CHAPTER 1

General introduction and aim of the thesis
Introduction: Chikungunya and Dengue virus cell entry

The work described in this thesis focuses on two important emerging arboviruses, chikungunya virus (CHIKV) and dengue virus (DENV). Both viruses are transmitted to humans via mosquitoes of the *Aedes* species and cause major epidemics in the tropical and subtropical parts of the world\(^\text{1,2}\). CHIKV was first isolated in 1952 and for a long time only caused small outbreaks in Africa and South-East Asia at irregular intervals\(^\text{3}\). At the end of 2004, the virus re-emerged and spread rapidly around the world. In 2013, a first local epidemic was reported in Central America. To date, over 40 countries in the region report CHIKV infections, leading to over 1.7 million suspected CHIKV cases\(^\text{4}\). Most individuals infected with CHIKV develop symptomatic disease, which is characterized by headache, fever, rash and pains in the muscles and joints. The joint pains are highly debilitating and may recur or persist for months to years after initial infection\(^\text{5–8}\). DENV is the most common arthropod-borne infection of the world, causing 390 million estimated infections annually world-wide\(^\text{9}\). Every year, approximately 96 million individuals develop symptomatic disease\(^\text{9}\). DENV exists as four different serotypes and each of the four serotypes can cause clinical disease, ranging from a relatively mild febrile illness to life-threatening manifestations as plasma leakage and hypovolemic shock\(^\text{10}\). Upon primary infection, life-long protection is elicited to re-infection with the same serotype. However, upon re-infection with a heterologous DENV serotype an increased risk for severe dengue is observed\(^\text{11–14}\). Increased disease severity is associated with pre-existing DENV antibodies and high circulating virus titers\(^\text{15–19}\), which suggests that antibodies directly influence the infectious properties of the virus. To date, no specific drug is available to treat CHIKV or DENV infection. For DENV, the first vaccine recently got licensed, yet this vaccine may only be used for children over the age of 9 which live in endemic countries\(^\text{20}\). For CHIKV, no vaccine is available.

Both CHIKV and DENV are enveloped viruses and consequently require a membrane fusion event to initiate infection of the host cell. Membrane fusion of enveloped viruses can be triggered by two different mechanisms. Viruses either trigger fusion with the cell surface via interactions with a cell surface receptor or fuse from within the acidic endosomes in response to a low pH trigger. At the start of this study, CHIKV was known to require exposure to low pH to initiate infection\(^\text{21–24}\), yet the endocytic pathway hijacked by the virus remained controversial. Both clathrin-mediated and clathrin-independent entry pathways were proposed\(^\text{24–26}\). We therefore revisited this topic and studied the CHIKV cell entry pathway using state-of-the-art-microscopy techniques. DENV was known to enter most cell types via clathrin-mediated endocytosis\(^\text{27,28}\). The mechanism by which antibodies influence the infectious properties of the virus was however unknown. Therefore, in this thesis we aimed to understand if antibodies control the endocytic pathway via which DENV enters the cell.
Endocytosis is an essential process via which extracellular and plasma membrane molecules are transported into the cell. It controls various complex physiological processes as hormone-mediated signal transduction, immune surveillance, antigen-presentation and homeostasis. To date, a number of distinct endocytic pathways have been described, including clathrin-mediated endocytosis, caveolar and lipid raft-mediated endocytosis, macropinocytosis and phagocytosis. Although clathrin-mediated endocytosis, caveolar endocytosis, lipid raft-mediated endocytosis and macropinocytosis may occur in all cell types, phagocytosis is typically restricted to specialized mammalian cells, such as macrophages and dendritic cells. In this section, we will describe the current knowledge on the different existing endocytic pathways. Moreover, the tools that can be used to study the route of virus cell entry will be discussed.

