CHAPTER 9
Summarizing Discussion and Future Perspectives
Introduction

Chikungunya virus (CHIKV) and dengue virus (DENV) are two mosquito-borne viruses which cause major epidemics in the tropical and the subtropical regions of the world\textsuperscript{1,2}. The majority of human CHIKV infections lead to chikungunya fever, a self-limiting febrile illness which is hallmarked by highly debilitating joint pains\textsuperscript{3}. In a substantial percentage of infected individuals these joint pains may persist or recur for months to years after initial infection; yet, the exact recurrence rates vary considerably between studies (12\% - 80\%)\textsuperscript{4–8}. In contrast, the majority of DENV infections are asymptomatic\textsuperscript{1}. Symptomatic DENV infections range from relatively mild dengue fever to severe manifestations as plasma leakage and hypovolemic shock\textsuperscript{1}. When not properly treated severe dengue can be fatal.

Studying the viral life cycle will help to identify host molecules that are important for virus infection. Consequently, these studies likely aid in the rational design of antiviral compounds. Special attention should be given to the early steps in virus infection. This as inhibition of virus cell entry prevents virus-induced cell-death and the presentation of viral antigens to the immune system. Furthermore, investigating how the human body reacts to and overcomes these viral infections, or why in some instances it fails to do so, will aid in the elucidation of the mechanisms behind severe/persistent disease. Only when we understand these mechanisms, we can start to effectively avert severe manifestations of disease.

In this thesis, we aimed to study the virus-host interactions of both CHIKV and DENV, with special attention to the first steps of the viral life cycle: virus cell entry. In this chapter, I will summarize and discuss the results obtained in our work. Similar to the structure of the thesis, in the first part of the summarizing discussion I will discuss our work on CHIKV, whereas in the second part I will focus on DENV.

Part I: Early events in Chikungunya infection

CHIKV initially caused minor outbreaks in Africa and South-East Asia and therefore not much was known about the virus when it rapidly emerged in 2004\textsuperscript{3}. Consequently, the first tentative descriptions of the CHIKV life cycle, including CHIKV cell entry, were primarily based on reports from the closely related and more thoroughly studied alphaviruses Semliki Forest virus (SFV) and Sindbis virus (SINV)\textsuperscript{3}. At the start of this thesis research, however, few reports on the CHIKV cell entry pathway were published which deviated from the standard view on alphavirus cell entry and more importantly contradicted with each other\textsuperscript{9–12}. Therefore, in part I of this thesis we aimed to dissect the CHIKV cell entry mechanism in detail.
The CHIKV cell entry pathway
Most alphaviruses, including SFV, SIN and Venezuelan Equine Encephalitis Virus (VEEV), are known to enter host cells via clathrin-mediated endocytosis\textsuperscript{13–15}. Already in the 1980s, Helenius \textit{et al.} described that SFV entered the cell in coated vesicles, which were later determined as clathrin-coated vesicles\textsuperscript{13}. For SIN, there was more debate on the cell entry mechanism. A few groups reported direct penetration of the virus at the plasma membrane\textsuperscript{16–18}, whereas others showed that SIN enters via receptor-mediated endocytosis and subsequently fuses from within the endolysosomal system in response to acidic pH\textsuperscript{19–21}. In the late 1990s, DeTulleo and Kirchhausen described that SIN, similar to SFV, enters cells via CME\textsuperscript{14}. Several later reports confirmed these findings\textsuperscript{11,22}.

At the start of my research the cell entry pathway hijacked by CHIKV was also under debate. CHIKV cell entry into the \textit{Aedes albopictus} cell line C6/36, the mammalian cell lines U2-OS and primary human umbilical vein endothelial cells was found to be mediated by clathrin\textsuperscript{11,12}. In contrast to this, Bernard \textit{et al.} proposed that CHIKV entered HEK cells via a non-classical endocytic pathway dependent on Eps15 and AP-2, but independent of clathrin\textsuperscript{10}. Importantly, all these studies solely relied on the use of perturbations to define the CHIKV cell entry pathway. As described in \textbf{Chapter 1}, many perturbations can exert off-target effects and the obtained results may be hard to interpret. This especially holds true when the effects of the perturbations are assessed at a late step of infection, as was done in these studies. The use of microscopy has the benefit that virus cell entry can be studied in a perturbation-free environment. Yet, as microscopy also has its own disadvantages (see \textbf{Chapter 1}), we believe that virus cell entry is best studied by using a combination of microscopy and perturbation approaches. Therefore, in \textbf{Chapter 3}, we re-evaluated the cell entry pathway of CHIKV. Live-cell tracking of single virions revealed that the majority of CHIKV particles enter mammalian BS-C-1 cells via CME. The most convincing evidence is based on the observation that clathrin moved together with the virus over time. Furthermore, perturbation of CME via pharmacological inhibitors and siRNAs was able to efficiently block CHIKV fusion and infection. Intriguingly, we observed that siRNA-mediated knockdown of CHC inhibited CHIKV infection in HeLa cells, whereas previously Bernard \textit{et al.} found no effect in HEK nor HeLa cells\textsuperscript{10}. Furthermore, and in line with our observations in Chapter 3, a very recent publication of Karlas \textit{et al.} also showed that clathrin is important for CHIKV infection of HEK cells\textsuperscript{23}. The observed differences between the studies are puzzling but might be explained by slight differences in the experimental set-up. For example, the exact lineage of HEK293 (HEK-293T versus HEK-293) and the used siRNAs differed. Moreover, the MOIs of infection might have differed, although this remains undefined as the MOI used in the study of Karlas \textit{et al.} was not reported\textsuperscript{10,23}. In my opinion, however, the most likely cause for these differences lies within the genetic make-up of the virus. RNA viruses readily adapt to their environment and therefore it is possible that although the same virus strain is used in the experiments, there are differences at the amino acid
level. These differences may in turn affect the ability of the virus to interact with a cell surface molecule and potentially influence the route of cell entry. Indeed, an earlier report showed that SINV adapted to heparin sulfate binding within three passages in cells. Furthermore, a DENV strain with a high affinity towards heparin sulfate was found to enter cells in a clathrin-independent manner, whereas DENV particles with a low affinity towards heparin sulfate entered the same cell type via CME.

