Host cell responses to dengue virus infection
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INTRODUCTION AND SCOPE OF THIS THESIS
1. Global burden of dengue virus infection

Dengue is the arboviral infection with the highest incidence worldwide. The etiological agent, dengue virus (DENV), is transmitted to humans by mosquitoes from the *Aedes* genus. The most important vectors are the mosquitoes *Ae. aegypti* and *Ae. albopictus*. There are four antigenically distinct serotypes of DENV, DENV1 – DENV4, and in many countries more than one serotype co-circulates in the same area. Since the 1950s the incidence of dengue has increased drastically and currently it is estimated that 390 million dengue infections occur every year, of which 96 million are clinically apparent with symptoms including fever, rash, muscle and joint pain. Of these, 500,000 people, mostly children, develop severe disease with hemorrhagic manifestations and/or hypotensive shock for which hospitalization is required, and about 2.5% of those affected die.

In the 1970s, only 9 countries reported dengue whereas it is now endemic in more than 100 countries. The Americas, South-East Asia and Western Pacific are the most seriously affected regions in the world. In the last three decades, a 4.6-fold increase in the number of reported cases has been observed in the Americas. Last year (2016), ~2.3 million cases were reported in this region with a total of 1,032 deaths. Colombia is among the countries most severely affected by dengue in the Americas, accounting for 103,822 cases and 199 deaths in 2016. In 2010, the country experienced the largest outbreak to date, with 157,152 cases and 217 deaths reported. Transmission also occurs in Africa and the Eastern Mediterranean, and new cases have been reported in Europe and the United states. Local transmission in Europe was first reported in France and Croatia in 2010 and several imported cases were detected in 3 other European countries. Moreover, in 2012 there was a large dengue outbreak on the Madeira islands of Portugal with over 2,000 cases. Figure 1 depicts the worldwide average number of reported dengue cases from 2010 to 2016.

Although several reasons account for the dramatic increase in the number of dengue infections around the globe, the geographical expansion of the vectors is one of great importance. *Ae. aegypti*, the primary vector of DENV, is believed to have originated in the jungles of Africa. It was introduced to the New World due to slave trade during the seventeenth to nineteenth centuries, from where it subsequently spread to tropical and subtropical regions of the world. *Ae. albopictus* is a secondary vector of DENV. Before 1979, it was only found in Asia and the Western Pacific, but in the last decades it has spread to North America and more than 25 countries in the European region. The geographic range of *Ae. albopictus* expands further north than that of *Ae. aegypti* and its eggs are resistant to subfreezing temperatures.

Intensified international trade and travel, changes in rainfall and temperature patterns, unplanned urbanization and poor measures for water management represent the
main reasons for the spreading of the mosquito vectors. A recent study on the global distribution of *Ae. aegypti* and *Ae. albopictus* predicts that these mosquitoes are currently present across all continents. Vector importation to new areas will eventually lead to autochthonous dengue transmission in those regions and therefore the incidence of dengue is expected to further increase.

**FIGURE 1 | Average number of worldwide dengue cases from 2010 till 2016.** DENV is endemic in most tropical and subtropical areas of the world. The average number of cases reported to the WHO from 2010 till 2016 are shown. In Africa, poor systems for DENV surveillance has made it difficult to assess the incidence in this region. Reproduced with permission from World Health Organization.

2. Viral structure and replication cycle

2.1. Virus particle and genome organization

DENV belongs to the Flaviviridae family. The viral particle consists of a host-derived lipid bilayer in which the viral membrane protein (M) and the envelope glycoprotein (E) are embedded. In the interior, the nucleocapsid is found, which comprises multiple copies of the capsid protein (C) associated with a positive sense single stranded RNA (+ssRNA) genome. The M protein is a small proteolytic fragment of its precursor form prM and is anchored into the viral membrane by two transmembrane helices. The ectodomain of the E protein has three structural domains, namely DI, DII and DIII. DI is the linker region connecting DII and DIII; DII is involved in the dimerization of
the E protein and it contains the fusion loop; DIII is an immunoglobulin-like domain that interacts with cellular attachment factors. The viral surface of mature DENV particles is composed of 90 homodimers of the E protein arranged in 30 rafts of three homodimers in a herringbone-like pattern. Immature virions, which are assembled in infected cells, are covered with a total of 60 spikes, each spike consisting of three prM/E heterodimers.

The DENV genome is ~11 Kb long, it carries a type 1 cap (m7GpppAmp) structure at the 5’ end and lacks a poly(A) tail at the 3’ end. The genome is recognized as a mRNA. The genome encodes a single open reading frame (ORF) that is translated into a large polyprotein of ~370 KDa. Further processing by cellular and viral proteases generates three structural proteins (C, M and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Besides encoding viral proteins, the genome contains highly structured 3’ and 5’ untranslated regions (UTRs) involved in the replication of the viral genome. The 5’UTR is relatively short (~100nt) while the 3’UTR is longer, ranging from ~340 to ~700 nt. The subgenomic flavivirus RNA (sfRNA), an important determinant of Flavivirus pathogenesis, is derived from the 3’UTR.

2.2. Entry and fusion

The E glycoprotein mediates the interaction with viral receptors expressed on the surface of susceptible cells (Fig. 2, step 2) including dendritic cells (DCs), B cells, T cells, monocytes, macrophages, hepatocytes and neuronal cells. There is not a unique DENV receptor, rather several molecules have been shown to promote infection. Well-characterized receptors include heparan sulfate, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, also known as CD209), mannose receptor (CD206), heat shock proteins 70 and 90 (HSP70/HSP90), binding immunoglobulin protein ((BiP) also known as 78 kDa glucose-regulated protein (GRP78)), laminin receptor, CD14-associated protein and the TIM/TAM proteins. Furthermore, during re-infection, virus particles opsonized with immunoglobulins interact with Fcγ receptors and this may lead to enhanced infection, which will be discussed in detail in a later section.

Upon binding to a virus receptor, the particle is internalized by receptor-mediated endocytosis (Fig. 2, step 3). DENV predominantly enters its host cells via clathrin-mediated endocytosis; however, clathrin-independent non-classical endocytic pathways have also been described. The entry pathway seems to be dependent on the cell type and the virus strain used. In the case of clathrin-mediated endocytosis, the receptor-bound virus diffuses towards pre-existing clathrin-coated pits, subsequently, the coat scissions from the plasma membrane in a dynamin-dependent manner. The clathrin-coated vesicle is transported away from the plasma membrane after which the vesicle loses its clathrin coat and delivers the virus to the early endosome. Later, the early endosome
matures into a late endosome where fusion of the viral and the endosomal membranes occurs. The low-pH environment within the endosomes triggers large conformational rearrangements of the E protein which cause dissociation of the E-homodimers. This leads to the outward projection of DII and insertion of the fusion loop peptide into the target cell membrane. At this moment three copies of E interact with one another until they are folded into a hairpin-like structure that forces the target membrane to bend towards the viral membrane, and eventually fusion takes place (Fig. 2, step 4 and 11,27). At this late step, negatively charged lipids also have been proposed to play a role.