**Virus cell entry pathways**

**Clathrin-mediated endocytosis**

Clathrin-mediated endocytosis (CME) was the first endocytic pathway described and is to date still the best understood endocytic pathway. CME is responsible for the uptake of a variety of plasma membrane receptors and occurs constitutively in all mammalian cells. In addition, de novo formation of clathrin-coated pits (CCPs) can be induced by certain cargoes, such as Influenza A virus. CME starts when adaptor protein 2 (AP-2) docks onto phosphatidylinositol-(4,5)-biphosphate (PtdIns(4,5)P2) at the plasma membrane (PM). Binding of AP-2 to PtdIns(4,5)P2 is thought to trigger conformational changes within AP-2, which facilitates binding of both clathrin and CME-specific cargo proteins to the docking site. Binding of clathrin stabilizes the docking of AP-2 onto the PM and leads to the growth of the clathrin coat by subsequent addition of more AP-2 and clathrin proteins. Next, endocytic accessory proteins, including among others Eps15 and epsin, are recruited to the edges of the assembling CCPs. Recruitment of these proteins further stabilizes the CCP and is essential for its growth. Ultimately, clathrin will form a basket-like structure around the membranous pit, leading to the formation and constriction of a vesicle neck. The GTPase dynamin is recruited to the vesicle neck, where it drives membrane scission. Although under normal conditions actin polymerization is not needed for membrane scission, it does assist in scission when the membrane is under tension. This might for example occur when large or irregularly shaped cargo is taken up by CME. Immediately after scission, uncoating of the clathrin-coated vesicles (CCV) is initiated by combined actions of phosphatase synaptojanin, auxilin and heat shock protein 70. Next, the vesicles are targeted to the early endosome and Fig. 1).
To investigate whether a virus enters the cell via CME, pharmacological inhibitors such as chlorpromazine, Pitstop2, dynasore and dyngo compounds are often used\(^{31}\). Both chlorpromazine and Pitstop2 are presumed to directly target the functions of clathrin\(^{35,36}\), while chlorpromazine might also target AP-2\(^{36}\). Dynasore and the dyngo compounds inhibit the function of the GTPase dynamin\(^{37,38}\). However, dynamin perturbations are not specific for CME, as this GTPase is involved in multiple endocytic pathways. Next to these pharmacological inhibitors, dominant negative mutants (DNMs) of Eps15 and dynamin and siRNAs against clathrin heavy chain, AP-2, Eps15 and dynamin-2 are often used. Although AP-2 and Eps15 have important functions in CME, also these proteins are not completely specific for CME\(^{34,39–41}\). Therefore, virus entry via CME cannot be concluded based solely on perturbations of these proteins. Of the described perturbations, siRNA-mediated knockdown of the clathrin heavy chain is the most powerful approach. Still, knockdown of this protein can be challenging due to its long half-life\(^{42}\) and knock-down levels should thus be carefully controlled. Moreover, interactions between the virus and the CCP can be visualized by microscopy, as CCPs can be clearly discerned by both electron and fluorescent microscopy techniques. In conclusion, to implicate CME in virus entry viral fusion or infection should be dependent on both clathrin and dynamin. In addition, it is recommended to visualize colocalization of the virus with CCPs by microscopy. Furthermore, strong additional evidence for virus entry via CME is the inhibition of infection upon treatment with either chlorpromazine or pitstop2 or upon perturbation of Eps15 or AP-2.

**Figure 1. Schematic overview of the endocytic pathways.** Although cargo can be internalized by several distinct endocytic pathways, almost all pathways converge at the early endosome. Whether macropinosomes and phagosomes fuse with the early endosome or these endocytic vesicles mature independently of the early endosome is still under debate. Figure was adapted from \(^{43}\) with permission from the Nature Publishing Group.
Caveolar and lipid raft-mediated endocytosis

Two types of endocytosis rely heavily on cholesterol- and sphingolipid-rich microdomains, so-called lipid rafts. The first type, caveolar endocytosis, is mediated by caveolae (‘little caves’). These are flask-shaped PM invaginations of 50-80 nm in size. Caveolae are found in many cell types and can constitute maximal 30% of the total PM \[^{29,30,43}\]. The second type is caveolae-independent and has no proper name but is often described as lipid raft-mediated endocytosis. Actually, these pathways are thought to be closely related and might actually be variations of one endocytic route \[^{44}\]. Therefore, these two pathways will be discussed together in the next paragraphs.

