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## Cell entry mechanisms of dengue virus

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# Summarizing discussion



## Introduction

Dengue virus (DENV) currently causes the most common mosquito-borne viral infection worldwide. Approximately 2.5 billion people live in areas where dengue is endemic. The four DENV serotypes each can cause a wide spectrum of disease in humans ranging from dengue fever, an acute febrile illness, to the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), characterized by potentially life-threatening bleeding manifestations. Interestingly, the individual's pre-existing immunity to DENV seems to play an important role in determining disease severity. Recovery from infection with one DENV serotype provides immunity against that serotype, while secondary infections with another serotype can result in severe disease. This phenomenon is explained by the widely accepted hypothesis of antibody-dependent enhancement (ADE) of DENV infection, which postulates that antibodies directly influence the cell entry mechanisms and infectious properties of the virus (Halstead, 2007).

The studies presented in this thesis elucidate the mechanisms involved in cellular entry of DENV. To this end, we have visualized the cell entry pathway of DENV and identified the organelle where membrane fusion occurs. Additionally, we have determined the effect of antibodies on the infectivity of a subpopulation of DENV particles, called immature particles. Ultimately, these fundamental insights into the cell entry properties of DENV particles may contribute to the development of vaccines and antiviral drugs to prevent or treat DENV infections.

## Cell entry of dengue virus

Previous studies have indicated that DENV infects its host cell via receptor-mediated endocytosis, a process which includes binding of the virus to a cellular receptor, internalization into the cell, membrane fusion from within an acidic endosome, and release of the viral genome into the cytosol (see **Chapter 1 and 2**). Viruses can hijack several internalization routes into the cell to reach endosomes. The first objective in this study was to resolve the exact cell entry pathway of DENV and to determine the kinetics and the efficiency of the different stages in cell entry.

Virus entry is initiated by binding of the virus particle to a cellular receptor. In **Chapter 3**, we have investigated the binding properties of DENV (serotype 2, PR159 S1 strain) to BS-C-1 cells that are highly permissive for DENV infection, and found that DENV has a limited capacity to bind to

these cells. Since unbound virus particles can initiate a productive infection when added to new cells, the low binding efficiency is not caused by the fact that a large fraction in the virus preparation is defective and therefore unable to bind to the cell. Therefore, the inefficient binding appears to be primarily related to the strength of the virus-receptor interaction. Several molecules on various cell types have been identified as candidate receptors or attachment factors for DENV. For entry into mammalian cells, such as BS-C-1 cells, glycosaminoglycans, exemplified by heparan sulfate, have been proposed to mediate cellular attachment of several flaviviruses (Chen et al., 1997; Chiou et al., 2005; Germi et al., 2002; Kroschewski et al., 2003; Liu et al., 2004). Yet, the DENV strain used in this study hardly binds to liposomes containing lipid-conjugated heparin (unpublished results I.A. Zybert, J. Wilschut, J.M. Smit). Thus, it appears that cellular binding of this DENV strain is mediated by an as yet unidentified receptor, which presumably is not abundantly expressed on the cell surface and probably also has a low affinity for DENV.

Following receptor binding, virus particles are internalized into the cell. There are several internalization routes that viruses can exploit to enter their host cell (Conner & Schmid, 2003; Marsh & Helenius, 2006). Furthermore, some viruses, such as influenza virus, are able to use more than one entry route (Rust et al., 2004). In **Chapter 4**, we demonstrate that DENV enters the cell exclusively via clathrin-mediated endocytosis, which is in agreement with other studies on flavivirus entry (Acosta et al., 2008; Chu & Ng, 2004; Nawa et al., 2003). There are various ways in which virus particles can enter clathrin-coated pits (CCPs). Viruses can either land on a CCP, join a CCP, or induce *de novo* formation of a CCP at the viral binding site (Rust et al., 2004). Our results show that DENV migrates on the cell surface until it is captured by a pre-existing CCP. The movement of DENV on the cell surface is characterized by random diffusion. Future studies should elucidate the role of receptors in the surface motion. Murine polyoma virus-like particles have been shown to randomly diffuse on the cell surface for 5 to 10 seconds. Subsequently, these particles abruptly lose their mobility due to clustering of the ganglioside molecules (i.e. the receptors) leading to confinement of the virus-receptor complex (Ewers et al., 2005). Likewise, a DENV particle could roll over different surface molecules until the particle is captured by multiple receptors present in a CCP, which mediate a multivalent binding interaction with the particle.