Furthermore, in Chapter 3 we described that not all CHIKV particles that fused first co-localized with clathrin. While 89% of the virus particles co-localized with clathrin before fusion, the remaining 11% of the virus particles did not show any clathrin co-localization before fusion. We thus wondered why in the latter cases no clathrin co-localization was observed. A possible explanation is that we missed co-localization between clathrin and CHIKV due to technical insufficiencies. Alternatively, the virus may be able to hijack multiple endocytic pathways to enter the cell. Indeed, other viruses including Influenza virus (IAV), Human Immunodeficiency Virus (HIV) and DENV, have been described to use multiple pathways to enter the cell. Moreover, in correspondence to our data, the group of Ari Helenius also found that a small percentage of SFV virions hijack other endocytic pathways than CME to enter the cell (personal communication). What then determines which cell entry pathway the virus hijacks? As above, I propose that the virus receptor, or in the case of CHIKV attachment factor, is the most logical candidate. As described in Chapter 2, multiple attachment factors have been defined for CHIKV, yet no bona fide entry receptor has been identified. Whereas multiple attempts have been undertaken to elucidate alphavirus entry receptors, to date only for SINV a bona fide entry receptor has been discovered. Therefore, I hypothesize that most alphaviruses do not need to bind a specific receptor to enter the cell. Binding of the virus to attachment factors and subsequent concentration of the virus on the plasma membrane will likely suffice to initiate entry. Most plasma membrane ‘receptors’ are preferentially internalized into the cell via a specific endocytic pathway, yet the preferred pathway differs per molecule and activation state. Therefore, binding to for example ‘attachment factor A’ might guide the virus to CME, whereas binding to others may direct the virus to another pathway. Indeed, this has been observed for DENV. To summarize, based on all the data that is available now, I propose that CME is the preferred route of entry for CHIKV. This conclusion is largely based on our live-cell imaging data obtained in a perturbation free-environment. Yet, I would like to stress the importance of confirming the CHIKV cell entry pathway in natural target cells using virus with low passage numbers. Furthermore, future studies should dissect if CHIKV cell entry is indeed dictated by the attachment factor and if the virus can productively infect cells via clathrin-independent pathways.

After cell entry, alphaviruses are delivered to the endolysosomal pathway. Alphaviruses primarily fuse from within mildly acidic early endosomes, with the exception of VEEV, which fuses from more acidic late endosomal compartments. Although multiple studies confirmed that CHIKV requires low pH exposure for fusion and infection,
the exact organelle with which CHIKV fuses has not been studied extensively. Bernard et al. described that CHIKV required Rab5-positive early endosomes but not Rab7-positive late endosomes for productive infection of HEK cells, indicating that CHIKV fusion occurs from within early endosomes. In the mosquito cell line C6/36, however, the integrity of the Rab7 compartment was required for CHIKV infection, which is suggestive for trafficking to late endosomes for fusion. The data described above was obtained using dominant negative mutants (DNMs) of Rab5 and Rab7. As described in Chapter 1 and Chapter 3 DNMs of the Rab proteins have two important drawbacks. Firstly, the extent of overexpression required for sufficient knockdown of the protein function is difficult to assess. Secondly, overexpression, especially of early endosomal marker Rab5, might be cytotoxic. In order to circumvent these drawbacks we tracked single DiD-labeled virions in cells overexpressing the natural form of Rab5 and Rab7 fused to GFP. The great benefit of this approach is that it not only provides us with the location and organelle of fusion, it also reveals the kinetics. In Chapter 3, we showed that in mammalian BS-C-1 cells CHIKV primarily fuses from within early endosomal compartments. Indeed, 95% of the CHIKV particles fused from a Rab5-positive compartment. A minority (17%) of CHIKV particles fused in co-localization with Rab7, implicating that these particles fuse from maturing (Rab5- and Rab7-positive) endosomes or late (Rab7-positive) endosomes. Together, the above studies show that in mammalian cells CHIKV primarily fuses from within early endosomes. Yet, based on the results of Lee et al. I propose that the organelle of fusion may differ between insect and mammalian cells. This notion might be related to reported differences in membrane composition between endosomes in insect and mammalian cells. However, additional studies, such as single particle tracking of CHIKV in mosquito cells, are required to confirm this hypothesis.

Interestingly, when assessing the kinetics of CHIKV fusion we noticed that 40% of CHIKV particles fused almost immediately upon entrance into the early endosome. The other 60% resided inside the endosome between 20 and 150 seconds prior to fusion. The difference in fusion kinetics might be caused by the acidity of the endosomal lumen at the moment the virus enters the endosome. When CHIKV enters an endosome with a pH of 5.9 or below, the virus will likely directly fuse as the pH threshold for CHIKV fusion is pH 5.9 (Chapter 3). However, when the virus enters an endosome with a pH above its threshold it has to await acidification before it can fuse. In line with this, it has been reported that different populations of early endosomes exist. Early endosomes can be subdivided into a ‘static’ and a ‘dynamic’ population, which show different rates of maturation. As endosomal maturation is linked to the pH inside the endosomal lumen, I expect that the pH inside rapidly maturing endosomes lowers quicker than the pH inside slowly maturing endosomes. Consequently, I propose that the observed differences in CHIKV fusion kinetics might be related to the maturation state of the endosome, and possibly the endosomal subpopulations. To address this hypothesis multiple-color tracking studies using fluorescently-labelled CHIKV and Rab5 in combination with pH-dependent fluorescent dyes may be performed.
**CHIKV infection and the cytoskeleton**

In Chapter 4 we described a relationship between CHIKV infectivity and the microtubule cytoskeleton. Microtubules were found to be important in the first 30 minutes of CHIKV infection, indicating that they might play a role during virus entry. Subsequent imaging of CHIKV cell entry revealed that half of the CHIKV particles indeed showed microtubule-dependent movement before fusion, while the other half of the CHIKV virions remained quite static until fusion. Microtubule-dependent movement primarily occurred after CME but before delivery to early endosomes. Interestingly, however, disruption of the microtubule-dependent CHIKV transport did not affect the extent of fusion. Yet, the cellular location of fusion was altered. Additionally, we observed that disruption of the microtubule cytoskeleton impaired CHIKV genome release.

Under normal infection conditions, CHIKV fusion was observed both in the periphery and the perinuclear region of the cell. Almost two-third of the particles that showed microtubule-dependent movement, fused in the perinuclear region. However, upon microtubule disruption, fusion in the perinuclear region was almost completely abrogated. These data suggest that fusion in the perinuclear region might be a perquisite for efficient genome delivery and infection. However, it seems counterintuitive that fusion in the perinuclear region is required for initiation of CHIKV infection. This as CHIKV replication occurs at plasma membrane, and the majority of the CHIKV particles fuse close to the plasma membrane in the cell periphery. Furthermore, albeit with low efficiency, artificial fusion of CHIKV with the plasma membrane was found to initiate a productive infection in HEK cells.