The events during caveolar and lipid raft-mediated endocytosis are complex and not very well understood. In contrast to CCPs, caveolae do not form \textit{de novo} at the PM, but formation is started at the ER. After transport to the PM, caveolae can remain stationary for an extensive period of time while being linked to the underlying actin cytoskeleton by EHD2 (Eps-15 homology domain containing protein 2) \[^{31}\]. The first step in both caveolar and lipid raft-mediated endocytosis is cargo-mediated clustering of lipid rafts. For caveolar endocytosis, lipid raft clustering appears to be required for sequestration of cargo into caveolae. This cargo sequestration is likely due to PM surfing of the cargo and subsequent entrapment inside the caveolae by high concentrations of cargo specific receptors. For lipid raft-mediated endocytosis, however, it is thought that clustering of lipid rafts increases the local concentration of cholesterol, which leads to spontaneous membrane curvature. The shape of the cargo, especially in case of a virus, likely aids in this process \[^{44}\]. Regardless of how the initial membrane invagination is formed, internalization is dependent on the phosphorylation of tyrosines. Different kinases, including the tyrosine kinase Src, the serine/threonine kinases KIAA0999 and MAP3K2 and the putative phosphatidylinositol 4-phosphate 5 kinase MGC26597 have been implicated in caveolar and lipid raft-mediated endocytosis, yet their functions are not completely understood \[^{31,44}\]. Scission of caveolae depends on dynamin and actin polymerization \[^{31}\], whereas scission of lipid raft-mediated endocytosis is reported to be dynamin independent \[^{45}\]. Furthermore, caveolar endocytosis is slow and asynchronous, while the kinetics of lipid raft-endocytosis seem to be somewhat faster \[^{46}\]. The endocytic vesicles generated in both caveolar and lipid raft-mediated endocytosis can fuse with early endosomes or caveosomes. Caveosomes are still poorly defined, but are thought to mainly interact with early endosomes, the ER and the Golgi. Indeed, cargo of these endocytic pathways frequently ends up in the ER \[^{29,39,43,44}\].

To conclude that a virus enters the cell via caveolar or lipid raft-mediated endocytosis, entry should be dependent on cholesterol, actin polymerization and tyrosine kinases. Cholesterol dependency can be studied by use of chemical inhibitors, such as methyl-bêta-cyclodextrin (MβCD), filipin, nystatin and progesterone. As high concentrations of MβCD may also affect CME and other endocytic pathways \[^{47}\] compounds as filipin, nystatin and progesterone should be favored over MβCD in studying caveolar and lipid raft-mediated endocytosis. Involvement of actin polymerization in entry can be studied
by use of latrunculin A and jaspakinolide. In addition, caveolar and lipid raft-mediated endocytosis can also be inhibited by use of the general tyrosine kinase inhibitor genistein, whereas akadaic acid and orthovanadate, inhibitors of Ser/Thr phosphatases and Tyr phosphatases, respectively, will accelerate internalization. In order to assess whether caveolar and lipid raft-mediated endocytosis is efficiently perturbed, cholera toxin B can be used as a control. To distinguish caveolar endocytosis from lipid raft-mediated endocytosis, dependency on caveolin-1, a protein important for the structural organization of caveolae, can be determined. Moreover, using fluorescent microscopy colocalization and co-internalization of the virus and caveolin-1 can be assessed. Furthermore, perturbations of dynamin-2 should only affect caveolar endocytosis, but not lipid raft-mediated endocytosis. Depletion of glycospingolipids has been reported to selectively block caveolar endocytosis and can be used to distinguish this pathway from novel endocytic pathways as RhoA and Cdc42-dependent endocytosis. It is likely that depletion of glycospingolipids also affects lipid raft-mediated endocytosis, although to our knowledge this has not been studied to date.

**Macropinocytosis**

Macropinocytosis is an endocytic mechanism quite distinct from CME and caveolar and lipid raft-mediated endocytosis. It involves large-scale cargo internalization through ruffling of the complete PM. These ruffles can take the shape of lamellopodia, circular cup-shaped projections—usually referred to as circular dorsal ruffles—, filopodia and blebs oriented towards the extracellular space (and Figure 2). Typically, macropinocytosis is a transient growth factor-induced process. Uptake of cargo is non-selective and often accompanied with increased fluid uptake. Next to growth factors, a variety of other structures, including apoptotic bodies, necrotic cells, bacteria and viruses, can induce macropinocytosis and consequently promote their uptake into the cell.