Little is known about the events that follow the fusion process, which involves the release of the viral genome from the nucleocapsid (uncoating) and its transport to the rough endoplasmic reticulum (ER) where translation takes place. Recently it was described that a non-degradative ubiquitination step carried out by ubiquitin E1-activating enzyme is required for the uncoating of the viral genome and that upon genome release the capsid is degraded by the proteasome system. Although the cytoskeleton transport system probably facilitates the transfer of the viral genome to the ER, the mechanisms involved remain unknown.

2.3. RNA translation

The viral RNA acts as mRNA which is translated into a single ER-bound polyprotein. The genome carries a type 1 cap structure and therefore translation of the viral polyprotein is thought to be cap-dependent, similarly as the vast majority of the eukaryotic mRNAs. During cap-dependent translation initiation, the cap-recognition complex (known as eIF4F) recognizes an m7GpppN cap structure at the 5’ end of viral and cellular mRNAs. Once the eIF4F is bound to the cap structure, the ribosomes are recruited to the mRNA and synthesis of the viral polyprotein is initiated at the 5’ proximal AUG codon. The poly(A)-binding protein (PABP) binds to the 3’UTR of DENV RNA which suggests that circularization of the genome via interaction with the PABP-eIF4F complex is required for efficient translation.

Cap-independent translation has also been described. However, the mechanisms by which this is achieved remain to be elucidated. Especially because DENV RNA does not contain an internal ribosome entry site (IRES) nor other structures capable of mediating translation initiation. Initially, it was postulated that cap-independent translation will occur under conditions in which cap-dependent translation is inhibited, such as when eIF4F levels are limiting; but, further studies have pinpointed cap-independent translation as a mechanism occurring during natural infection. It is possible that DENV makes use of both systems such that translation of viral mRNAs has a mechanistic advantage over translation of cellular mRNAs. Such advantage would be particularly useful during variable intracellular conditions as a consequence of cellular stress driven by the infection.
Translation of the large DENV ORF generates a polyprotein which is cleaved to generate the three structural proteins C, prM/M and E and seven non-structural (NS) proteins (Fig. 2, step 5). The NS proteins include the large, highly conserved proteins NS1, NS3, and NS5, and the four small hydrophobic, less conserved, NS2A, NS2B, NS4A, and NS4B proteins. The processing of the DENV polyprotein is carried out co- and post-translationally by a combination of cellular proteases of the furin-type or other Golgi-localized proteases and the viral serine protease NS3, which requires NS2B for its activity.
2.4. Genome replication

Replication of DENV occurs in close association with virus-induced ER membrane invaginations, known as vesicle packets (VPs), which wrap around the replication complex. The VPs contain viral proteins, viral RNA, and probably host cell factors (Fig. 2, step 6). The formation of VPs promote efficient viral replication by anchoring the replication machinery in membrane compartments and shield the virus from host defense mechanisms. VPs have been shown to contain NS2B, NS3, NS4A, NS4B and NS5. NS2A, NS4A and NS4B contain membrane-spanning domains that anchor to the ER membrane thereby promoting VPs formation. NS3 and NS5 harbor the catalytic activities required to replicate the viral RNA and cap it; i.e. NS3 acts as a triphosphatase and helicase while NS5 is a methyltransferase and the RNA-dependent polymerase (RdRp). Nucleotides and other factors needed for replication enter the VPs via a pore that connects with the cytoplasm.

The RdRp activity of NS5 begins the synthesis of full-length complementary minus strand RNA (−)RNA, thereby generating replicative intermediates of double stranded RNA (dsRNA). The −)RNA serves as a template for the synthesis of additional plus strand RNA (+(+)RNA). Viral RNA synthesis is asymmetric, with +(+)RNA strands accumulating in around 10-fold excess over −)RNA strands. The newly synthesized +(+)RNA functions as a template for further −)RNA synthesis, mRNA for viral protein translation, precursor for the sRNA and genomic RNA to form new virus progeny.

2.5. Particle assembly, maturation and egress

Newly synthesized RNA likely exits the VPs through the pore that connects with the cytoplasm. Its interaction with mature molecules of the C protein is thought to be mediated by virus-induced lipid droplets which provide a scaffold for genome encapsidation. Encapsidation occurs at the ER membranes to which the C proteins associate through four hydrophobic α helices (Fig. 2, step 7 and 43). Then, the non-structured nucleocapsids bud into the ER lumen, thereby acquiring a lipid membrane that contains heterodimers of the structural proteins E and prM (Fig. 2, step 8). These newly assembled virions are immature and have a spiky appearance.

Immature particles are transported through the cellular secretory pathway where the mildly acidic pH of the Golgi apparatus leads to the dissociation of the E/prM heterodimers. At this point, 90 E homodimers are formed lying smoothly on the surface of the viral particle, while prM continues to be associated to the E fusion loop. These conformational changes expose the cleavage site of prM and during the transport of the viral particle through the trans-Golgi network, the cellular protease furin cleaves the pr-peptide from the M protein (Fig. 2, step 9 and 45). The pr-peptide, however, continues to be associated with the virion to avoid premature
fusion of the E protein with the exosomal membrane during the transit of the particle through the acidic environment of the trans-Golgi network. The viral particles are subsequently transported to the plasma membrane by exocytosis and released to the extracellular milieu where the neutral pH facilitates the release of the pr-peptide. Unlike immature particles, mature infectious particles have a smooth appearance.

prM processing is essential to the formation of mature infectious particles; however, furin cleavage is not efficient and varies depending on the cell type. Therefore, infected cells produce a mix of fully mature, partially mature and fully immature particles (Fig. 2, step 1 and 47). In the immature particles, E/prM heterodimers are re-formed given the viral particle a spiky appearance. Fully immature particles are in essence non-infectious, except in the presence of anti-prM antibodies that can facilitate their entry into Fc receptor-bearing cells. Partially immature virions can be infectious, but the threshold of prM content that allows infectivity is currently unknown.

3. Clinical manifestations and pathogenesis

It has been estimated that ~75% of dengue infections are asymptomatic. Clinical dengue involves a wide spectrum of symptoms ranging from mild fever to severe plasma leakage leading to life-threatening shock. In 1997, symptomatic infections were classified by the World Health Organization (WHO) into three categories: undifferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF). DHF was further classified into four severity grades, with grades III and IV being defined as dengue shock syndrome (DSS). In 2009, the WHO proposed a new classification due to the difficulty in applying the previous classification system in clinical settings and because of increasing reports of severe disease that did not fit the criteria for DHF. The new classification is based on the levels of disease severity: dengue, dengue with warning signs, and severe dengue.