Yet, as described above, two subsets of early endosomes have been described to date, which differ in their motility and maturation kinetics. Approximately 65% of the early endosomes are described to be quite ‘static’ as they do not move on microtubules and mature relatively slowly. The other 35% are considered ‘dynamic’, as these organelles move along microtubules and rapidly evolve into maturing endosomes. Cargo-containing vesicles that show microtubule-dependent movements were found to specifically target their cargo towards dynamic early endosomes. Consequently, microtubule-dependent trafficking might target CHIKV particles to dynamic endosomes, an organelle that may promote efficient genome release. This concept, however, holds one flaw. CHIKV infection in HEK cells was found to be dependent on the clathrin adaptor AP-2, whereas microtubule-dependent targeting of cargo towards the dynamic early endosome was found to correlate with AP-2-independent cell entry. To address this hypothesis, we first need to determine whether CHIKV infection in BS-C-1 cells is dependent upon AP-2 and whether the virus is delivered to the dynamic population of early endosomes. Moreover, as most of the knowledge on the AP-2 sorting mechanism and the endosomal subpopulations is based on one study, a more thorough characterization of these mechanisms is required to proof or reject this hypothesis. A first and more direct way to proof the concept that the organelle
and/or location of fusion is important for genome release is by tracking single CHIKV particles of which both the envelope and the viral genome or capsid are fluorescently labelled. In this way, the fate of the viral genome or capsid can be tracked post-fusion and it can thus be assessed whether the viral genome is more efficiently released in the perinuclear region than in the periphery.

Another and in my opinion more rational explanation to our findings is that microtubules are needed for uncoating of the CHIKV nucleocapsid. Nucleocapsid uncoating of multiple viruses, including HIV and IAV, was described to rely on an intact microtubule network. HIV primarily enters the cell via direct penetration at the plasma membrane. Once inside the cell, the viral core needs to be transported towards the nucleus and uncoated. Uncoating of the HIV core was found to be mediated by the microtubule motors dynein and kinesin-1. The authors proposed three different mechanisms via which microtubule motors could induce nucleocapsid uncoating. Firstly, as dynein and kinesin-1 traverse the microtubule in opposite directions, the HIV core could be ‘pulled apart’ by the mechanical forces generated in opposite directions. Secondly, interactions with the motor proteins may situate the core in the optimal location for uncoating. Finally, uncoating might be mediated by a microtubule-associated proteins (MAP). In this scenario, microtubules are required for facilitating the interactions between the MAP and the viral capsid. Comparable processes were described during the early steps of IAV infection. Upon fusion from within the late endosome, the IAV capsid recruits components of the aggresome processing machinery, including the microtubule motor dynein and the actin-traversing protein myosin 10, to aid in capsid uncoating. Disruption of both the microtubule and actin cytoskeleton was found to affect uncoating. Similar to HIV, it was suggested that the IAV capsid was broken apart by the opposing physical forces generated by the motor proteins. In Chapter 4 we showed that the microtubule cytoskeleton is primarily required in the first 30 minutes of infection. Furthermore, our results show that microtubule disruption does not affect the overall extent of membrane fusion. The latter is in line with Bernard et al. who suggested that microtubules act at a post-fusion step of the CHIKV life cycle. In fact, our data suggests that microtubules are required in nucleocapsid uncoating. Previous reports on alphavirus uncoating described that interactions between the nucleocapsid are ribosomes are sufficient to induce uncoating. Interestingly, a considerable proportion of the ribosomes are associated with the microtubule cytoskeleton, implying that for CHIKV microtubules might facilitate the interactions between the nucleocapsid and microtubule-associated ribosomes. Confocal or electron microscopy studies can be performed to visualize whether microtubules, ribosomes and the CHIKV nucleocapsid are indeed associated. Alternatively, it would be interesting to see if disruption of the interactions between ribosomes and microtubules would affect CHIKV genome release.
Concluding remarks

Due to the continued expansion of the CHIKV vector, the naïve immune state of the human population in most parts of the world and the debilitating symptoms of CHIKV infections, development of a vaccine or antiviral treatment is of great importance. Rational design of anti-CHIKV therapeutics requires good understanding of the virus-host interactions. In Chapter 3, we determined that CHIKV, like most other alphaviruses, primarily enters its target cells via CME. Subsequently, the virus is transported towards the Rab5-positive early endosome from where CHIKV fuses. Neither clathrin nor the early endosomal marker Rab5 are good targets for the design of therapeutics, due to their essential roles in cell homeostasis. However, as described in Chapter 1 endocytic pathways are not static. Instead, the endocytic pathways make use of different modules and adaptors to efficiently internalize their cargo. I believe that the ‘choice’ of these modules might be based on the receptor to which the cargo binds. It would thus be worth-while to further look into the CME-adaptors required for CHIKV infection. Firstly, this would enhance our understanding on clathrin-mediated endocytosis in general. Secondly, if these adaptors are not essential for CME as such, they may proof suitable targets for the development of antivirals.

That early endosomes might be a viable target for the development of antivirals was recently demonstrated. Mainou et al. found that serotonin receptor agonists block CHIKV infection, likely by temporarily dispersing early endosomes. However, as endosomes are critical for endocytosis the effect of prolonged serotonin receptor agonists treatment, and thus perhaps prolonged endosome dispersion, on cell viability and infection still need to be addressed. In Chapter 4 we suggested that CHIKV may need to enter a specific early endosome subtype for efficient genome release. Although different subsets of early endosomes were described, they remain poorly characterized. To date, it is unknown whether and how these subsets differ from each other in their membrane composition and use of adaptors. If our hypothesis could be confirmed, it would be important to further characterize this endosomal subtype and the endosomal targeting events, as they can perhaps be selectively blocked to prevent CHIKV infection.

The requirement of microtubules early in infection is highly intriguing, yet demands further characterization. My best guess would be that microtubules are required for capsid uncoating by facilitating interactions between ribosomes and the CHIKV nucleocapsid. As requirement of microtubules for infection does not seem to be a general feature of alphaviruses, the next step would be to confirm our findings in other cell lines and primary target cells. If the dependency on microtubules is a general characteristic of CHIKV infection, short-term treatment with microtubule depolymerizing agents might be considered as a potential anti-CHIKV treatment. This, as microtubule-targeting agents are already used as anti-parasitic and anti-cancer targets and are being tested as therapeutics against neurodegenerative diseases. However, as CHIKV infection
is generally self-limiting, and long-term treatment with microtubule-targeting drugs affects the proliferative effects of the cells and might thus cause adverse side effects\textsuperscript{53}, it is obvious that the benefits of the use of these therapeutic agents should outweigh the downsides.

To conclude, Chapter 3 and 4 once again underline the benefits of single virus tracking studies over perturbations studies. Whereas, also in our hands, perturbations can sometimes give ambiguous results, studying CHIKV cell entry in a perturbation-free environment gives a more clear view on the events that occur during CHIKV cell entry. Moreover, additional information, including kinetic parameters of virus entry, can be obtained.

**Part II: DENV infection of natural target cells in absence and presence of antibodies**

To date, the four different dengue virus serotypes (DENV-1-4) cause the most common arthropod-borne viral infection in the world\textsuperscript{1}. While approximately 75\% of all DENV infections are subclinical, an estimated 96 million individuals experience symptomatic disease per year\textsuperscript{54}. Clinical dengue ranges from relatively mild flu-like symptoms to severe and potentially life-threatening manifestations like plasma leakage and hypovolemic shock\textsuperscript{1}.