Macropinocytosis is induced when a ligand binds to receptor tyrosine kinases (RTKs) at the cell surface, including EGFR, integrins and some phosphatidylinerine receptors. Signaling of these RTKs activates a complex and interconnected signaling cascade that changes the actin dynamics and triggers membrane ruffling. In this signaling cascade Ras family GTPases play a crucial role. Activation of Ras induces multiple parallel signaling cascades involving among others Rac1, Cdc42, PI3K, Arf6 and Rab. These kinases function together to modulate the ruffle formation, the subsequent macropinosome closure and downstream vesicle trafficking. Rac1 and Cdc42 trigger membrane ruffling by altering the actin dynamics. Both molecules relay signaling to downstream effectors that promote actin polymerization, such as the WAVE complex and the actin-related protein complex (Arp2/3). Activity of Rac1 and Cdc42 is affected by cholesterol and Na+/H+ exchangers. Both Rac1/Cdc42 signaling as the initial RTK signaling can recruit PI3K to the PM. Recruitment of PI3K promotes ruffling by indirect activation of phospholipase C (PLC) and protein kinase C (PKC). Moreover, Rac1/Cdc42
activate PAK$^{51,52}$, a protein necessary for the induction of macropinocytosis$^{53}$. Like Rac1/Cdc42, Arf6 is directly activated by Ras. Arf6 can promote both Ras-dependent and -independent macropinocytic programs. As the effector functions of Arf6 and Ras largely overlap, synergistic roles during macropinocytosis are suggested. Irrespective of the type of ruffling, ruffles trap any cargo which is located between themselves and the cell body when they fold back onto the PM. Subsequently, the ruffle fuses with the PM and an endocytic vesicle called the macropinosome is formed. Different factors, such as CtBP1/Bars, dynamin, PKC, PI3K, myosins and Rab34 are implicated in macropinosome closure. Yet, the specific requirements seem to depend on the type of ruffle. For example, lamellopodial ruffles need CtBP1/Bars for closure whereas circular dorsal ruffle closure is dynamin-dependent$^{31,51,52}$. After macropinosome formation, vesicles depend on Arf6 and Rab5 for transport deeper into the cytosol. There, macropinosomes undergo maturation steps comparable to endosomes, including the gradual decrease in pH$^{39,51}$.

Studying virus entry via macropinocytosis can be quite challenging, as none of the
many proteins involved is exclusive. Consequently, several approaches are needed. Inhibitors of actin (e.g. cytochalasin D) and Na+/H+ exchangers (amiloride or is derivative EIPA) are good initial screening tools to assess entry via macropinocytosis. If entry is blocked by these inhibitors, microscopy techniques can be used to assess whether PM ruffling is apparent. As ruffling of the complete cell body is specific for macropinocytosis\(^5\), this characteristic can be used to discriminate macropinocytosis from other endocytic pathways. In addition, a transient 5- to 10-fold increase in fluid phase uptake\(^5\), which can be measured by assessing uptake of high molecular weight dextrans, is a good parameter for macropinocytosis and defines it from other endocytic pathways. Furthermore, entry should depend on either Rac1 or Cdc42, which can be assessed by use of DNMs or the Rac1 inhibitor NSC23766. Additionally, inhibitors of PI3K (wortmannin or LY294002) and PKC (rottlerin, calphostin C and bisindolylmaleimide) should block entry\(^31,39\). EM can be used to screen for virus particles inside macropinosomes. In EM macropinosomes are visible as large irregular-shaped uncoated vesicles, reaching diameters of up to 10 µm. Other factors worth testing include dependency on Arf6 and cholesterol, yet the requirement of Arf6 in macropinocytosis has been found to vary\(^5\).

**Phagocytosis**

Phagocytosis is a highly regulated endocytic mechanism employed by specialized cells of the immune system. It functions to clear large cargo, such as bacteria, yeast and cell debris. In contrast to macropinocytosis, phagocytosis is strictly dependent on receptor-cargo binding\(^5\). Phagocytosis includes a variety of related molecular mechanisms. In this paragraph I will solely focus on Fc-receptor mediated phagocytosis, as this phagocytic pathway is the most relevant for this thesis. This type of phagocytosis can mediate the engulfment of antibody-opsonized particles\(^29,31,54\).

The process of Fc-receptor mediated phagocytosis is initiated by binding of a ligand to the receptor, and subsequent receptor clustering. Although receptor clustering most likely occurs by diffusion, the GTPase RhoA is suggested to facilitate this process\(^55\). Upon receptor clustering, the immune-receptor tyrosine-activated motifs (ITAMs) of the Fc-receptors associate with each other and become phosphorylated by members of the Src kinase family. Next, Syk is recruited to the phosphorylated FcR, where it phosphorylates additional neighboring ITAMs. Syk phosphorylation leads to recruitment of additional signaling proteins, which appear critical for downstream signaling during phagocytosis. The initiated signaling cascade leads to activation of Cdc42, Rac1 and Rac2, which indirectly mediate actin rearrangements. Next to actin rearrangements, membrane rearrangements mediated by among others PI3K and PLC are essential to phagocytosis\(^31,54\). For the exact mechanisms via which the Rho GTPases, kinases and lipases lead to actin and membrane rearrangements, respectively, I would like to refer to the extensive review on phagocytosis of Flannagan *et al.*\(^54\). Together, both the actin and membrane rearrangements lead to the formation of the pseudopods, which form the phagocytic cup. Also myosins, actin-associated contractile proteins, participate in this
process. Moreover, as formation of the phagocytic cup requires a considerable amount of membrane area, additional membrane is needed. This additional membrane likely comes from late and recycling endosomes which fuse with the growing phagocytic cup. Furthermore, Arf6 and dynamin were found to be important for membrane delivery to the nascent phagocytic cup. Although contractile forces of myosins are thought to be required for efficient ingestion of particles, the exact mechanism by which the phagocytic cup closes is still unknown. Newly formed phagosomes likely fuse with early endosomes, acquiring early endosomal markers. Phagosome maturation is thought to be comparable to endosome maturation.

