection should be re-defined in retro perspective based on the results of the recent clinical trials, since the current correlate of protection of DENV is based on early vaccine-related studies on the flaviviruses JEV, YFV, and TBEV; but does not take the genetic variability between and within the different serotypes of DENV into account [110].

In summary, dengue is a complex disease and multiple factors are important in controlling disease outcome. More fundamental research into the virus life cycle and host immune response is imperative to fully understand disease pathogenesis and should guide the rational design of antiviral therapies and vaccines.
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