Intriguingly, whereas the majority of primary DENV infections are relatively mild, secondary infections with a heterologous DENV subtype are correlated with severe disease. The increased occurrence of severe disease during a heterologous secondary DENV infection can be explained by the phenomenon ‘original antigenic sin’. During a heterologous secondary DENV infection, memory B- and T-cells are rapidly activated, yet their antiviral response is more directed to the DENV serotype of the primary infection, the ‘original antigen’, than to the DENV serotype of the current infection. This aberrant immune response leads to high numbers of cross-reactive antibodies and low-affinity T-cells that do not efficiently clear the virus\textsuperscript{55–59}.

Already in the 1970s, Halstead \textit{et al}. observed that declining levels of maternal anti-DENV antibodies correlated with severe disease in infants during primary DENV infection\textsuperscript{60}. This together with the observation that individuals experiencing a heterologous secondary infection have an increased risk of developing severe disease led to the hypothesis of “antibody-dependent enhancement” (ADE) of DENV infection\textsuperscript{60}. Multiple studies confirmed that non-neutralizing concentrations of anti-DENV antibodies can indeed enhance DENV infection \textit{in vitro} and \textit{in vivo}\textsuperscript{61–64}. Consequently, ADE is now considered an important contributor to severe disease. At the start of this thesis, the exact mechanisms of ADE of DENV infection were however still elusive. Therefore, the main aim of part II of this thesis was to study how DENV enters natural target cells in
presence and absence of enhancing concentrations of antibodies. Moreover, we aimed to dissect the molecular mechanism behind ADE of DENV infection.

**DENV cell entry in macrophage-like cells**

As outlined in *Chapter 5*, the cell entry pathway of DENV has been extensively studied in a variety of cell lines \[^65-73\]. DENV was described to enter its target cells predominantly via CME, though some cell type- and virus-specific variations have been observed\[^25,66,71\]. However, the DENV cell entry pathway was never thoroughly studied in natural target cells such as monocytes, macrophages or DCs. In *Chapter 8*, we studied the DENV cell entry pathway in the mouse macrophage-like cell line P388D1 using a combination of perturbation and live-cell imaging techniques. We observed that DENV cell entry into P388D1 cells strongly depends on Eps15 and dynamin, yet was independent of cellular cholesterol and PI3K. Overall, the perturbation profile pointed towards cell entry via CME. However, as described in *Chapter 1*, a perturbation profile as such is insufficient to make a strong conclusion about the virus cell entry pathway. This as dynamin is important in many cell entry pathways (*Chapter 1*) and Eps15 is not strictly related to CME\[^31\]. Therefore, we also attempted to perform single virus tracking in the presence of fluorescently-labelled endocytic marker proteins. Unfortunately, the low transfection efficiency of the plasmids in the cells, which led to rapid bleaching of the fluorophore, in combination with the thickness of the cells made it impossible to study the DENV cell entry pathway into P388D1 using live-cell single particle tracking techniques.

Intriguingly, we also observed deviations from classical CME in the perturbation profile. For example, DENV cell entry was found to be dependent on the actin cytoskeleton. Although actin dynamics are usually not required during CME, actin has been reported to support in the formation and scission of the clathrin-coated vesicles when elongated or odd-shaped particles are taken up\[^74-76\]. However, as DENV is a small spherical particle of approximately 50 nm in size, the involvement of actin in CME of DENV is not expected. Instead, when we zoomed in at the plasma membrane using live-cell fluorescent microscopy we observed that actin actively forms membrane protrusions which seem to aid in the uptake of the virus particles. As described in *Chapter 1*, the formation of membrane protrusions are a hallmark of macropinocytosis and phagocytosis. However, direct involvement of both pathways could be excluded based on the independency on kinase PI3K and the absence of fluid phase uptake. The ability of viruses to enter via non-classical entry pathways, which display features of different established endocytic pathways, has been described before\[^77,78\]. Hence, based on these data we propose that DENV cell entry in P388D1 cells involves a novel endocytic mechanism with elements of both clathrin-mediated endocytosis and phagocytosis.

Like most endocytic cargo, DENV is delivered to the endolysosomal pathway after internalization. We and others showed before that DENV primarily ends-up in late endosomal compartments were membrane fusion occurs\[^69,70,73,79\]. DENV fusion from
within early and recycling endosomes have also been reported\textsuperscript{65,79}. In Chapter 8, we determined the organelle from which DENV fusion occurs in P388D1 cells by use of dominant negative Rab mutants. In line with literature, we found that DENV fusion was reliant on functional Rab7, indicating that the virus fuses from the late endosome. Intriguingly, however, dominant negative Rab5 had no effect on DENV fusion, suggesting that the virus follows a non-classical intracellular trafficking route in which it bypasses the early endosomal compartment. These results are in contrast with the previous studies on DENV intracellular trafficking, which report that DENV passes through the early endosomal compartment during intracellular trafficking\textsuperscript{65,69,70,73,79}. Moreover, they contradict with the standard view of endocytic trafficking, in which cargo first has to be transported through the early endosomes in order to reach the late endosomal compartment (Chapter 1). We attempted to confirm this finding using dual-color single particle tracking but due to the low transfection efficiency and thickness of the cells this was impossible. As described before in this thesis (Chapter 1), usage of DNMs of Rab proteins comes with some important drawbacks. One of those drawbacks is that it is hard to determine how much overexpression is required to efficiently knock-down protein function. It is thus possible that due to the low transfection efficiency of P388D1 insufficient Rab5 knock-down was achieved and therefore no effect was seen. However, as the Rab5 construct did cause inhibition of fusion when we infected the cells with antibody-opsonized virus (Chapter 8), this scenario is unlikely. An alternative explanation is that DENV indeed uses a ‘novel’ intracellular trafficking pathway. To our knowledge, to date Rab5-independent endosomal trafficking has only been described for Lymphocytic Choriomeningitis Virus (LCMV)\textsuperscript{78}. Interestingly, the cell entry pathway of LCMV, like DENV, cannot be categorized to one of the known endocytic pathways. Therefore, it is tempting to speculate that entry via non-classical endocytic pathways is related to Rab5-independent endocytic trafficking. Future studies should reveal whether these variations on the classical endocytic pathways naturally exist or are perhaps virus-induced.