Phagocytosis and macropinocytosis share many important features, such as the dependency on actin, the involvement of Cdc42/Rac1 and the relatively large vacuole size. Yet, there are some important differences. Firstly, phagocytic uptake is particle specific. Although the vacuole might be large, it fits tightly around the phagocytosed particle and no additional cargo or fluid is taken up. Secondly, as phagocytosis is particle-driven the membrane activation is more localized compared to macropinocytosis. Lastly, Fc-receptor mediated phagocytosis can only be seen in specialized cell types, whereas macropinocytosis can occur in virtually all cell types. To study whether a virus enters via phagocytosis entry has to be dependent on actin dynamics, dynamin, PI3K and Arf6. The actin dynamics can be studied using previously described inhibitors as latrunculin A, cytochalasin B and jaspakinolide. More specifically, the Rac1 inhibitor NSC23766 or DNM of RhoA, Rac1 and Cdc42 can be used. Dynamin can be inhibited by use of dynasore and the dyngo compounds or the DNM Dynamin-K22A. PI3K can be perturbed using wortmannin or LY294002. Finally, Arf6 involvement can be assessed by use of dominant negative Arf6 mutants (either Arf6-T27N or Arf6-Q67L). Of the perturbations described above only dependency on RhoA can discriminate phagocytosis from macropinocytosis. Other techniques to discern phagocytosis from macropinocytosis include EM and certain powerful light microscopy techniques. With these techniques local formation of the phagocytic cup can be visualized. Finally, as phagocytosis is strictly receptor-mediated, blockage of the Fc-receptor should inhibit virus uptake via Fc-receptor-mediated phagocytosis.

Other (virus) entry pathways
Next to the above described ‘classical’ endocytic pathway, an increasing number of novel endocytic pathways are being described. These pathways are clathrin and caveolin-independent, usually rely on a functional actin cytoskeleton, but differ in their dependency on cholesterol, sphingolipids and dynamin. Some of these pathways are constitutive, whereas others are triggered by specific signals or perhaps even pathogens. Furthermore, they differ in their kinetics, associated molecular machinery and cargo destination. Factors that have been linked to these pathways are flotillin, Graf1 (CLIC/GEEC), IL-2 and Arf6. Although flotillin, CLIC/GEEC and Arf6-dependent endocytosis are described as different endocytic pathways, other reports suggest that they could also
be variations of one type of endocytic pathway\textsuperscript{30,31,43}. To date, none of these endocytic pathways has been associated with entry of enveloped viruses\textsuperscript{39}.

Moreover, viruses have been described to enter cells via pathways that do not fit any classical endocytic pathway. For example, Human Papilloma Virus (HPV) is described to enter via a pathway reminiscent of macropinocytosis. HPV entry requires a set of kinases similar to those involved in macropinocytosis, but unlike entry via macropinocytosis HPV entry was independent of cholesterol and Rho-GTPases. In addition, no outward protrusions, which are typical for macropinocytosis were observed\textsuperscript{60}. Likewise, the pathway via which Lymphocytic Choriomeningitis Virus (LCMV) enters the cell cannot be categorized into one of the known endocytic pathways. Microscopic data showed that LCMV entered the cell via membrane invagination-derived endocytic vesicles, but neither cholesterol, actin and dynamin nor more specific proteins as clathrin, Arf6 and flotillin were required for LCMV cell entry\textsuperscript{61}. It remains to be studied whether the entry pathways hijacked by HPV and LMCV are novel, possibly virus-induced, endocytic pathways or natural variations to existing pathways.

**Endosomal transport of viruses**

Most endocytic pathways converge at the early endosome (EE)\textsuperscript{43}. After scission from the cell surface, the endocytic vesicles derived from clathrin- or caveolar endocytosis travel towards the EE, with which they subsequently fuse. For macropinosomes and phagosomes, it is still unclear whether they completely fuse with EEs or acquire endosomal markers via another mechanism, for example by interacting with EEs in a ‘kiss-and-run’ fashion\textsuperscript{52,62}. Irrespective of the manner by which endosomal markers are acquired, the functions and maturation steps of endosomes, macropinosomes and phagosomes are comparable\textsuperscript{54,57}. The main steps of endosomal maturation will be discussed in this paragraph.

The endolysosomal system is a complex cellular trafficking network responsible for sorting, recycling, degrading, storing, processing and transcytosis of molecules. It consists of different vesicular organelles such as early endosomes, recycling endosomes, maturing endosomes, late endosomes and lysosomes. Upon endocytosis cargo is rapidly delivered to the EE\textsuperscript{63,64}. EEs are vesicular structures with vacuolar and long extended tubular elements, which are responsible for the initial sorting of incoming cargo. The EE lumen is slightly acidic and its membranes are marked by Rab5 and early endosomal antigen 1 (EEA1). Rab5 is crucial for delivery of cargo to EEs by aiding fusion between endocytic vesicles and EEs. Moreover, the EE membrane is marked by several other Rab molecules, which aid in cargo selection, in a patch-like manner. For example, Rab4 aids in transport back to the PM, Rab7 traffics cargo to late endosomes (LEs), Rab15 is involved in transport to recycling endosomes and Rab22 is important for transport to the trans-Golgi network (TGN)\textsuperscript{65}. Viruses are generally targeted to LEs and lysosomes for degradation. Transport of cargo from EEs to LEs can occur by two distinct mechanisms. In the first mechanism, a small vesicle, positive for both Rab5 and Rab7, scissions from
the endosome. This vesicle subsequently transports its cargo to the LE, with which it fuses. In the second mechanism, the EE gradually loses its early endosomal markers and completely matures into a LE. During endosomal maturation the following steps take place. First, the pH inside of the endosome gradually drops from a pH between 6.8 and 5.9 in EEs to a pH ranging from 6.0 to 4.9 in LEs and lysosomes. Subsequently, a switch in the Rab subsets marking the endosome occurs. Whereas EEs are mainly positive for Rab4 and Rab5, LEs are marked by Rab7 and Rab9. The change in Rab subsets indirectly allow endosomes to increasingly interact with microtubules. Consequently, Rab7-positive LEs migrate from the periphery to the perinuclear region, where the LEs will eventually fuse to lysosomes. In lysosomes, acidic organelles filled with digestive enzymes, cargo will be ultimately degraded (Figure 3).

**Figure 3. Schematic overview of the endolysosomal pathway.** Upon endocytosis, cargo is typically transported to Rab5-positive early endosomes. The early endosome functions as a cargo sorting station, either selecting cargo for recycling back to the plasma membrane or for degradation in the lysosome. Cargo selected for degradation is sequentially transported through maturing and late endosomes before reaching the lysosome. During this trafficking the endosomes gradually lose the early endosomal marker Rab5, but acquire amongst others the late endosomal and lysosomal markers Rab7 and LAMP1. Figure was adapted from 75 with permission from the Nature Publishing Group.
The endosomal system is highly connected and interdependent⁶⁷,⁶⁸, which makes it hard to study. Long-term perturbation of a single endosomal function might lead to disruption or retardation of the whole system⁶⁸. To date, dominant negative Rab mutants, such as Rab5-S34N and Rab7-T22N, are still the ‘golden standard’ to perturb the endosomal pathway. Rab5-S34N blocks fusion between endocytic vesicles and endosomes and consequently the formation of normal-sized EEs⁶⁹. Rab7-T22N blocks the transition of cargo from early to late endosomes and therefore, cargo accumulates in Rab5-positive EEs⁷⁰. Mercer et al. previously reviewed biochemical inhibitors that are used to study the endolysosomal pathway³⁹. Bafilomycin A1 is reported to inhibit the V-ATPase, which is important for endosomal acidification, and thereby blocks the formation of maturing endosomes⁷¹–⁷³. Wortmannin causes delays in transport from early to late endosomes⁷⁴. Furthermore, nocodazole, a microtubule-disrupting agent, is observed to block both LE formation and cargo transport to recycling endosomes⁷². Yet, both wortmannin and nocodazole can also affect other steps in virus entry. A more direct and elegant way to study from which endosomal compartment viral fusion occurs is to track single viral particles in live cells expressing fluorescent Rab proteins. To conclude from which endosomal compartment viral fusion occurs, ideally both live cell microscopy and DN Rab mutants are combined. Yet, due to technical issues a lot of studies still base their conclusions solely on the use of DN Rab mutants.