Upon internalization via CCPs, cargo is transported to the endocytic pathway. DENV exhibits two different types of intracellular transport behavior (**Chapter 3**). Approximately one-third of the virus particles remains relatively

stationary and fuses in the cell periphery, whilst the majority of the virus particles adopts a long-range, three-stage movement towards the perinuclear region. In stage I, the virus particles move slowly in the cell periphery. Then, the virus travels rapidly on microtubules to the perinuclear region (stage II), where the particles move intermittently and often in a bidirectional manner (stage III) before fusing from within the acidic endosome. These different types of transport behavior might imply distinct entry pathways for DENV. To verify this possibility, we illuminated various organelles of the endocytic network by use of plasmids encoding fluorescently labeled Rab5, an early endosome marker, and Rab7, a late endosome marker (**Chapter 4**). By tracking single DiD-labeled DENV particles in these cells, we observed that all DENV particles travel to Rab5-positive early endosomes following cellular uptake, and subsequently progress to Rab7-positive late endosomes, where the vast majority of DENV particles fuses. Thus, with respect to the itinerary of endocytic compartments, DENV does not utilize distinct entry pathways as the different types of transport behavior might have implied.

Internalization of DENV particles into cells occurs quickly, since 50% of the particles localize to early endosomes at 3.5 minutes post-attachment to the cell surface. Thereafter, the particles start to associate with Rab7-positive endosomes. The first membrane fusion events take place at 5 minutes post-infection, whereas nearly all fusion events have occurred within 17 minutes (**Chapter 3 and 4**). These results are in agreement with a previous study on West Nile virus (WNV) entry into Vero cells (Chu et al., 2006). Using cryo-immunoelectron microscopy, these authors have observed that WNV particles reside in endocytic vesicles 5 minutes after initiating infection. At 10 minutes post-infection, the endocytic vesicles each containing one virus particle fuse with one another to form larger vesicles that are primarily located in close association with the ER in the perinuclear region of the cell. WNV particles fuse from within these large vesicles at 15 minutes post-infection. Subsequently, the authors have determined the identity of the endocytic vesicles by use of indirect immunofluorescence staining of endosomal markers. At 3-5 minutes post-infection, WNV particles colocalize with early endosome markers and at 12 minutes the particles are situated in late endosomes and lysosomes (Chu et al., 2006).

Early endosomes have previously been divided into two populations with respect to their maturation time, which defines the time period until early endosomes become intermediate endosomes through acquisition of Rab7 (Lakadamyali et al., 2006). The largest group (65%) comprises dynamic early

endosomes, which are quite mobile on microtubules and mature rapidly by accumulation of Rab7, while the other population is static and does not gather Rab7 within a time frame of 100 seconds. Interestingly, cargo internalized via clathrin-mediated endocytosis appears to be differentially sorted into the two populations of early endosomes depending on its destiny. Cargo intended to be degraded in lysosomes at the end of the endocytic pathway is targeted to dynamic early endosomes, while cargo destined to return via recycling endosomes to the plasma membrane for reuse prefers the static population of early endosomes. Notably, this differential sorting of cargo was found to correlate with the presence of the adaptor protein AP-2 in the CCP, with cargo not requiring AP-2 for cell entry being preferentially targeted to dynamic early endosomes (Lakadamyali et al., 2006). Surprisingly, DENV is non-selectively sorted into both populations of early endosomes, while it is being transported to the endocytic pathway (**Chapter 4**). Also, preliminary data have suggested that AP-2 is not essential for DENV cell entry (unpublished results H.M. van der Schaar, J. Wilschut, X. Zhuang, J.M. Smit). It remains to be investigated which other factors, for example adaptor proteins present in CCPs, are involved in the sorting of DENV.