Taken together, in Chapter 8 we showed that DENV enters macrophage-like cells via a novel endocytic pathway resembling both CME and phagocytosis. Next, the virus is directed towards the late endosome, from where it fuses. As both the entry and trafficking pathways deviate from the original ‘textbook view’, more work is required to confirm and further characterize these mechanisms. To assess whether CME is indeed involved in DENV entry into P388D1 cells, the interactions between DENV and clathrin-coated pits (CCPs) may be visualized using EM. Additionally, EM can possibly also be used to assess whether membrane protrusions deliver the ‘captured’ virus particles to adjacent CCPs. Alternatively, confocal laser scanning microscopy or TIRF may be used to assess clathrin-DENV co-localization on fixed samples. This as both techniques have an improved resolution an cause less photobleaching compared to epi-fluorescence microscopy. Likewise, to confirm that DENV indeed bypasses the early endosome, the
co-localization of early endosomal markers and DENV can be determined using confocal microscopy in fixed samples. Moreover, although cumbersome to work with, my next step would be to corroborate our findings in primary human target cells.

**The role of immature DENV particles in primary and secondary DENV infections**

Both DENV-infected mammalian and insect cells secrete a mixture of mature, immature and partially immature particles (Chapter 5 and 80). It has been published that the proportion fully immature particles can reach up to 45% in the Aedes albopictus-derived cell line C6/3680. Furthermore, over 90% of the DENV particles secreted from C6/36 cells have some degree of immaturity, as they can be captured by anti-prM antibodies80. However, the exact proportion of immature virions varies between cell types80–84. We and others previously showed that DENV maturation is important for infectivity63,84,85. Whereas mature DENV virions are infectious, fully immature particles are unable to bind to many cell types and are consequently virtually non-infectious. Partially immature virions on the other hand can still be infectious86, yet the degree of maturation required for infectivity is unknown.

Interestingly, it was recently shown that immature West Nile Virus (WNV), a flavivirus closely related to DENV, can infect cells in a DC-SIGN-dependent manner86. DC-SIGN is also described to be an important attachment factor for DENV (Chapter 5 and 87,88) and is highly expressed on immature Dendritic Cells (DCs)89, which are natural target cells of DENV infection90. Additionally, other DENV target cells like mature DCs and specific subpopulations of tissue-specific macrophages also express DC-SIGN, albeit to a lower extent91. When assessing the infectivity of immature DENV in DC-SIGN-expressing cells we observed that immature DENV particles can infect cells in a DC-SIGN-dependent manner (Chapter 6). DC-SIGN-mediated infection was observed for immature preparations of DENV-1, 2 and 4, suggesting that it is a general property of all four DENV serotypes. Yet, the extent by which DC-SIGN enhanced infection differed between serotypes; whereas expression of DC-SIGN enhanced productive infectivity of immature DENV-1 by almost 5 orders of magnitude, the infectivity of immature DENV-2 and DENV-4 were only enhanced by 2 to 3 orders of magnitude. The observation that DENV-1 is more dependent on DC-SIGN for infection has been reported before and was suggested to be related to different glycosylation patterns between the DENV serotypes88. It has been suggested that the E protein of DENV strains with high affinity to DC-SIGN (DENV-1 and DENV-3) are glycosylated at Asn-67 and Asn-153, whereas the E protein of DENV strains with lower affinity to DC-SIGN (DENV-2 and DENV-4) are only glycosylated at Asn-6788,92. However, other studies did not find differences in DC-SIGN dependency between the DENV serotypes93,94 and a more recent study on DENV glycosylation suggests that all DENV serotypes have similar glycosylation patterns95. Therefore, it remains to be determined why we and others observed a more extensive enhancement of virus production for DENV-1 compared to DENV-2 and -4.
Immature particles were previously considered irrelevant for disease pathogenesis during primary infection. However, our findings combined with previous reports that immature DENV can also use claudin-1 as an attachment factor\textsuperscript{96,97} imply that immature particles might contribute to viremia during primary infection. Immature particles, which are produced to a large extent in insect cells\textsuperscript{100}, can likely initiate infection via binding to DC-SIGN expressed on immature DCs. Indeed, several publications confirm that immature DCs are the most susceptible cell type for mosquito-derived DENV\textsuperscript{89,90,98}. DENV-infected immature DCs however only secrete a limited number of immature particles and therefore in subsequent rounds of replication fewer immature particles are present. Furthermore, the overall lower infectivity of immature particles when compared to standard DENV preparations and the notion that immature DENV is non-infectious in monocytic and macrophage-like cell types\textsuperscript{83,85} together suggest that the overall contribution of fully immature particles to the viral load during primary infection is minimal. In line with this hypothesis, completely immature WNV did not cause clinical disease in mice\textsuperscript{99}.

Upon secondary DENV infection, the role of immature particles is likely more important. Once the virus is inoculated it encounters pre-existing DENV antibodies. Most anti-DENV antibodies are cross-reactive and poorly-neutralizing and consequently have the capacity to enhance infection of a heterologous DENV serotype\textsuperscript{56,85,100,101}. Rodenhuis-Zybert et al. previously showed that next to mature DENV particles also fully immature DENV particles can trigger ADE of infection when opsonized with antibodies\textsuperscript{63}. Infectivity was facilitated through the interaction of the virus-antibody-complex with Fc-receptors (FcR) expressed on immune cells. Moreover, upon entry, immature DENV required enzymatic activity of the protease furin for maturation of the viral particle. In Chapter 6, we showed that not only immature DENV-2, but also immature DENV-1 and -4 can trigger ADE. Unfortunately, we were unable to test the ADE-evoking potential of immature DENV-3 due to difficulties in virus propagation. Nevertheless, our results suggest that ADE of immature DENV is a general property of all DENV serotypes and consequently that immature forms of all DENV serotypes may contribute to severe disease. Especially, as ADE of immature DENV can be observed in monocytic and macrophage cell types, cell types that are known to be important in ADE of DENV infection. Immature DCs, on the other hand, do not support ADE of DENV infection, irrespective of the DENV maturation status (Chapter 6 and \textsuperscript{89,102}). The extent of DENV-ADE seems to be inversely correlated with the level of DC-SIGN binding\textsuperscript{89,103}. Immature DCs express both DC-SIGN and FcRs to high levels\textsuperscript{89}. Therefore, viruses with a high affinity to DC-SIGN will cause no net increase in internalization nor infection upon antibody opsonization. However, DENV produced on immature DCs has low affinity to DC-SIGN and thus does not effectively interact with immature DCs\textsuperscript{102}. Consequently, antibody opsonization likely leads to more efficient interactions with immature DCs and a net increase in infection.

As high levels of prM antibody responses are reported upon secondary DENV
infections, Rodenhuis-Zybert et al. recently investigated the contributions of immature DENV particles and the antibodies recognizing these particles to severe disease\textsuperscript{104}. Importantly, immature DENV particles within standard DENV preparation were shown to contribute significantly to DENV-ADE. Nevertheless, when comparing acute serum of patients, experiencing either dengue fever, dengue hemorrhagic fever or dengue shock syndrome, no differences were found between the proportion and avidity of antibodies recognizing immature DENV. Moreover, all sera equally well promoted ADE of immature DENV. Together, these data imply that immature particles do contribute to the viral load during secondary infection, thereby aiding to severe disease outcomes. Yet, antibody recognition of immature DENV on itself does not alter DENV pathogenesis.