**Advantages and disadvantages of the tools used to study virus cell entry**

Tools such as pharmacological inhibitors, (dominant negative) protein mutants and RNA interference are frequently used to study the viral cell entry pathway⁴¹. The main advantages of pharmacological inhibitors are their ease of use and the relative low costs involved. A well-known downside of pharmacological inhibitors is that most compounds are reported to have pleiotropic effects⁷⁶. One strategy to minimize pleiotropic effects is to carefully choose an assay by which solely virus entry is assessed. In this thesis we studied the effects of pharmacological inhibitors on virus entry by use of a microscopic viral fusion assay. This assay is ideal to pinpoint which host proteins are required for viral entry, as only the effects that inhibitors have on viral entry and fusion are determined⁷⁷. Moreover, the effective concentrations of pharmacological inhibitors should be carefully controlled. For example, it was described in literature that the cholesterol-depleting agent methyl-bêta-cyclodextrin (MβCD) affects CME although CME is not dependent on cholesterol⁴⁷. It is believed that high concentrations of MβCD changed the fluidity of the PM thereby avoiding sufficient invagination of the CCPs. Finally, in this thesis we have observed that inhibitors such as chlorpromazine and Pitstop2 might – depending on the cell line on which they are used – have a very narrow window of use in terms of cytotoxicity. Cytotoxicity and efficacy should thus always be tested by use of viability assays and cargo controls. In order to minimize pleiotropic effects it is advised to select the lowest effective concentration for experimentation.
Compared to pharmacological inhibitors the use of dominant negative or constitutively active protein mutants is more specific. These protein mutants are either defective (dominant negative) or overactive (constitutively active) in their functions\(^{31}\). Upon overexpression in the cell, these proteins overrule the functions of the natively expressed protein. However, an important drawback of the use of DNMs is that the level of overexpression that is required for the mutant to exert its function is often poorly defined. In the review of Kühling and Schelhaas an extensive list of described protein mutants and their effects is given\(^{31}\).

The most powerful and specific perturbation approach to study the virus cell entry pathway is the use of RNA interference (RNAi). Either small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) are used for this purpose. RNAi is mostly used in small-scale experiments silencing pathway-specific proteins such as clathrin and caveolin or in targeted screens involving different siRNAs against important endocytic proteins. Using RNAi, it is important to control for false-positive and false-negative hits, which are results of off-target effects and insufficient silencing, respectively. Off-target effects can be minimized by the use of multiple independent siRNAs, whereas the insufficient silencing can be prevented by the simultaneous use of multiple different siRNAs against the specific target. Also upon the use of siRNAs it is advised to control the silencing efficiency by use of functional controls or western blot.

In addition to the techniques described above, different microscopy techniques are used to study virus cell entry. These techniques include electron microscopy (EM), colocalization studies using fluorescence microscopy and single particle tracking of fluorescently-labelled viruses in live cells. The great positive of EM is that the type of vesicular structures via which viruses enter the cell can be directly observed. Negatives, however, are that EM data can be hard to interpret and a trained eye is often needed to classify structures in EM images. Furthermore, in order to pick up virus entry events by EM cells are often need to be loaded with high numbers of infectious virus particles, which can possibly induce artefacts.

In fluorescence microscopy both the location of the virus and specific endocytic markers can be assessed. Extensive colocalization of the fluorescent virus particle and endocytic markers can indicate virus entry via a specific endocytic pathway. However, it must be taken into account that the resolution of standard fluorescent microscopes is below 200 nm\(^{78}\). As many viruses are smaller than 200 nm, optical colocalization does not necessarily imply physical colocalization. Specific microscopic techniques, such as fluorescence resonance energy transfer (FRET)\(^{79}\) and Stochastic Optical Reconstruction Microscopy (STORM)\(^{80}\) have been developed to visualize protein interaction and improve resolution, respectively. In single particle tracking (SPT) of virions smaller than 200 nm, however, co-migration of virions and endocytic markers gives a good indication of physical colocalization. This as the chance that two non-interacting structures spontaneously co-migrate is limited. Moreover, using SPT the exact moment of viral fusion can be determined\(^{77}\). Consequently, it is possible to determine whether entry via a specific pathway leads to viral fusion. However, as specialized
equipment is needed to perform live cell SPT, this technique is still relatively underused. All of the above described techniques have their own advantages and disadvantages. For example, using microscopy virus entry can be directly visualized, yet whether entry leads to productive infection cannot be determined. Thus, the best way to study virus entry is to combine multiple approaches. Favorably, both perturbations and microscopic techniques are combined, but the value of the techniques does differ for the different endocytic pathways.