Membrane fusion of DENV predominantly occurs from within late endosomes. Moreover, expression of the dominant-negative Rab7 mutant Rab7T22N significantly decreases the membrane fusion activity and the viral infectivity, which demonstrates that there is a functional requirement for Rab7 in DENV (serotype 2, PR159 S1 strain) infection (**Chapter 4**). Surprisingly, the infectivity of the New Guinea C (NGC) strain of DENV serotype 2 is not impaired by a functional repression of Rab7 (Krishnan et al., 2007, **Chapter 4**). Which factors define the organelle of fusion and could explain this discrepancy between the two virus strains? One could speculate that the pH-threshold for fusion of the virus determines the cellular location of membrane fusion, as the lumen of late endosomes is slightly more acidic (pH ~5.5) than that of early endosomes (pH 6-6.5) (Kielian et al., 1986; Mellman et al., 1986; Schmid et al., 1989). DENV S1 strain has a pH-threshold for fusion of 5.8, whereas the NGC strain already fuses at pH 6.4 in a fusion-from-without assay (personal communication with dr. P. Young, University of Queensland, Australia), which could explain the difference in the organelle promoting fusion. On the other hand, influenza virus particles (X-31 strain) fuse from within intermediate Rab5- and Rab7-copositive endosomes (Lakadamyali et al., 2006). In a plasma membrane fusion assay as well as in a liposomal model system, the pH-threshold for fusion of this influenza strain is pH 5.4-5.6 (Beyer et al., 1986; Chams et al.,

1999), which suggests that the lumen of intermediate endosomes is sufficiently acidic to trigger membrane fusion of DENV S1 strain. Future studies should reveal whether the pH-threshold for fusion determined in indirect assays can predict the organelle of fusion. To resolve this issue, one might use pH-sensitive dyes to monitor the pH values that the virus encounters during entry into living cells (Cooper et al., 2002).

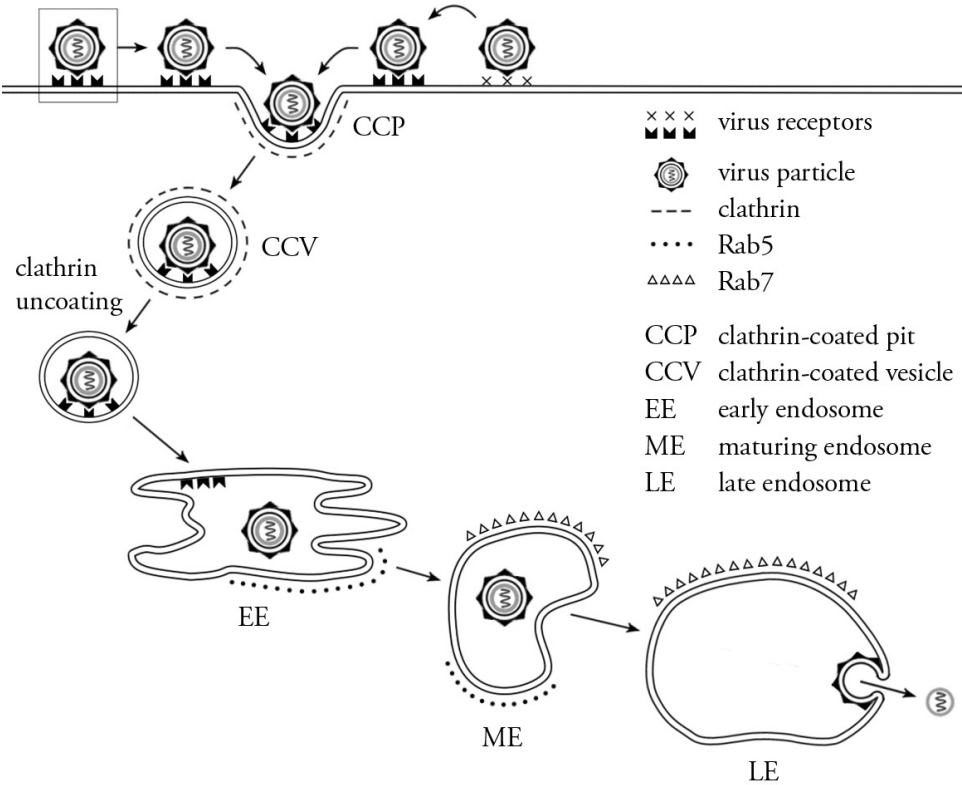
Another factor which might influence the location of fusion is the lipid composition of the organelle. Some viruses require the presence of specific lipids in the target membrane in order to undergo membrane fusion. For example, cholesterol and sphingolipids are essential for the membrane fusion reaction of the alphaviruses Semliki Forest virus (SFV) and Sindbis virus (SINV), as they promote the association of the spike subunit E1 with the target membrane (Bron et al., 1993; Klimjack et al., 1994; Nieva et al., 1994; Phalen & Kielian, 1991; Smit et al., 1999; White & Helenius, 1980). Cholesterol and sphingolipids are present in the membranes of early endosomes, while being nearly absent in the limiting membrane of late endosomes (Kobayashi et al., 1998; Kobayashi et al., 2002; Mobius et al., 2003). The specific lipid-dependency of SINV and SFV, in addition to their capability to fuse at a mildly acidic pH, therefore supports fusion from within early endosomes (Sieczkarski & Whittaker, 2003). When late endosomes do contain significant amounts of cholesterol and sphingolipids, which is the case in fibroblasts from patients with the lipid storage disease type A Niemann-Pick disease, SINV replicates faster and to higher titers (Ng et al., 2008). Interestingly, Venezuelan equine encephalitis virus, another alphavirus, is relatively insensitive to cholesterol depletion from cells and requires functional late endosomes in its infectious entry (Kolokoltsov et al., 2006). Furthermore, single amino acid substitutions in E1 of SFV can suppress the requirements for cholesterol and sphingolipids (Ahn et al., 1999; Chatterjee et al., 2002; Vashishtha et al., 1998). It is conceivable that DENV strains that vary in only a few residues have differential requirements for certain lipids in order to undergo membrane fusion, which might perhaps then also explain the discrepancy in the organelle of fusion between DENV S1 and NGC.

Strikingly, DENV particles spend a substantial time period in the late endosome before membrane fusion occurs (**Chapter 4**). In a liposomal model system on the other hand, the flaviviruses tick-borne encephalitis virus (TBEV) and WNV fuse immediately upon acidification (Corver et al., 2000; unpublished results B. Moesker, J. Wilschut, J.M. Smit). Furthermore, exposure of TBEV and WNV to a low pH for more than 30 seconds in the absence of target membranes results in a complete loss of fusogenicity (Corver et al.,

2000; unpublished results B. Moesker, J. Wilschut, J.M. Smit). Yet, DENV particles reside on average 5.5 minutes in a late endosome without being inactivated and still undergo membrane fusion. Perhaps this period reflects the time a late endosome needs to lower its pH to the threshold for fusion of DENV S1 strain. As mentioned before, this possibility can be investigated by use of pH-sensitive dyes to monitor the pH values that a virus particle is exposed to during cell entry. A more intriguing scenario however is that DENV awaits an additional cellular trigger to activate its membrane fusion machinery. This possibility is supported by the fact that DENV does not fuse with plain liposomes of various lipid compositions (unpublished results I.A. Zybert, J. Wilschut, J.M. Smit). Fusion with the plasma membrane on the other hand can be achieved upon low pH exposure in a fusion-from-without assay (Randolph & Stollar, 1990), which indicates that all factors necessary for fusion other than a low pH are present on the plasma membrane. Perhaps interaction with the receptor induces an initial reorganization of the structural proteins to a more favourable conformation. Yet, as receptor-interaction occurs on the cell surface, it cannot explain why membrane fusion of DENV particles occurs relatively late after entry into the late endosome. Some enveloped viruses require endosomal proteolysis as an additional trigger in order to infect their host cell, as exemplified by the endosomal cleavage of SARS coronavirus and ebolavirus by cathepsins (Chandran et al., 2005; Huang et al., 2006). Future research should reveal whether additional cellular factors other than the low pH in the endosomal lumen, such as enzymatic processing, are involved in DENV cell entry.

What is the chance that a DENV particle succeeds in initiating a productive infection? The efficiency of the cell entry process is reflected in the ratio between infectious units and physical particles, which is approximately 1,000 to 10,000 for DENV S1 strain depending on the specific assays used (**Chapter 3**). Similar ratios have been observed for DENV serotype 2 strain JAM1409 and yellow fever virus strain 17D, while the ratio of DENV serotype 2 strain 16681 is around 100 (Bae et al., 2003; Richardson et al., 2006; Zybert et al., 2008). The main hurdle DENV S1 strain needs to overcome during cell entry seems to be attachment to the cell surface. Once a DENV particle is bound to the cell surface, the chance is around 20% that it eventually will undergo membrane fusion. Interestingly, Thomas and coworkers reach a similar conclusion for the disproportionate numbers of apparently defective HIV-1 virions, which was mainly attributed to a low binding efficiency to the cell in titration assays (Thomas et al., 2007). Still, we do not know whether all





**Figure 1. Model of dengue virus cell entry.** See text for details.

membrane fusion events will result in a productive infection. The DENV S1 strain is highly attenuated and therefore might have a relatively large fraction of particles with a defective genome such that not all particles that fuse will initiate infection. Furthermore, membrane fusion should occur at a site where viral RNA replication can proceed, since simple diffusion of the nucleocapsid or viral genome in the cytoplasm is hampered by the cytoskeleton (Marsh & Bron, 1997; Sodeik, 2000). Also, the translation of the viral RNA after uncoating of the nucleocapsid is fairly inefficient for certain DENV strains due to a specific difference in the 3' untranslated region (Edgil et al., 2003). These steps as well as other stages in the viral life cycle occurring after translation, such as replication, assembly, and exocytosis of progeny virions, could also fail and contribute to the high infectious unit-to-physical particle ratio.

Collectively, our results lead to the following model for cellular entry of DENV S1 strain, see Figure 1. First, the virus particle lands on the cell surface

and migrates in a diffusive manner towards a pre-existing CCP, either by rolling over different surface molecules or by moving as a virus-receptor complex. Upon capture by the CCP, the virus particle loses its mobility. Then, the CCP matures, encloses the virus particle, and pinches off into the cell cytoplasm to deliver the virus particle to a Rab5-positive early endosome. In general, the early endosome carrying the virus matures into a late endosome by gradual accumulation of Rab7 and subsequently a gradual loss of Rab5. Finally, the DENV particle resides in a Rab7-positive late endosome, which moves through the cytoplasm of the cell until the onset of membrane fusion allows the delivery of the nucleocapsid into the cytoplasm. Notably, the finding that DENV particles utilize pre-existing CCPs for internalization and are subsequently non-selectively targeted to early endosomes seems to indicate that DENV exploits constitutive clathrin-mediated endocytosis rather than inducing this entry pathway itself.

### **Infectious properties of immature dengue virus particles**

The severity of disease caused by DENV infection seems to be controlled by pre-existing antibodies. While antibodies specific to the E protein have been studied extensively, the role of antibodies directed against the prM in the disease pathogenesis remains elusive. Anti-prM antibodies are frequently found in the sera of patients, which is in agreement with multiple *in vitro* studies that demonstrate that cells infected with DENV secrete high numbers (~30%) of immature particles containing unprocessed prM (Anderson et al., 1997; Bray & Lai, 1991; Cardoso et al., 2002; Henchal et al., 1985; Randolph et al., 1990; Zybert et al., 2008). Furthermore, DHF/DSS patients have elevated antibody responses against prM (Lai et al., 2008), which suggests that immature DENV particles, although appearing non-infectious in classical titration assays (Zybert et al., 2008), might be involved in the pathogenesis of DHF.

The second objective in this study was to investigate the effect of anti-prM antibodies on the infectivity of immature DENV particles, generated in furin-deficient LoVo cells with the DENV-2 16681 strain (Zybert et al., 2008). In **Chapter 5**, we demonstrate that immature DENV particles opsonized with monoclonal antibody 70-21 (mAb 70-21) directed against prM productively infect Fc receptor-expressing K562 cells. In fact, the infectivity of immature DENV is nearly restored to the level of wild-type DENV upon addition of mAb 70-21. Remarkably, this antibody-dependent enhancement (ADE) of infection is observed in a broad range of antibody concentrations, even at conditions of antibody excess.

To elucidate how mAb 70-21 renders the immature particles infectious, we have investigated different stages in the cell entry process (**Chapter 5**). First, the binding properties of immature particles to K562 cells were examined in the presence and absence of mAb 70-21. When opsonized with mAb 70-21 antibodies, the binding of immature particles to the cells significantly increases by a factor of 30, which is most likely due to the interaction with the Fc receptors. Indeed, treatment of cells with anti-CD32 antibodies to block Fc $\gamma$ R2 completely abrogates ADE of infection, indicating that the antibodies facilitate cell entry of immature particles via Fc receptor-mediated phagocytosis. Yet, immature particles are not fusogenic as prM withholds the E protein from undergoing the conformational changes that enable membrane fusion (Elshuber et al., 2003; Yu et al., 2008). Hence, Fc receptor-mediated delivery of the immature particles into the cell does not entirely resolve how immature particles become infectious. In an infected cell, furin cleaves the prM proteins of immature particles into M proteins and pr peptides in the trans-Golgi network (TGN), which generates mature and infectious virus particles. Interestingly, furin, although predominantly located in the TGN, also shuttles between early endosomes and the plasma membrane (Molloy et al., 1999). To investigate the potential involvement of furin in the infectious entry of immature particles, we have evaluated the effect of a furin inhibitor on ADE of infection. Inhibition of furin entirely abolishes the infectivity of antibody-opsonized immature particles, which demonstrates that maturation through furin cleavage is an obligatory step in the infectious entry pathway of immature particles.

Furin cleavage of immature particles during cell entry presumably occurs within acidic endosomes, since immature particles first need to undergo a conformational change induced by exposure to low pH in order to be cleaved by furin (Yu et al., 2008). Probably, the anti-prM antibodies have dissociated from the immature particles when furin cleavage takes place, as they most likely would sterically hinder this process. Interestingly, a recent study has shown that following furin cleavage, the pr peptides remain associated with the E proteins, until the virus particles are exposed to a neutral pH (Yu et al., 2008). By this mechanism, the virus particles are protected from undergoing premature fusion in the mildly acidic secretory pathway, which would abort the viral life cycle. Upon cell entry however, the virus particles do not encounter a neutral pH anymore once they are cleaved by furin in acidic endosomes. In order to explain how the virus particles can rearrange their E proteins to the fusion-active conformation, one may speculate that the pr peptides dissociate from the virus

upon further acidification later in the endocytic pathway. Another interesting possibility is that the pr peptides stay attached to the anti-prM antibodies, thereby releasing a fusogenic particle. Future studies should resolve the precise sequence of these events during the infectious cell entry of immature particles.

In summary, our results suggest that immature DENV particles, although generally considered as an irrelevant byproduct of infection, in fact behave as veiled pathogens that are highly infectious when opsonized with antibodies. The main reason for the lack of infectivity in the absence of antibodies appears to be attachment of the immature particle to the cell. Once cellular binding and internalization is facilitated by antibodies, immature particles are processed by furin and subsequently productively infect the cell.

## **Perspectives**

The first studies described in this thesis elucidate the cell entry pathway of DENV. We have shown that DENV requires clathrin-mediated endocytosis and needs to travel to late endosomes to undergo membrane fusion for delivery of its genetic material into the cell cytosol. Moreover, these studies have provided unique information on the mechanisms and kinetics involved in DENV cell entry. It will be of particular interest to investigate whether DENV also utilizes this route of entry into human target cells, such as dendritic cells, macrophages, and monocytes. Furthermore, the entry characteristics of DENV appear to be strain-dependent (**Chapter 4**, Krishnan et al., 2007), which will be very interesting to further explore at a single-particle level.

During secondary dengue episodes, pre-existing anti-E antibodies are believed to alter the cell entry pathway of DENV in human target cells and thereby control the outcome of viral infection, as discussed in **Chapter 2**. Antibodies might neutralize infection of Fc receptor-bearing cells by blocking the conformational changes in the E proteins required for membrane fusion from within the endosome, which consequently leads to degradation of the virus particles further down the endocytic pathway. During enhancement of infection on the other hand, the virus particles must escape from degradation in the endocytic pathway. ADE occurs at subneutralizing antibody concentrations, and under this condition not all E proteins of the virus particles might be occupied by antibodies. Also, antibodies with a low affinity might mediate enhancement of infection through detachment from the virus in the acidic lumen of the endosome. In both scenarios, the unoccupied E proteins can undergo the conformational changes resulting in membrane fusion and ADE.

Further research should provide a better insight into the internalization route, endocytic trafficking, and, when appropriate, the organelle composition promoting membrane fusion of antibody-opsonized DENV particles, which is essential for understanding the difference between virus neutralization and ADE leading to DHF.

Additionally, it will be of particular interest to further explore the cellular responses mediated by DENV either through signaling via the virus receptor or via the Fc receptor. Following attachment of the virus, the different receptors might guide the particle along divergent endocytic pathways and activate distinct signalling cascades leading to different cellular responses. Recent studies have demonstrated that, compared to unbound DENV, antibody-opsonized particles stimulate the secretion of TNF $\alpha$ , IL-6, and IL-10, and suppress the nitric oxide (NO) production, while the percentage of infected cells was similar (Boonnak et al., 2008; Chareonsirisuthigul et al., 2007). Notably, these *in vitro* data mirror the clinical observations that the NO concentration is reduced in the sera of DHF patients, whereas the cytokine levels are elevated (Chareonsirisuthigul et al., 2007). A decrease in the NO production might contribute to the high viral load in the prelude of DHF, as this mediator impairs viral replication through inhibition of NS5 activity (Charnsilpa et al., 2005; Takhampunya et al., 2006). The cytokines on the other hand most likely operate in the strong immune response and the development of plasma leakage.

Immature particles have often been perceived as a non-infectious, irrelevant byproduct of DENV infection. Indeed, in individuals that do not have pre-existing immunity to DENV, immature particles presumably are of minor significance in the course of infection as they lack the ability to infect cells (Zybert et al., 2008). Yet, our observation that an antibody directed against prM can render immature particles infectious has major implications for the pathogenesis of disease during secondary or sequential dengue episodes. Anti-prM antibodies, including those recognizing the same epitope as mAb 70-21, are frequently detected in sera of DHF/DSS patients (Bray & Lai, 1991; Cardoso et al., 2002; Lai et al., 2008; Se-Thoe et al., 1999). When the subpopulation of immature DENV particles is mobilized by the anti-prM antibodies, the number of infected cells most likely increases, which might contribute to progression towards severe disease. To verify whether immature particles indeed participate in the disease pathogenesis, future studies should determine the content of immature particles in humans during DENV infections and further explore the antibody responses to prM. Additionally, it will be very interesting

to investigate if anti-E antibodies can mediate ADE of infection of immature particles. Also, future research should illustrate whether antibody-mediated uptake of immature particles leads to different cellular responses than that of mature particles.

Knowledge on the cell entry properties of a virus can contribute to the rational design of therapeutics. Based on the studies presented in this thesis, an ideal drug for treatment of primary and sequential DENV infections should interfere with both clathrin-mediated uptake as well as Fc receptor-mediated phagocytosis of virus particles. As it has a crucial role in the life cycle of DENV, furin could be a strategic target for a therapeutic agent. In the first round of infection, a furin inhibitor would block maturation of the virus particles in the secretory pathway, which consequently leads to the release of immature particles exclusively. Subsequently, a furin inhibitor would prevent the maturation of immature particles during cell entry in the second round of infection, and thereby abrogate the productive life cycle of DENV. Since furin inhibitors affect a cellular protein, it is less likely that the virus becomes drug-resistant through mutations. Interestingly, a broad spectrum of furin inhibitors have already been reported in literature (Jiao et al., 2006), since this endoprotease has also been implicated to play a role in Alzheimer's disease, cancer, and the activation of bacterial and viral pathogens (Thomas, 2002). It will be interesting to test the effect of these compounds on DENV infection.

Our observation that the anti-prM antibody mAb 70-21 enhances the infectivity of immature particles, even at conditions of antibody excess, might have important repercussions for vaccine development. The presence of mAb 70-21, irrespective of the concentration, might have adverse effects on disease protection. However, some studies have reported that other anti-prM antibodies, although lacking neutralizing activity *in vitro*, can protect mice from a lethal challenge with DENV (Falconar, 1999; Kaufman et al., 1989). Future studies are required to reveal the precise correlates of protection in DENV infections, which is crucial for the development of a safe, tetravalent dengue vaccine.

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