Taken together, we can now conclude that immature DENV contribute to viral load both in absence and presence of antibodies. The contributions during primary infection are likely marginal. Contributions during secondary infection are significant yet do not seem to affect clinical outcome on its own. Consequently, immature virions are not the sole factor determining disease pathogenesis. Nevertheless, as on the one hand immature DENV was described to induce antiviral responses\textsuperscript{105}, yet on the other hand can lead to aberrant immune responses due to induction of cross-reactive, poorly-neutralizing anti-prM antibodies\textsuperscript{56,85,100,101}, it would be interesting to further define how immature particles affect the host immune response.

### Mechanisms of antibody-dependent enhancement of DENV infection

At the start of this thesis it was hypothesized that two concurring mechanisms contributed to ADE of DENV infection. Firstly, antibodies direct the virus towards FcR-bearing cells, which include natural DENV target cells like DCs, monocytes and macrophages, and evoke increased viral binding, uptake and consequently infection\textsuperscript{106–109}. This process is known as ‘extrinsic’ ADE. Next to increased infection, the number of secreted particles per infected cell (‘burst size’) was also found to be elevated under ADE conditions\textsuperscript{89,110}. This was based on the observation that the fold-increase in produced viral particles outweighed the fold-increase in the number of infected cells. The increased burst size was thought to be due to a suppression of antiviral signaling and is known as ‘intrinsic’ ADE\textsuperscript{111–113}. Together, both extrinsic and intrinsic ADE are thought to give rise to strongly increased DENV titers, and supposedly to more severe dengue disease. The above studies had however some caveats. Although the notion of the extrinsic ADE hypothesis was generally assumed, scientific data was scarce. Additionally, studies on intrinsic ADE compared DENV infectivity in the absence and presence of antibodies at a similar multiplicity of infection (MOI) rather than an equal number of infected cells. The latter is of great importance as a different ratio of infected and ‘bystander’ cells may bias the results. Furthermore, ADE was often studied in cell lines rather than primary cells.

In Chapter 7 and 8 we dissected the mechanism behind ADE of DENV infections in primary human monocyte-derived macrophages and murine macrophage-like cells
(P388D1). In human monocyte-derived macrophages, optimal ADE conditions led to a 2-fold enhancement of infected cells and a 4-fold enhancement of produced infectious viral particles per cell, suggesting that indeed extrinsic and intrinsic factors play a role during ADE in macrophages. Comparable results were found in the murine macrophage cell line, although the fold enhancement was much higher. In the primary human monocyte-derived macrophages, no enhanced DENV binding was observed under ADE conditions, which is in line with a previous study in the monocyte/macrophage-like cell line THP-1114. Nevertheless, we did observe a net increase in the number of fusion-positive cells. Based on these results, we postulate that the increased number of infected cells is primarily due to an increased chance of the virus to fuse under ADE conditions. Intriguingly, and in contrast to human monocyte-derived macrophages, however, DENV binding and entry in P388D1 cells was enhanced under ADE conditions. Moreover, DENV membrane fusion was enhanced in P388D1 cells yet to a similar extent as binding and entry, which suggests that the enhanced fusion was due to increased binding. Importantly, these results indicate that the precise mechanism via which antibodies lead to a higher percentage of infected cells is cell type-specific. This difference is likely related to the extent of virus cell binding in the absence of antibodies. Indeed, in the absence of antibodies, DENV binds relatively poor to P388D1 cells when compared to monocyte-derived macrophages. This may be related to the host species as P388D1 is a mouse cell line and mice are not a natural host of DENV infection. In the presence of antibodies, however, it is known that DENV is targeted to FcR, which are expressed in both mice and humans. As binding of the DENV-immune complex to the FcR is expected to be more efficient, an enhanced extent of DENV binding is observed. In contrast, binding of DENV to human monocyte-derived macrophages is likely already quite efficient and accordingly binding of the DENV-immune complex to the FcR is not able to enhance the total binding extent. This notion has been observed before in DCs (Chapter 6 and 89,103). Here, the expression levels of the receptor molecule DC-SIGN inversely correlated with the extent of DENV-ADE. In order to assess whether differences in virus receptor binding are indeed responsible for the observed differences, it should first be defined which attachment factor is responsible for DENV binding to mouse/human macrophages and whether the expression levels on these cell types are distinct or not.

Early studies on ADE of DENV infection suggested that antibody-opsonized DENV particles enters cells via FcR-mediated phagocytosis115–117, however the cell entry route of antibody-opsonized DENV was never studied in detail. In Chapter 8, we confirmed that antibody-bound standard and immature DENV preparations mainly enter the cell via phagocytosis. Perturbation of molecules such as dynamin, actin and PI3K effectively inhibited fusion of antibody-opsonized particles. Moreover, using live-cell microscopy actin protrusions resembling phagocytic cups were observed. Interestingly, antibody-opsonized DENV particles were also found to induce extensive membrane
ruffling. Although this may imply that under ADE conditions DENV enters cells via macropinocytosis, membrane ruffles did not support uptake of fluid phase markers - a hallmark for macropinocytosis, as described in Chapter 1 - nor DENV. Moreover, treatment with EIPA, an inhibitor of macropinocytosis, inhibited fusion of Ab-opsonized DENV only mildly. Together, these data suggest that macropinocytosis is not involved in productive cell entry of DENV. A mild inhibition of fusion was also observed upon perturbation of Eps15, an adaptor of CME, suggesting that next to phagocytosis some particles might also enter P388D1 via CME. These particles may include non-opsonized particles or particles that can still bind to and enter via the ‘natural’ attachment factor. Alternatively, the dependence of Eps15 can be explained by the possibility that Eps15 aids in phagocytosis. Indeed, other important molecules of the clathrin machinery, such as clathrin and AP-2, were found to be occasionally involved in phagocytosis.