Dengue is mainly characterized by fever, nausea, vomiting, rash and leukopenia; the warning signs include abdominal pain, persisting vomiting, fluid accumulation, mucosal bleed, lethargy and restlessness, liver enlargement (>2 cm) and an increase in the hematocrit concurrent with a rapid decrease in platelet count. The criteria for severe dengue include severe plasma leakage leading to shock or fluid accumulation with respiratory distress, severe bleeding and severe organ involvement, affecting the liver, the central nervous system, heart and other organs. The pathogenesis of severe disease is poorly understood, but most of the manifestations are associated to abnormal immune responses involving high production of pro-inflammatory cytokines and chemokines, activation of T-lymphocytes and disturbed homeostasis of the blood clotting system.
Human infection begins in the skin with the injection of DENV by the bite of an infected mosquito. Langerhans cells – skin resident dendritic cells (DCs) – skin macrophages and skin mast cells are thought to be among the first cells infected\textsuperscript{51–53}. Infected cells subsequently migrate to the regional lymph nodes where they facilitate the spread of the virus to other susceptible cells such as resident DCs, monocytes and macrophages. As infected monocytes and macrophages migrate through the lymphatic system, DENV spreads to multiple organs such as the liver, lymph nodes, spleen and lung (Fig. 3 and\textsuperscript{54–56}). The liver is the most commonly affected organ and several lines of evidence have shown that hepatic cells and Kupffer cells (resident-liver macrophages) are prime targets of DENV\textsuperscript{56–59}. Involvement of the liver leading to hepatic dysfunction is clinically characterized by hepatomegaly and increased serum levels of liver enzymes\textsuperscript{60}.

Infected or bystander monocytes, macrophages and DCs, are stimulated to produce the bulk of cytokines and chemokines involved in the immune response to the infection. The more cells that are infected, the higher the viremia. Higher viral loads correspond with an exacerbated immune response – cytokine storm – which is believed to play a direct role in the pathogenesis of severe dengue disease\textsuperscript{61–63}. Hence, compared to DF patients, severe dengue patients have higher levels of pro-inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNF-\(\alpha\)), IL-6, IL-8, IL-10, chemokine (C-C motif) ligand 2 (CCL-2, also known as monocyte chemoattractant protein 1 (MCP1)), CCL-3, and interferon (IFN)-\(\gamma\). The final target of these mediators is the vascular endothelium, thereby inducing vascular permeability, hemorrhagic manifestations and hypotensive shock\textsuperscript{63}. For this reason, dengue is considered to be an immunopathological disease. How this massive cytokine production is induced and controlled is not entirely understood.

Several factors are associated with the development of severe dengue. For example, host factors such as age, gender, genetic background, nutritional status and comorbidities and viral factors related to the genetic variability among DENV serotypes and strains\textsuperscript{64–66}. However, all these factors are contingent by whether the patient is experiencing a primary or a secondary infection. A large body of evidence, however, demonstrates that severe dengue predominantly occurs in individuals experiencing a secondary infection with a heterologous serotype and during primary infection of 6-to-12-month old infants born to dengue immune mothers\textsuperscript{67,68}. Infection with one serotype induces life-long protection against homologous serotypes, but cross-protection against other serotypes is short-lived, lasting from 2 to 3 months\textsuperscript{69}. During secondary infection with a heterologous DENV serotype, memory B and T cells are rapidly activated, but their responses are skewed towards the initial DENV serotype, a phenomenon referred to as \textit{original antigenic sin}\textsuperscript{70}. As a result, low-affinity T-cells are expanded and high levels of cross-reactive antibodies
are produced. The majority of these antibodies are non-neutralizing and they have been found to enhance infection, a phenomenon known as ADE\textsuperscript{19}. Furthermore, since severe dengue has been observed during primary infection of newborns with declining levels of maternal antibodies, antibodies alone can also aggravate dengue disease\textsuperscript{67,68}.

**FIGURE 3 | DENV dissemination in the human host.** The bite of an infected mosquito leads to the infection of skin-resident macrophages, dendritic cells (DCs) and mast cells. Infected cells migrate to regional lymph nodes where new target cells are infected increasing the viral load. DENV is disseminated through the lymphatic system to organs such as liver, lymph nodes, spleen and liver. Reproduced with permission from Nature Publishing Group.

Non-neutralizing cross-reactive antibodies bind to the viral particle and facilitate infection of Fc-receptor-bearing cells. Furthermore, virus infection through antibodies has been shown to increase the number of virus particles produced per infected cell.
Collectively, a higher viral load is seen under these conditions. Higher viral burden early in infection has been postulated to eventually induce the cytokine storm associated with severe disease. Whether antibodies are neutralizing or enhancing depend on several factors, among them, the concentration, the avidity and the accessibility of the epitope on the viral particle. All anti-E antibodies tested so far elicit ADE when the antibody concentration is lower than the threshold required for neutralization. In addition, anti-prM antibodies enable uptake of immature DENV particles which are normally not infectious in the absence of antibodies. Next to its role in facilitating uptake of viral particles, antibody-mediated DENV entry is thought to suppresses the host antiviral immune responses, thereby promoting virus replication and virus particle production. However, the mechanisms behind this phenomenon, regarded to as intrinsic ADE, are not yet completely elucidated.

4. Cellular responses to DENV infection

Viruses, as obligatory parasites, rely on the host cell to ensure productive infection. Because RNA viruses carry relatively small genomes (in comparison with DNA viruses), a myriad of host cell factors are hijacked by these viruses to ensure viral multiplication. From the start of the infection until the secretion of newly produced viral particles, host cells engage in a fight to clear the virus and try to protect non-infected cells. Therefore, numerous different cellular pathways are triggered in virus-infected cells and summarizing them all is beyond the scope of this introduction. Instead, we want to describe the three cellular responses to DENV infection that are important in this thesis research.

We will begin with the IFN response which is the main antiviral weapon of mammalian cells. IFN is essential for the clearance of the viruses and the initiation of adaptive immune responses. We will continue with a description of the stress pathways that contribute to the restoration of cellular homeostasis upon insults such as viral infection. Importantly, these pathways have been previously linked to viral sensing and suppression of viral replication. Finally, we will describe the microRNA machinery. By fine-tuning gene expression, microRNAs are able to restore or reprogram gene expression patterns which, in turn, can determine the outcome of infection.