**Perspectives**

In conclusion, virus cell entry can occur via different endocytic pathways, which can be studied by different perturbation and microscopy techniques. Although the different endocytic pathways were described as separate mechanisms, it must be taken into account that these pathways are highly interdependent cellular mechanisms. Upon blockage of one endocytic pathway, other endocytic pathways might be upregulated and take over functions of the blocked endocytic pathway. Consequently, results of perturbation experiments can be confusing, especially for viruses that do not strictly depend on one endocytic pathway for endocytosis. Furthermore, it is becoming more apparent that endocytic pathways are probably not static, but dynamic mechanisms which can share and use different adaptors. For example, CME has been reported in absence of AP-2, Eps15 can mediate clathrin-independent endocytosis; and AP-2 and clathrin were reported to be involved in phagocytosis. Thus, dependency on an endocytic protein can be a good indication for virus entry via a specific entry pathway, but should not be used as definite proof.

**Scope of the thesis**

The work presented in this thesis focuses on the early events in CHIKV and DENV infection, with a special interest in virus cell entry and membrane fusion.

CHIKV is central to the first part of this thesis, specifically Chapter 1 to Chapter 4. In Chapter 2 the literature on the early events in CHIKV infection is reviewed. In this review, a detailed insight into the molecular structure of CHIKV is provided. Subsequently, the cell tropism, receptor binding and the putative CHIKV receptors are discussed. Furthermore, the current knowledge on the CHIKV cell entry pathway, including the data from Chapter 3, and the CHIKV fusion mechanism is described. Finally, we discuss the use of entry inhibitors to prevent CHIKV infection.

In Chapter 3 the CHIKV cell entry pathway is unraveled by single particle tracking in live cells. For this purpose, CHIKV was fluorescently labelled with the fluorescent probe DiD. Tracking of DiD-labelled CHIKV was performed in cells expressing fluorescent marker proteins, such as clathrin, Rab5 and Rab7. Using this approach the cell entry
pathway, the cell entry kinetics and the organelle of viral fusion were determined. These results were confirmed by alternative approaches including the use of pharmacological inhibitors, siRNAs and DN mutants. Furthermore, we assessed the effects of the E1-A226V mutation, which first arose during the 2005/2006 CHIKV outbreak in La Réunion84, on cholesterol dependency of CHIKV entry and fusion.

When analyzing the CHIKV single particle tracking data, it was observed that approximately half of the viral particles showed fast-directed movements during virus cell entry. Therefore, Chapter 4 focusses on the transport behavior of CHIKV particles. Specifically, we aimed to elucidate the relationship between microtubules and CHIKV trafficking and infection.

DENV is the main subject of Chapter 5 to 7. Chapter 5 provides a general introduction on this virus. This chapter mainly focuses on DENV pathogenesis and the DENV life cycle, with special attention to cell tropism, receptor binding, entry and fusion. Furthermore, the concept of antibody-dependent enhancement (ADE) of infection, which is key to Chapter 7 and 8, is discussed.

During DENV infection, a large proportion of immature DENV particles are produced next to mature fully-infectious DENV particles85,86. Immature DENV particles were previously demonstrated to be essentially non-infectious in various cell types87–89. However, DC-SIGN, a receptor molecule which is expressed on the natural target cells of DENV, was recently described to promote infection of both mature and immature West Nile Virus particles90,91, a closely related flavivirus. In Chapter 6 we aimed to investigate the infectious properties of immature DENV particles in cells expressing DC-SIGN. For this purpose, we assessed the infectious properties of both mature and immature DENV-1, DENV-2 and DENV-4 particles on cells that do or do not express DC-SIGN. Moreover, the relation of DC-SIGN to the effects that antibodies exert on DENV infectivity were assessed.

In Chapter 7 the molecular mechanisms underlying antibody-dependent enhancement of DENV infection were identified. To this end, DENV infection was studied in primary human macrophages, a natural target cell of DENV infection. The effects that antibodies have on the different stages of DENV infection were gradually quantified in these cells. Furthermore, a microarray study was employed to gain insight into the intracellular response upon DENV infection.

In Chapter 8, we zoomed in on the cell entry pathway DENV hijacks under ADE conditions. According to literature, DENV-antibody complexes direct the virus towards Fc-receptor-bearing cells, such as macrophages. In this chapter, we performed a detailed study on the DENV cell entry pathway into the mouse macrophage cell line P388D1. Using a combination of biochemical inhibitors, dominant negative mutants and single particle tracking, the entry pathways of DENV under ‘standard’ and antibody-opsonized conditions were studied for both mature and immature particles.

Finally, the results obtained in this thesis are summarized and discussed in Chapter 9.
References

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GENERAL INTRODUCTION AND AIM OF THE THESIS

CHAPTER 1


GENERAL INTRODUCTION AND AIM OF THE THESIS