Post-internalization, antibody-opsonized DENV required both functional Rab5 and Rab7 for infection, indicating that antibody-opsonized DENV did follow the classical endolysosomal trafficking pathway (Chapter 1). Furthermore, and in line with literature, requirement of Rab7 implied that the virus fused from within the late endosome. Although we would have liked to confirm these findings using single particle tracking, we were unable to perform these experiments due to the thickness of the cells and the low transfection efficiency, as described before. Interestingly, endocytic trafficking of antibody-opsonized DENV differed from non-opsonized DENV, yet this difference did not affect the DENV fusion kinetics (Chapter 8). Instead, the maturation status of the internalized virion did. Fusion kinetics of antibody-opsonized immature DENV were on average 10 minutes delayed with respect to mature DENV particles. This difference in kinetics is most likely reflects the time that is needed to cleave prM to M. As described in Chapter 5, DENV maturation normally occurs during viral egress in the mildly acidic Trans Golgi Network (TGN). After furin-mediated cleavage of prM, the pr-peptide stays attached to DENV-E in order to prevent ‘premature’ fusion in the mildly acidic lumen of the TGN. The pr-peptide is only released when the virion is excreted to the neutral pH extracellular environment. Although furin cleavage in the endosomal pathway is known to be essential for the infectivity of immature DENV, it is unknown how pr-peptide dissociation is triggered during entry of immature DENV particles. It was speculated that immature virions might be recycled back to the PM, losing the pr-peptide when reaching the extracellular environment. We cannot exclude this possibility, as direct recycling from the early endosome to the plasma membrane was reported to have a half-life of 5 minutes. However, a more rational explanation is that the pH in the endolysosomal pathway, which becomes substantially lower than the mildly acidic pH in the TGN, might also cause dissociation of the pr-peptide. Interestingly, the pr-peptide of prM has also been shown to interact with the vacuolar ATPase, but whether this interaction affects endosomal acidification and/or pr-peptide release during entry is unknown.
The above described results indicate that the presence of antibodies changes the entry pathway and intracellular trafficking behavior of DENV in the cell. As I anticipate that the receptor, in this case FcR, dictates the route of virus cell entry, no differences between P388D1 and human monocyte-derived macrophages are expected. Yet, it would be good to confirm this hypothesis using inhibitor studies. Live cell microscopy studies, such a single particle tracking, are probably not suitable as transfection likely activates human monocyte-derived macrophages thereby changing -amongst other things - receptor expression levels. Furthermore, the transfection efficiency is extremely low in these cells. The different DENV cell entry route and endocytic trafficking in absence and presence of antibodies might explain the enhanced fusion observed in human monocyte-derived macrophages. This as upon phagocytosis the virus in transported in phagosomes instead of endosomes (see Chapter 1). Although phagosomes largely resemble endosomes, phagocytosis is specifically designed for uptake and subsequent degradation of pathogens and cell debris. Therefore, it is not unlikely that phagosomes differ slightly from endosomes, e.g. in pH and lipid composition, creating a more favorable environment for DENV fusion. However, if this hypothesis holds true, why did we not observe enhanced fusion relative to the already enhanced binding in P388D1?

A potential caveat in our studies was that the fusion extent of DENV in P388D1 may have already reached plateau level under our experimental ADE conditions due to the enhanced binding. Therefore, and in order to test this hypothesis, binding and fusion experiments in P338D1 cells should be performed at lower MOIs. In any case, the observed discrepancies in the mechanism of ‘extrinsic’ ADE between the human macrophages and P388D1 deserve further attention.

Although the increased number of infected human monocyte-derived macrophages under ADE conditions can be largely explained by the observed increase in fusion, the 4-fold enhancement in burst size cannot. In contrast to the dogma, we did not observe an antibody-induced immune suppressive state nor any other antibody-specific transcriptional profiles under ADE conditions (Chapter 7). Moreover, although we did observe enhanced transcription and translation of viral proteins, the replication efficiency was not altered. As enhanced transcription and translation was also observed at higher MOIs, we propose that a relatively small increase in the extent of fusion induces a cascade leading to enhanced replication and consequently enhanced burst size of infected cells. Interestingly, however, although the burst size at MOI-1-ADE and MOI 5 were similar, more fusion yet a lower replication efficiency was observed for MOI 5. These observations indicate that the observed enhancement of fusion is not the only factor that contributes to DENV-ADE. Although all infection conditions caused an antiviral state early post-infection, more interferon-related genes were induced upon infection at MOI 5. DENV is known to be sensitive to antiviral effects of interferon early in infection\(^{122}\), yet once infection is established the sensitivity to interferon becomes limited. We observed that infection at high MOIs induced an enhanced antiviral
response early in infection, thereby inhibiting replication efficiency. In contrast, the IFN response induced upon MOI 1-ADE was similar to the response induced for MOI 1 in the absence of antibodies and thus no enhanced antiviral responses were evoked under these conditions. Consequently, we propose that the ‘success’ of DENV-ADE in human monocyte-derived macrophages is related to enhanced fusion activity without triggering an enhanced antiviral response early in infection.

If the cellular response early in infection is similar in presence and absence of antibodies, why do we see enhanced translation during ADE? The most likely explanation is the phenomenon of superinfection\(^1\); simultaneous infection of one cell by multiple virus particles. Indeed, in Chapter 7 we observed that not only the number of fusion-positive cells, but also the extent of fusion per cell was enhanced. The latter observations suggest that superinfection might indeed occur. In theory all incoming genomes may independently translate and replicate, leading to exponential enhancement of viral genomes and proteins in the cell. This increase in viral proteins and genomes will plausibly be followed by enhanced production of viral particles per cell. Although few studies report that DENV-infected cells cannot be superinfected, these studies were both performed in cells were DENV infection was already established\(^12,125\). As under our experimental conditions multiple virus particles can infect the cell (almost) simultaneously, I believe that such restrictions will not apply in our case. Moreover, based on the correlation between MOI and burst size, I believe that superinfection is a natural phenomenon which also occurs in absence of antibodies. Whether indeed enhanced replication and thus burst size is related to superinfection remains to be investigated.

**Involvement of actin membrane protrusions in DENV infection**

A novel, yet puzzling finding was the widespread activation of the actin cytoskeleton upon addition of DENV to P388D1 cells (Chapter 8). Although actin was previously reported to be involved in DENV entry and infection\(^126–128\), large scale changes in cell morphology and actin redistribution were not reported before. In Chapter 8, we described three different responses: (i) a generalized membrane ruffling response resembling macropinocytosis, (ii) phagocytic cup-resembling actin protrusions forming around the viral particles and (iii) sheet-like actin protrusions actively trying to capture the virus. All three responses will be discussed below.

A generalized membrane ruffling response was seen for both non-opsonized and antibody-opsonized DENV, although the extent of ruffling was considerably higher in presence of antibodies. Even though membrane ruffling is generally related to macropinocytosis, no enhanced macropinocytic uptake was observed upon DENV-induced membrane ruffling. Similar ‘unproductive’ membrane ruffling was previously observed for adenylate cyclase toxin, a virulence factor of the *Bordetellae* bacteria, when added to macrophages\(^129\). The obvious question that originates is: why do we observe ruffling, yet no macropinocytic uptake? Several controls showed that membrane ruffling
was at least partially initiated by antibodies binding the FcR. Interestingly, phagocytosis and macropinocytosis share similar signaling pathways and FcRs can induce both phagocytosis and membrane ruffling via PI3K, Rac1 and/or Cdc42 signaling\textsuperscript{130,131}. A possible explanation is therefore that DENV-induced ruffling is triggered by the activation of FcR signaling. Indeed, ruffling is only initiated several minutes after addition of the virus, indicating that signaling events might be involved. Another explanation for the unproductive membrane ruffling might come from the notion that ruffling is also involved in several non-macropinocytic functions, including receptor internalization, cell motility and cell migration\textsuperscript{132}. It is thus plausible that the ruffling might be a cellular response to the sensing of a pathogen, in this case DENV, in order to evoke increased cell motility and immune surveillance. Still, the exact mechanism via which ruffling is induced and whether ruffling has a pro- or antiviral role in DENV infection remains unknown and deserves further attention.