4.1. The IFN response

DENV is first recognized via the endosomal toll-like receptors (TLRs) TLR-3, TLR-7 and TLR-8, and the cytoplasmic RIG-I-like receptors (RLRs) retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5). TLR-3 recognizes dsRNA whereas TRL-7 and TRL-8 recognize ssRNA. On the other hand,
RIG-I recognizes 5’-triphosphorylated blunt ends of viral genomic dsRNA and MDA5 binds internally to long dsRNA with no end specificity. Activation of these receptors leads to the nuclear translocation of interferon regulatory factor 3 and 7 (IRF3 and IRF7), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1). NF-κB and AP-1 induce pro-inflammatory cytokines whereas IRF3 and IRF7 induce the transcription of type I IFN (IFN-α/β).

IFN is key to the antiviral response. Newly synthesized IFNs are secreted from the infected cell and act in an endocrine and paracrine manner. IFNs have three major functions: establishment of an antiviral state; modulation of innate immune responses by promotion of antigen presentation and activity of natural killer cells; and the development of high affinity antigen-specific T and B cell responses. Binding of IFN-α/β to their cognate receptors triggers signaling through the JAK/STAT pathway which results in the transcription of IFN-stimulated genes (ISGs), the effector molecules of the IFN response. Although hundreds of ISGs have been described, the function of most of them remains unknown. Examples of well characterized ISGs are 2′-5′-oligoadenylate synthetase 1 (OAS1) and protein kinase R (PKR). Both recognize the presence of dsRNA. OAS1 mediates activation of RNase L which is implicated in RNA degradation, whereas PKR inhibits protein translation. Intriguingly, not all ISGs have antiviral properties. For example, adenosine deaminase acting on RNA 1 (ADAR1), family with sequence similarity 46 member C (FAM46C), lymphocyte Antigen 6 Complex (LY6E) and Mucolipin 2 (MCOLN2) were recently shown to enhance replication of Yellow fever virus, Chikungunya virus and West Nile virus in cells. The observation that some ISGs actually promote infection adds another layer of complexity to the IFN response and future studies should address the actions of individual ISGs thoroughly as it may serve as a therapeutic strategy.

It has been reported that activation of TLRs and RLRs aids in restricting DENV replication and that pre-treatment of cells with IFN-α/β prevents DENV infection. Therefore, DENV has evolved mechanisms to both prevent IFN production and suppress IFN signaling. Indeed, DENV does not induce IFN-α/β production in human primary DCs and once infected these cells are unable to produce IFN upon stimulation with strong type I IFN-inducers, like polyI:C. In contrast, DCs from non-human primates were shown to produce IFN-α in response to DENV infection yet this species does not develop severe manifestations of dengue disease. Lastly, gene expression analysis of dengue patients revealed that the extend of ISG expression is lower in patients with DSS compared to patients without DSS.

Several strategies have been described by which DENV dampens the antiviral effects of IFN. First, DENV was shown to avoid recognition by cytosolic RLRs. This is facilitated by the induction of intracellular membranes that conceal viral dsRNA intermediates thereby preventing their exposure to the cytosol. Furthermore, 2′-O
methylation of the viral mRNA cap structure evades MDA5 recognition\textsuperscript{94,95}. Second, it has been postulated that the NS2/NS3 viral protease actively inhibits IFN production by preventing nuclear translocation of IRF3 and inducing degradation of mediator of IRF3 activation (MITA), two important signaling molecules in the TLR3 and RIG-I-triggered pathways\textsuperscript{96,97}. Third, DENV hinders the IFN-triggered signaling pathway through inhibition of STAT1 phosphorylation and degradation of STAT2, which was shown to be mediated by NS4B and NS5 respectively\textsuperscript{90,98–100}. Finally, it was recently reported that DENV represses translation of several ISGs due to the sequestration of the RNA-binding proteins Ras-GTPase-activating-protein binding protein 1 (G3BP1), G3BP2 and CAPRIN1 by DENV sfRNA\textsuperscript{101}.

The hallmark response of mammalian cells to infection is the production of type I IFNs. Their autocrine and paracrine actions seek for the containment of the infection and the protection of non-infected cells. As we have described, DENV is sensitive to IFN; yet, it has evolved several mechanisms to avoid its antiviral actions in naturally targeted cells. Further deepening of our knowledge on the IFN-related pathways that are hijacked by DENV are important as it may guide the development of interventions that promote virus clearance.

4.2. Cellular stress responses

Viruses rely entirely on the host to produce viral proteins and therefore place a lot of pressure on the translation and protein folding machineries of host cells. In an attempt to recover cell homeostasis, cells respond to viral infection by decreasing the levels of protein synthesis and/or by enhancing the protein folding capacity of the ER. A decrease in protein synthesis is achieved through the activation of the integrated stress response (ISR), whereas an increased folding capacity is mediated by the unfolded protein response (UPR). Both signaling pathways are aimed at the recovery from stress, but when this cannot be accomplished, the ISR and UPR activate cell death pathways. In the following section, we will discuss these two pathways in the context of DENV infection.

The integrated stress response

In 2003, Harding and colleagues coined the term \textit{integrated stress response} (ISR) as a way to organize the effect of multiple stresses that converge in the phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2\(\alpha\)), which leads to a global decrease in translation\textsuperscript{102}.

ISR signaling (Fig. 4) starts with the recognition of stress stimuli by four kinases: PERK that is activated during ER stress, PKR recognizes foreign dsRNA, heme-regulated eIF2\(\alpha\) kinase (HRI) senses oxidative stress, and general control nonderepressible 2 (GCN2) which is triggered by nutrient deprivation\textsuperscript{103}. Activation of these kinases
subsequently lead to increased levels of the phosphorylated form of eIF2α (pS51-eIF2α). eIF2α is part of the eIF2 complex, which binds to GTP and the methionine-charged initiator tRNA (tRNA_{Met}^{iMet}) to form what is known as ternary complex (eIF2-GTP-tRNA_{Met}^{iMet}). The ternary complex delivers the tRNA_{Met}^{iMet} to the 40S ribosomal subunit in order to start translation at the AUG codon. This process depends on the exchange of GTP to GDP mediated by eIF2. Phosphorylation of eIF2α therefore impedes the formation of the ternary complex and causes stalling of translation initiation\textsuperscript{104}. Stalled translational initiation complexes are assembled into cytoplasmic protein aggregates called stress granules (SGs)\textsuperscript{105}. Consequently, SGs are aggregates of non-translating mRNA, 40S ribosomal subunits, several eukaryotic initiation factors such as eIF3, eIF4F and eIF4B and a variety of RNA binding proteins with functions in mRNA stability/decay and cell signaling. The SG’s composition varies depending on the cell type and the nature of the stress; however, T cell internal antigen-1 (TIA-1), TIA-1-related (TIAR) and G3BP1 are well-accepted SG markers\textsuperscript{105,106}.

Upon activation of the ISR and the formation of SGs, the translation of most cellular mRNAs is inhibited; nevertheless, selective translation of transcripts such as the ones for activating transcription 4 (ATF4), CHOP and DNA damage inducible 34 (GADD34) is promoted. The translation of these mRNAs relies on a re-initiation mechanism due to the presence of upstream open reading frames (uORFs) or direct recruitment of ribosomes to internal ribosome entry sites (IRES)\textsuperscript{107,108}. ATF4 is the main effector of the ISR and its translation is regulated by two uORFs. The first uORF (uORF1) encodes a polypeptide which is only three amino acid residues in length. The second uORF (uORF2) is 59 amino acid residues in length and it is regarded as inhibitory uORF because it overlaps out of frame with the first 83 nucleotides of the ATF4 coding DNA sequence (CDS). Under normal conditions, ribosomes initiate scanning at uORF1 and, due to abundance of the ternary complex, rapidly re-initiate at the inhibitory uORF2. In stressed cells, the limiting availability of the ternary complex favors re-initiation at ATF4 CDS instead of the inhibitory uORF2\textsuperscript{107,109}.

ATF4 induces the transcriptional regulation of stress-responsive genes, but the set of genes expressed varies depending on the stress conditions that lead to eIF2α phosphorylation. For example, the pattern of gene transcription directed by ATF4 is different between ER stress and amino acid deficiency; thus, the partnership with other transcription factors is important for the induction of a tailored response. Indeed, activating transcription factor 6 (ATF6) and X-box binding protein 1 (XBP1) function in combination with ATF4 to direct the UPR thereby increasing the expression of chaperons that help in decreasing the load of misfolded proteins\textsuperscript{109}. The formation of heterodimers with C/EBP homologous protein (CHOP), which is a downstream target of ATF4, is associated with the induction of autophagy upon starvation\textsuperscript{110}. A more general target of ATF4 is GADD34, a protein that functions as the regulatory subunit
of protein phosphatase 1 (PP1). PP1 is the phosphatase that dephosphorylates eIF2α allowing restoration of the cellular translational rates. Hence, expression of GADD34 is an important negative feedback loop that allows termination of the ISR\textsuperscript{111,112}.

FIGURE 4 | The integrated stress response signaling. The ISR integrates the signaling of four kinases that recognize specific stressful stimuli. GCN2 recognizes amino acid deprivation, PKR is activated by exogenous dsRNA (e.g. in the presence of viral infection), HRI senses heme-deprivation and oxidative stress and PERK is activated during ER stress. The activation of these kinases leads to the phosphorylation of eIF2α which induces a general decrease in cap-dependent translation with the concomitant selective translation of ATF4. ATF4 induces a tailored gene expression response depending on the presence of other transcription factors. However, a common target of ATF4 is GADD34, which regulates PP1. PP1 promotes the dephosphorylation of eIF2α resulting in the restoration of the translational rates and the termination of the ISR. Reproduced with permission from John Wiley and Sons.

Viruses have developed mechanisms to hijack the activation of the ISR and secure viral protein translation. Antagonizing eIF2α phosphorylation is a common strategy deployed by several viruses such as influenza, vesicular stomatitis virus, human papilloma virus and herpes simplex virus\textsuperscript{113–116}. The ISR to DENV infection is complex and there is no consensus as to whether cells infected with DENV activate the eIF2α/ATF4 pathway or not.
It was initially described that DENV inhibits eIF2α phosphorylation. In this report, Emara and Brinton investigated pS51-eIF2α levels upon infection of baby hamster kidney cells clone 21 (BHK-21) with DENV-2. Their results showed that the pS51-eIF2α levels of infected cells do not differ from mock-infected cells at very late time points post-infection (36 and 72 hours post-infection; hpi). In line with these observations, no SGs were assembled during the course of infection. Recently, Fraser and collaborators described that infection of human lung epithelial A549 cells with DENV-2 did not alter the levels of pS51-eIF2α at 1, 2 and 6 hpi; yet despite the absence of eIF2α phosphorylation, DENV induced activation and nuclear localization of ATF4.

In contrast, other reports showed increased eIF2α phosphorylation following DENV infection. For example, Pena and Harris investigated the pS51-eIF2α levels at 3, 6, 9 and 12 hpi in human fibrosarcoma cells (2fTGH) and revealed a transient increase in pS51-eIF2α levels at 6 hpi. Further analysis, discarded the activation of PKR and GCN2 and attributed the increased phosphorylation to the activity of PERK. In a concomitant study, a time-dependent increase in pS51-eIF2α levels was observed in A549 cells infected with DENV-2 and DENV-1. In this case, higher pS51-eIF2α levels were observed from 24 to 72 hpi and this correlated with augmented levels of total eIF2α. The authors suggested that the phosphorylation of eIF2α is probably due to the activation of PKR and PERK, although their experimental data does not support this hypothesis. In a subsequent report, Xia et al. confirmed that DENV-2 induces eIF2α phosphorylation in A549 cells at 24 hpi and found G3BP1-positive SGs in infected cells.

The unfolded protein response

The ER is responsible for protein folding and secretion, lipid biosynthesis and calcium homeostasis. Alterations in any of these cellular processes can induce ER stress. To cope with stress, cells activate the UPR, which results in i) attenuated translation to reduce the protein load in the ER; ii) increased folding capacity due to the synthesis of chaperons and iii) increased ER-associated degradation (ERAD) of misfolded proteins. Depending on the magnitude and duration of the stress, the UPR might also induce cell death.

The UPR is initiated by three different ER-resident proteins: PERK, inositol requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6). In homeostatic conditions, these proteins are associated with the folding chaperone BiP/GRP78. However, when ER-stress occurs and there is an accumulation of misfolded proteins in the ER lumen, BiP/GRP78 is released from its binding partners causing the activation of the different UPR signaling axes. As discussed before, activation of PERK leads to phosphorylation of eIF2α and decreased protein translation; therefore, PERK
constitutes the convergent point of the UPR and the ISR. On the other hand, the activation of IRE1 and ATF6 results in the transcription of genes involved in the quality control of proteins, ER expansion, and export/degradation of misfolded proteins, all seeking for restoring cellular homeostasis. Nevertheless, also pro-apoptotic genes are expressed and the balance between cell survival and cell death depends on the extent and duration of the stress\textsuperscript{124}.

A great deal of the DENV replication cycle occurs in association with ER membranes. Viral mRNA translation generates large amounts of viral proteins thereby putting a high pressure on the protein folding machinery of the ER. Also, replication takes place in VPs, for which large ER rearrangements are needed. These membrane rearrangements are driven by viral proteins and lead to changes in the protein composition and lipid content of the ER membranes. Lastly, progeny virions acquire their membrane by budding into the ER lumen\textsuperscript{38,125}. For all these reasons, DENV infection is known to induce ER stress and to activate the UPR. Indeed, consistent results in a variety of cellular systems indicate that DENV activates the IRE1 and ATF6 arms of the UPR, although variations in the time of induction with regard to cell type and differences between serotypes have been reported\textsuperscript{119,120,126–128}. Importantly, the activation of IRE1 has been associated with DENV-induced ER expansion and with cellular resistance to DENV-related cytotoxicity due to the expression of genes involved in the ERAD\textsuperscript{126}. In addition, DENV blocks the expression of apoptotic mediators downstream of IRE1, which leads to enhanced cell survival\textsuperscript{119}. Therefore, the role of the UPR during DENV infection is associated with changes in the cellular environment that favor viral replication and spread.

The ISR and UPR are closely related and both have been studied in the context of DENV infection. However, an integrated view of both pathways in dengue-infected cells is lacking. Thus, unraveling the interactions between the signaling cascades of the ISR and the UPR in the context of DENV infection, will help to solve the puzzle of the stress response in infected cells. This is turn, will reveal the pathways that DENV regulate in order to maintain cells in a ‘functional’ state and secure viral particle production.

4.3. Human cellular miRNA response

Since their discovery in 1993\textsuperscript{129,130}, miRNAs have changed our understanding of gene expression control. MiRNAs are non-coding RNAs that target mRNAs in a sequence specific manner, thereby inducing mRNA degradation or translational repression. Because miRNAs target more than 60% of all mammalian mRNAs, they aid in shaping the protein landscape of the cell\textsuperscript{131,132}.

The biogenesis of miRNAs is depicted in Fig. 4.1 of chapter 2. Most mammalian miRNAs are transcribed by RNA polymerase II\textsuperscript{133}. The miRNA-coding genes are mostly
located in introns of protein-coding genes. Their transcription is either initiated by the same promoter as the gene in which they reside, or by independent promoters, controlled by separated mechanisms\textsuperscript{134-136}. Transcription of miRNA genes generates a primary miRNA (pri-miRNA) with a cap at the 5' end and a 3' polyadenylated tail. Pri-miRNAs are frequently several kilobases in length and contain one or multiple hairpin structures which can be cleaved into one or several distinct miRNAs (polycistronic transcription unit)\textsuperscript{137}. This cleavage is carried out by the RNAase Drosha and its co-factor DiGeorge critical region 8 (DGCR8) and leads to the formation of a second transcript known as precursor miRNA (pre-miRNA). Pre-miRNAs are approximately 70 nt in length and are exported to the cytoplasm through the nuclear transport receptor exportin-5 in a Ran-GTP dependent manner\textsuperscript{138}.

Once in the cytoplasm, pre-miRNAs are further processed by the RNase Dicer and TAR RNA-binding protein (TRBP). Dicer cleavage occurs near the terminal loop of the pre-miRNA thereby generating a small RNA duplex (~22 nt) that is subsequently loaded onto the RNA-induced silencing complex (RISC). The core component of RISC is a member of the Argonaute (Ago) subfamily of proteins. In mammalian cells, there are four Ago proteins (Ago1-4). The RNA duplex is subsequently separated into ssRNAs by the Ago proteins, a process known as unwinding, after which one strand (the passenger strand) is discarded and the other strand (the guide strand) is retained in a mature functional RISC\textsuperscript{139,140}. The guide miRNA binds to miRNA recognition elements (MRE) which are mostly located in the 3’ UTR of mRNAs. The interaction between miRNAs and mRNAs is largely dependent on nucleotides 2 – 8 from the 5’ end of the miRNA, which is known as the seed region\textsuperscript{141}. Upon binding of target mRNAs, Ago proteins induce translational repression and/or de-adenylation that leads to mRNA decay. Furthermore, when there is high complementarity between the miRNA and the mRNA, Ago2 can induce endonucleolytic cleavage of mRNAs\textsuperscript{142}.

miRNAs regulate a variety of cellular processes from differentiation to oncogenesis, and are part of the complex network of interactions between viruses and their host cells\textsuperscript{132,143,144}. Virus infection induces extensive changes in the protein landscape of the host cells; thus, the expression of miRNAs is also expected to be altered. The regulation of miRNAs during viral infection is either due to a direct effect of the virus on miRNA coding genes or is caused by an indirect effect by changes in protein expression upon infection. The consequence of virus-mediated miRNA regulation depends on the virus and the cellular context. Indeed, human miRNAs have been described to both restrict and aid in viral replication. In chapter 3, we discuss the RNAi response (that includes miRNAs and siRNAs) to several flaviviruses in mammalian and arthropod hosts. Here, we will describe the current knowledge of the miRNA response to DENV infection in the mammalian host.
At the beginning of my thesis research there were no studies on the miRNA profile of DENV-infected cells. In 2013, the first study was published by Qi and collaborators who reported the miRNA profile in peripheral blood mononuclear cells (PBMCs) infected in vitro with DENV-2. The function of the differentially expressed miRNAs (Table 1) was not evaluated, yet, bioinformatics tools predicted that their potential targets were cytokines and chemokines whose expression was also altered upon infection. For example, let-7e might target IL-6 and CCL-3, while miR-451, miR-4279 and miR-106b may target migration inhibitory factor (MIF), CCL5 and chemokine (C-X-C motif) ligand 1 (CXCL1), respectively. Additional studies have been conducted in human hepatic Huh7 cells and in vascular endothelial EAhy926 cells.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>miRNA expression profile in cells infected with DENV.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>System</strong></td>
<td><strong>Differentially expressed miRNAs</strong></td>
</tr>
<tr>
<td>PBMCs</td>
<td>↑ miR-4290, miR-4279, miR-625*, let-7e, miR-1290, miR-378, miR-1246, miR-767-5p, miR-320c, miR-720, miR-491-3p, miR-3647, miR-451, miR-4286. ↓ miR-106b, miR-20a, miR-30b, miR-3653.</td>
</tr>
<tr>
<td>EAhy926 cells</td>
<td>↑ miR-3178, miR-324-3p, miR-937, miR-3195, let-7a-2*, miR-20b. ↓ miR-21, miR-2116, miR-142-3p, miR-223.</td>
</tr>
<tr>
<td>Huh7</td>
<td>↑ let-7c, miR-92b, miR-130b, miR-140-3p, miR-149. ↓ miR-181c, miR-374a, miR-453, miR-1268.</td>
</tr>
<tr>
<td>Whole blood from dengue patients</td>
<td>↑ miR-145-3p, miR-19a-3p, miR-19b-3p, miR-299-3p, miR-33b-5p, miR-374a-5p, miR-5583-5p, miR-614, miR-634. ↓ miR-320a, miR-423-3p, miR-4451, miR-4503, miR-4529-3p, miR-4788, miR-548e, miR-576-5p, miR-584-5p, miR-652-3p.</td>
</tr>
</tbody>
</table>

Red arrows: upregulated miRNAs; blue arrows: downregulated miRNAs.

The abovementioned studies and others identified individual miRNAs that influence DENV infection in a negative as well as a positive way (Table 2). miR-146a was the first miRNA described to have an effect on DENV replication. Its expression is increased in human primary monocytes and THP1 cells infected with DENV-1, DENV-2 and DENV-3. miR-146a was found to promote viral replication by reducing IFN-β production. The antiviral activity of 4 other miRNAs, namely, let-7c, miR-223, miR-30e* and miR-548g-3p, was reported shortly thereafter and we recently showed that miR-133a also interferes with infection. MiR-548-3p was described to directly target the 5'UTR of DENV-2. The other miRNAs likely target cellular components whose altered expression is detrimental for viral replication. Lastly, Smith et al. revealed that the miR-34 family act as potent inhibitors of several flaviviruses including DENV, West Nile virus, Japanese encephalitis virus and Zika virus. The authors propose that the miR-34 family promotes IRF3 phosphorylation thereby causing enhanced production of type I IFN.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>System</th>
<th>Expression upon infection</th>
<th>Effect on infectivity</th>
<th>Function</th>
<th>Year and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a</td>
<td>Primary human monocyes and THPI cells</td>
<td>Increased</td>
<td>Proviral</td>
<td>Downregulates TRAF6 which results in lower IFN-β production</td>
<td>2013, 148</td>
</tr>
<tr>
<td>Let-7c</td>
<td>Huh7, U937-DC-SIGN</td>
<td>Increased</td>
<td>Antiviral</td>
<td>Downregulates BACH1 causing upregulation of HO-1 which antagonize the oxidative stress response</td>
<td>2014, 146</td>
</tr>
<tr>
<td>miR-223</td>
<td>EAhy926 cells</td>
<td>Reduced</td>
<td>Antiviral</td>
<td>Downregulates STMN1 expression a microtubule-destabilizing protein</td>
<td>2014, 147</td>
</tr>
<tr>
<td>miR-30e*</td>
<td>HeLa, U937 and PBMCs</td>
<td>Increased</td>
<td>Antiviral</td>
<td>Promotes NF-kβ activation and production of IFN-β</td>
<td>2014, 149</td>
</tr>
<tr>
<td>miR-548g-3p</td>
<td>U937</td>
<td>Increased</td>
<td>Antiviral</td>
<td>Targets the viral 5’UTR</td>
<td>2014, 150</td>
</tr>
<tr>
<td>mir-150</td>
<td>PBMCs from DHF patients</td>
<td>Increased</td>
<td>Biomarker for severe disease</td>
<td>Downregulates SOCS1 resulting in lower IFN-γ production</td>
<td>2014, 153</td>
</tr>
<tr>
<td>miR-133a</td>
<td>Vero</td>
<td>Reduced</td>
<td>Antiviral</td>
<td>Downregulates PTB, a protein needed for viral RNA synthesis</td>
<td>2016, 151</td>
</tr>
<tr>
<td>miR-34a</td>
<td>HeLa</td>
<td>Unaffected</td>
<td>Antiviral</td>
<td>Downregulates Wnt signaling potentiating type I IFN response</td>
<td>2017, 152</td>
</tr>
</tbody>
</table>

TRAF6: TNF receptor associated factor 6; BACH1: transcription regulator protein BACH1; HO-1: heme-oxigenase 1; STMN1: Stathmin; SOCS1: suppressor of cytokine signaling 1; PTB: polypyrimidine-tract-binding protein.

A role for miRNAs during DENV pathogenesis has also been proposed. Chen and colleagues described that miR-150 expression is inversely correlated with mRNA expression of suppressor of cytokine signaling 1 (SOCS1) in leukocytes from DHF and DF patients\textsuperscript{153}. SOCS1 functions as a negative regulator of cytokine signaling. In DHF patients, SOCS1 expression was downregulated, whereas the expression of miR-150 was upregulated. In DF patients, the opposite was observed. The authors suggested that augmented miR-150 expression and decreased SOCS1 levels was associated with the pathogenesis of DHF\textsuperscript{153}. A subsequent study identified miRNAs that are differentially expressed in blood samples from DENV-infected patients and healthy individuals (Table 1 and\textsuperscript{154}). Furthermore, the authors described sets of miRNAs that can potentially distinguish between different disease presentations of dengue, yet, no functional experiments were conducted\textsuperscript{154}. Table 2 summarizes the effect of miRNAs on DENV infection and for further reading on this topic I would like to refer to two comprehensive reviews that were published recently\textsuperscript{155,156}.
5. Vaccination and therapy

The development of a vaccine to DENV has been challenging mainly due to the existence of four DENV serotypes and the phenomenon of ADE. Basically, the later implies that the vaccine needs to simultaneously induce robust and durable immunity to all serotypes. The six-decade long wait ended in December 2015 when the chimeric yellow fever-dengue virus tetravalent dengue vaccine (CYD-TDV, also known as Dengvaxia®, Sanofi Pasteur) was licensed in Mexico\textsuperscript{157}. To date, CYD-TDV is registered for use in individuals aged 9 to 45 years. Currently, the vaccine is also licensed in The Philippines, Brazil, El Salvador, Costa Rica, Paraguay, Guatemala, Peru, Indonesia, Thailand and Singapore\textsuperscript{158}. In mid-2016, WHO recommended that countries should consider introduction of CYD-TDV only in geographic settings (national or subnational) where epidemiological data indicate a high burden of disease\textsuperscript{159}. According to WHO, the introduction of a routine CYD-TDV vaccination program at 9 years of age in these regions is expected to result in a 10–30% reduction in symptomatic and hospitalized dengue illness over 30 years\textsuperscript{159}.

Although the licensing of CYD-TDV opened a window of optimism, it must be considered that the highest burden of dengue disease is observed in infants 4-9 months of age and in children 5-9 years old\textsuperscript{160}. Moreover, several concerns regarding the efficacy of CYD-TDV against DENV2, the most prevalent serotype worldwide, and the safety of the vaccine when it is administered to seronegative individuals, have been raised\textsuperscript{161,162}. Therefore, the need to explore other vaccine alternatives persists and the prevention of DENV transmission remains primarily limited to vector control interventions, including the use of larvicides, adulticides, biological control and environmental management\textsuperscript{163}.

There is no specific antiviral therapy for dengue disease, therefore treatment of patients is limited to symptoms relief and the only strategy available to treat severe cases is supportive fluid therapy in medical facilities\textsuperscript{159}. The observation of high levels of viremia in DHF/DSS patients compared to DF patients suggests that a drug able to decrease viremia during the early stages of disease could lead to favorable prognosis\textsuperscript{164}. The ideal DENV drug must have i) action against all serotypes, ii) capacity to reduce viral load 2 logs, iii) low or no significant toxicity, iv) high genetic barrier to emergence of resistance, v) stability to high temperature and humidity, and vi) affordability in dengue-endemic countries\textsuperscript{165}.
### TABLE 3 | Completed clinical trials for dengue therapy reported from 2010.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type</th>
<th>Mode of action</th>
<th>Main Findings</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>Antimalarials and amebicides.</td>
<td>Interferes with acidification of intracellular organelles, preventing fusion and furin-dependent virus maturation. Downregulates pro-inflammatory cytokines</td>
<td>The first trial conducted in Vietnam showed no effect on viremia, NS1 antigenemia, fever duration, cytokine and T cell response. A second smaller trial conducted in Brazil, confirmed the lack of effect of the drug. However, the majority of the dengue patients treated with chloroquine reported experiencing less pain.</td>
<td>169,170</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Anti-inflammatory. Treatment of hypercholesterolemia</td>
<td>Inhibition of cholesterol synthesis. Minimize the inflammation-induced damage to capillary endothelium.</td>
<td>Lovastatin treatment did not have effect on RNA levels, virus clearance or symptomatic relief.</td>
<td>171</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Anti-inflammatory. Use to treat diseases such as allergic reactions and autoimmunity disorders.</td>
<td>As a corticosteroid it may alleviate symptoms related to severe dengue as capillary permeability, hemorrhagic shock, thrombocytopenia and hemorrhage</td>
<td>The study was conducted to assess safety and not powered to assess efficacy. However, the results suggested that although safe to use, prednisolone did not lower viremia or increase viral clearance and it was actually associated with attenuation of the host antiviral response. Currently WHO does not advocate corticosteroid therapy for dengue.</td>
<td>172</td>
</tr>
<tr>
<td>Balapiravir</td>
<td>Nucleoside analog. Initial trials against chronic HCV were dropped due to high cytotoxicity</td>
<td>Upon uptake by the host cell, it is phosphorylated to its 5’-triphosphate metabolite, inhibiting the viral RdRp</td>
<td>No effect on viremia and NS1 antigenemia. Also it did not attenuate the kinetics of plasma cytokine concentrations and whole blood transcriptional profile.</td>
<td>173</td>
</tr>
<tr>
<td>Celgosivir</td>
<td>Iminosugar. Tested for HCV in phase 1 and phase 2 trials but dropped as it did not perform better than existing therapies.</td>
<td>Inhibits ER alpha-glucosidases thereby impairing proper processing of DENV E, prM and NS1 proteins. Could also affect the host UPR pathway</td>
<td>Treatment for 5 days at 12h intervals failed to reduce viral load and fever burden in dengue patients. A new trial administering the drug at shorter intervals has been scheduled.</td>
<td>174</td>
</tr>
</tbody>
</table>
The dengue drug discovery initiatives are underway for over a decade and great progress has been made in recent years in developing DENV inhibitors. Most of them target viral components (mainly the NS3 protease, the NS4B and the NS5 RdRp), but also host factors crucial for the viral replication cycle\textsuperscript{166,167}. However, none of these compounds have reached clinical trials due to a variety of reasons, including limited pan-serotype activity, poor pharmacokinetic properties and adverse effects in animals\textsuperscript{168}. Alternatively, a handful of small molecules indicated for other medical conditions have entered clinical trials for their assessment as anti-dengue compounds\textsuperscript{164}. The results of the six clinical trials completed so far (Table 3 and\textsuperscript{164,168}) are rather disappointing and with only a few more trials currently ongoing, it seems clear that the availability of an effective anti-dengue drug is still far away.

The lack of dengue-targeted drugs making it to clinical trials highlights our limited understanding of dengue biology and pathogenesis. Therefore, in the pursuit of an effective anti-dengue drug, it is of the utmost importance to better understand the naturally occurring interactions between DENV and its human host cells.

**Scope of this thesis**

The research presented in this thesis revolves around the response of human host cells to dengue virus (DENV) infection. The interaction between DENV and its host cells is highly complex and we zoomed in on various aspects of the infection. Emphasis was on the cellular tropism of DENV, the molecular mechanism of antibody dependent enhancement of infection, the influence of cellular microRNAs on DENV replication and the stress pathways triggered in infected cells. A detailed study of these aspects will allow us to better understand the replication cycle of the virus, pathogenesis of disease and will guide the rational design of anti-dengue therapies. Indeed, during our studies, we discovered a potential anti-DENV drug and decided to explore its antiviral activity in more detail.

Flaviviruses are the most common arthropod-borne viruses worldwide yet there are no specific antiviral therapies available to treat infection. In chapter 2, we review the potential of RNAi as a therapeutic against the most commonly distributed flaviviruses and its vectors. We also summarize the challenges that must be tackled in order to use RNAi as a therapy.

In chapters 3–5, we focus on the interactions of DENV with primary macrophages and dendritic cells (DCs) derived from human blood donors. As macrophages and DCs are important targets for DENV, deciphering the response of these cell types to infection will help us to understand the natural occurring interactions between DENV and the human host.
In chapter 3, we investigate the infectious properties of DENV in macrophages and DCs and show how cellular susceptibility relates to the infectivity of progeny viruses. This was done to better understand the sequential rounds of replication in the human host. Paired comparisons of susceptibility and infectivity in macrophages and DCs derived from the same blood donor, allowed us to identify which cells are likely the first target of DENV and which cells contribute predominantly to subsequent rounds of infection. Furthermore, we examined whether the cellular origin of the virus influences its tropism in the presence of antibodies.

In chapter 4, we focus on deciphering the molecular mechanism of ADE in human primary macrophages. To this end, we investigated the effect of enhancing antibodies on the different steps of the DENV replication cycle. Furthermore, we addressed the phenomenon of intrinsic ADE.

In chapter 5, the contribution of microRNAs to DENV infection is examined. We challenged macrophages with DENV and separated the population of cells that were infected from the cells that remained refractory to infection in the same culture conditions. The miRNAs differentially expressed in both populations were identified and their effect on DENV infectivity was determined by overexpression analysis. Proteomic analysis was performed to identify the targets of one miRNA and the impact of one of its targets on DENV infectivity was assessed.

In chapter 6, the antiviral effects of tomatidine are described. The influence of tomatidine on the percentage of infection, virus particle production and infectivity was determined in vitro. Additionally, time-of-addition experiments were conducted to receive an insight in the mode of action of tomatidine. Also, we investigated whether tomatidine acts by downregulation of activating transcription factor 4 (ATF4), an important response factor to cellular stress.

In chapter 7, we study the pathways leading to ATF4 expression in DENV-infected cells and the contribution of this transcription factor in controlling the stress responses induced by DENV.

Lastly, in chapter 8 we summarize and discuss the results obtained in this thesis.
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Introduction and scope of this thesis