Next to the generalized membrane ruffling, local actin protrusions were observed upon the addition of DENV to macrophages. Considering the morphological resemblance, the efficiency by which particles are taken up and the suspected involvement of phagocytosis in DENV entry of P388D1 cells, we propose that the observed Type I actin protrusions are in fact phagocytic cups. Type II protrusions seemed to be specifically generated to capture viral particles located away from the plasma membrane. Although actin structures were previously described to be involved in virus cell entry and were observed to capture virus particles near the cell surface\textsuperscript{133}, the actin structure described by Lehmann\textsuperscript{133} and type II protrusions do not morphologically resemble each other. Moreover, the notion that a single protrusion repeatedly attempts to capture a single virus particle, has to our knowledge not been described before. Therefore, I reckon that this is the first description of sheet-like actin protrusions which actively ‘hunt’ and capture virus particles. Since the phenomenon was almost exclusively observed for antibody-opsonized DENV, we proposed that type II protrusions were directed towards antibodies instead of the virus. Antibodies that dissociate from the virus particle likely provide a chemotactic signal to the cell, via which the cell can sense and clear foreign particles located away from the cell body. Although the observation of active virus capture by type I and type II protrusions is highly intriguing, it must be taken into account that we did not establish a causal relation between virus capture and infection. For both DENV infection in presence and absence of antibodies the actin cytoskeleton was required for infection. However, perturbations affecting actin polymerization disrupt the complete actin cytoskeleton, and it thus remains to be determined whether actin protrusions or other functions of the actin cytoskeleton are required for DENV entry.

**Concluding remarks**

Due to the high disease burden of DENV and a continued expansion of its vector\textsuperscript{54,134,135}, there remains a high need for specific antiviral treatments and/or an universal DENV
vaccine. For this purpose a better understanding of DENV infections under standard and ADE conditions is required. In the second part of the thesis, we investigated DENV infections of natural target cells in absence and presence of antibodies. We observed that immature DENV particles of all serotypes can contribute to disease both during primary and secondary infection. Additionally, we described that the binding affinity of DENV to its wildtype receptor likely determines whether ADE can be observed or not. Moreover, we elucidated the DENV cell entry pathway into macrophage-like cells under standard and ADE conditions. Finally, we clarified the mechanism behind DENV-ADE in human macrophages.

One of the most striking observations from our work was that DENV enters mouse macrophages via a non-classical endocytic mechanism resembling both CME and phagocytosis and subsequently by-passed the early endosomal compartment (Chapter 8). Although I would still like to confirm our results using alternative approaches, this notion further emphasizes that endocytic pathways should not be viewed as rigid, set mechanisms. Instead endocytic pathways can be highly variable, sharing modules and proteins between pathways in order to adapt themselves to their cargo. This variability makes them hard to study and stresses the need for a combination of inhibitor studies and advanced microscopy techniques to discern all details of the endocytic mechanisms. Moreover, it once again proves that the study of virus cell entry also provides valuable insight in endocytosis itself.

We moreover observed that DENV cell entry in absence and presence of antibodies was found to be highly reliant on the actin cytoskeleton (Chapter 8). Unfortunately, targeting the complete actin cytoskeleton as a DENV therapeutic is not viable option. This as a multitude of essential processes inside the human body, including cardiac function, rely on actin dynamics\textsuperscript{136}. However, both in absence and presence of antibodies actin-mediated membrane protrusions were observed to actively capture virus. As membrane protrusions were previously reported to be essential for DENV cell entry into HMEC-\textsuperscript{1126}, it would be of interest to see whether the observed protrusions are also required for DENV infection into macrophages. If this hypothesis could be confirmed, specific actin adaptors that initiate the formation of membrane protrusion can perhaps be targeted to inhibit DENV cell entry both during primary and secondary infections.

In contrast to earlier reports, we found no evidence for intrinsic DENV-ADE mechanisms in Chapter 7. Instead a tight balance between a higher fusion extent - likely caused by the alternative entry pathway and endocytic trafficking (Chapter 8) - and avoidance of a strong antiviral response early in infection contributes to the ‘success’ of ADE. Upon infection with high MOIs, a strong antiviral response, mainly of interferon-related genes, was correlated with a reduced replication efficiency. Consequently, interferons may be used as a therapeutic against DENV. Indeed, IFN\textalpha has already been reported to relieve dengue fever in humans\textsuperscript{137}. However, as we reported that IFN\textalpha is only effective at early stages of infection (Chapter 7) and unpublished data reveals that IFN\textalpha is not
very effective against DENV-3 and DENV-4\textsuperscript{138}, more research has to be performed to investigate whether interferons can indeed be used as an effective DENV therapeutic.

Although our results do not directly translate to any therapeutics or vaccines, a thorough understanding of DENV-ADE is of utmost importance to develop an universal DENV vaccine. For related flaviviruses such as yellow fever virus, Japanese encephalitis virus and tick-borne encephalitis virus vaccines have been found to be very effective in preventing disease\textsuperscript{139}. Yet for DENV, the existence of four serotypes and the possible induction of ADE has hampered vaccine development. Recently, however, the first tetravalent DENV vaccine has been licensed. Still, as vaccinated individuals with no pre-existing immunity against DENV were found to be at greater risk of hospitalization due to severe dengue manifestations\textsuperscript{140}, the vaccine could only be registered for individuals between the age of 9 and 45 living in endemic areas\textsuperscript{141}. The recent spread of Zika virus (ZIKV) will likely add another dimension to the inability to develop an universal and safe DENV vaccine. It has recently been shown that pre-existing antibodies against DENV can bind to ZIKV and enhance ZIKV infections\textsuperscript{142}. It has thus been proposed that the high incidence of severe Zika manifestations, including microcephaly in unborn children\textsuperscript{143}, in parts of Brazil might be related to the high pre-existing anti-DENV immunity in those regions. Consequently, cross-reactive antibodies between ZIKV and DENV must be considered when producing ZIKV and DENV vaccines. The safest option would thus be to develop a pentavalent vaccine, including all four DENV strains and ZIKV. Consequently, future research should continue to address how antibodies influence DENV (and ZIKV) infections. A better understanding of what types of antibodies enhance and neutralize flavivirus infections will ultimately guide the rational design of a vaccine that induces a potently neutralizing antibody response against all four DENV serotypes and ZIKV.
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SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES


