Dengue and Chikungunya virus
van Duijl-Richter, Mareike

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Chapter 9

Summarizing Discussion and Perspectives
Introduction

Dengue virus (DENV) and Chikungunya virus (CHIKV) are rapidly emerging, arthropod-borne viruses causing febrile disease. Both viruses are transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes and are therefore predominantly circulating in the tropical and subtropical regions of the world [1,2]. CHIKV (an alphavirus) and DENV (a flavivirus) are enveloped single-stranded positive-sensed RNA viruses. Not only the genome structure, but also the reproduction cycle of these viruses displays parallels. For example, both viruses enter their target cell via receptor-mediated endocytosis followed by fusion from within acidic endosomes [3-6]. Although the fusion proteins of DENV and CHIKV do not share conservation in amino acid sequence, the secondary and tertiary structure show remarkable overlap. Because of this homology, the fusion proteins E1 in alphaviruses and E in flaviviruses have been categorized as “class II” fusion proteins [7,8]. In this thesis, I strived to receive more insight into the entry mechanism of both DENV and CHIKV, and how antibodies can influence viral infectivity. Detailed knowledge of the cell entry process is not only important for our understanding of the viral life cycle, it also guides the development of antivirals that interfere with the early stages of infection.

In part (A), I will focus on the role of the DENV precursor membrane (prM) protein in virus infectivity and how antibodies control the infectious properties of the virus. In part (B), the CHIKV entry pathway and the molecular mechanisms of membrane fusion will be discussed. A promising approach to prevent or treat infection is the administration of highly neutralizing antibodies that interfere with these early steps of the viral life cycle. Therefore, in this chapter I will also focus on the working mechanism of potent, fusion-inhibitory monoclonal antibodies that have been described recently by us and others [9-11].

**Part A**

**Dengue virus heterogeneity and infectivity – the importance of the receptor molecule DC-SIGN and pre-existing antibodies**

Infections with dengue virus (DENV) have rapidly increased in number and geographical distribution during the last and current century. All four serotypes (DENV 1-4) can cause disease ranging from rather mild dengue fever to severe dengue, which can be fatal [12]. About 100 million symptomatic infections occur annually worldwide. Of these patients, about 500,000 – 1 million suffer from severe disease, and in 25,000 cases dengue infection is fatal [13]. Severe disease is almost exclusively seen in patients suffering from a secondary infection with another DENV serotype and in infants with waning maternal antibody titers. These observations led to the well-accepted theory of antibody-dependent enhancement of disease (ADE).
During ADE, cross-reactive and weakly neutralizing antibodies facilitate rather than neutralize DENV infectivity in natural target cells (including macrophages, monocytes and dendritic cells) via interaction with Fc-receptors [14,15]. In addition, during secondary heterologous infections “original antigenic sin” of B and T cells occurs. Effectively, this implies that the immune response is skewed towards the primary infecting DENV serotype (original antigen), resulting in high numbers of cross-reactive antibodies and low-avidity T cells. The phenomenon of ADE together with less efficient clearance of infected cells leads to a high viral load and infected cell mass early in infection. As a consequence, excessive cytokine production occurs, which in turn can induce endothelial cell damage thereby causing plasma leakage and hemorrhage [16-20].

In recent years, we and others focused on the role of the precursor membrane (prM) protein in DENV infectivity and disease. During virus reproduction, the prM protein acts as a chaperone to stabilize the membrane fusion protein E during exocytosis. Within infected cells, progeny virions are assembled in an immature form with the pr segment of prM capping the fusion loop on E [21]. The newly assembled prM-containing virions subsequently mature while passing through the Golgi and Trans Golgi Network (TGN). The slightly acidic lumen in these compartments induces a global conformational re-arrangement within the virion, through which a furin cleavage site at the pr-M junction is exposed. The pr segment is cleaved from the M protein by the host protease furin. At the slightly acidic pH of the TGN, the pr peptide stays associated with the particle to prevent pre-mature rearrangements of the E glycoprotein. Upon release of progeny virions in the pH-neutral extracellular space, the pr peptide dissociates from the viral particle [22-24]. This maturation process is required to render the particle infectious [25,26]. Interestingly, antibody precipitation studies and electron-microscopy analysis of DENV particles revealed that prM cleavage is inefficient. Mosquito cells were shown to secrete a mixture of prM-containing fully immature, M-containing mature, and prM/M-containing partially immature virions. Here, at least 40% of the secreted particles were partially immature, and approximately 3% of all particles were fully immature [27,28]. Partially immature particles have a mosaic presence with defined mature and immature areas rather than randomly cleaved prM proteins across the surface [29]. The maturation status of DENV particles secreted from human cells is poorly understood. Virus produced in immature dendritic cells contains less prM than mosquito-derived virions, whereas virus derived from primate endothelial cells and human embryonic kidney cells have prM contents comparable to those of mosquito-derived virus [30,31]. Here, we will describe the current knowledge on the role of prM-containing virus particles during primary and secondary DENV infection.
Role of immature and partially immature particles during primary infection

Until recently, fully immature DENV particles were thought to be essentially non-infectious in various cells, including endothelial cells, monocytic and macrophage-like cell lines and human PBMCs, and were therefore considered irrelevant in disease pathogenesis [26,31,32]. Likewise, fully immature particles of West Nile virus (WNV) – which is closely related to DENV – were found to be essentially non-infectious both in vitro and in an in vivo mouse model [33-35].

Previous research showed that fully immature DENV-2 preparations displayed a strong reduction in cell binding compared to mosquito cell-derived virus preparations (also referred to as standard [std] DENV). Binding is probably reduced because the receptor-binding site is less accessible on the immature prM-containing virion [32,36,37]. Although natural infection is presumably stopped at the level of virus-cell binding, immature particles were also found to lack the capacity to induce membrane fusion [25,33]. Membrane fusion is likely inhibited by the pr peptide, which caps the fusion loop on E [22,25]. In chapter 3, we show that besides DENV-2, also immature DENV virions of the other serotypes are essentially non-infectious in endothelial cells and macrophage-like cells.

Intriguingly, however, immature WNV was found to be infectious in cells engineered to express DC-SIGNR [38]. Other C-type lectins – amongst which DC-SIGN – are known to facilitate infection by std DENV [39,40]. DC-SIGN is expressed in high levels on immature dendritic cells (imDCs) [41]; cells that are encountered early in infection and are highly permissive to std DENV infection [42]. These findings prompted us to evaluate the infectious properties of immature DENV particles in imDCs.

In chapter 3, we show that immature DENV particles indeed infect imDCs via interaction with DC-SIGN, albeit to a much lower extent when compared to infection by std DENV. Infection is presumably mediated by binding to the N-linked glycans present on prM or E [21,23,38,43]. The extent to which DC-SIGN contributes to DENV infection was variable between the serotypes, with DENV-1 infectivity being most dependent on DC-SIGN expression. DC-SIGN expression enhanced productive DENV-1 infectivity of both std and immature virus preparations by four to five orders of magnitude. This difference was much less pronounced for the other two serotypes tested, DENV-2 and DENV-4.

Other groups also found a more profound effect of DC-SIGN on DENV-1 infectivity [40,44], whereas others did not [45,46]. The reasons for this discrepancy are not clear especially as most studies used the same DENV-1 strain and mosquito cells for reproduction. Furthermore, the glycosylation sites of the envelope proteins are conserved within the DENV species [47,48], thus other yet unknown factors must be involved in the serotype specific differences of DC-SIGN-dependent infectivity of DENV. Binding of a pathogen (in this case, DENV) to DC-SIGN induces intracellular signaling that contributes to the host defense and modulates activation of Toll like
receptors (TLRs) [49,50]. TLRs mediate activation of the innate antiviral response, amongst others by inducing the expression of antiviral interferons (IFNs). However, it is also known that DENV can counteract this antiviral response [51]. One of the possible explanations for the differences in DC-SIGN dependency is that the host response is suppressed to varying extents by different DENV strains, or that replication is differentially affected by the cellular antiviral mechanisms.

Upon binding to DC-SIGN, the immature virion will be internalized and delivered to the acidic endosome. Here, presumably post-entry maturation by furin cleavage takes place, followed by fusion from within the endosome [32]. Indeed, we show that furin activity is a prerequisite for productive infection by immature DENV. Though the pr peptide stays associated with the M/E protein at the mildly acidic pH in the TGN, it has been shown that the pr peptide dissociates from the virion at pH 5.0, a condition that is met in the late endosome [22]. Interestingly not only fully immature virus preparations but also std DENV showed a ten-fold reduction in infection when furin activity was inhibited (chapter 3). This again confirms that prM-containing particles that require post-entry cleavage are present in std DENV preparations. The majority of partially immature particles in std DENV preparations do however not require furin-mediated cleavage for infectivity. The threshold required for prM cleavage in relation to infectivity is currently unknown. Partially immature particles have one or more immature patches on their surface, while other regions are mature [29,52]. The E homodimers in the mature patches can interact with receptor or attachment factors to facilitate entry. Simultaneously, prM may act as receptor binding protein, so particles that do not have a sufficient degree of maturation to interact via the E protein, can still attach to cells expressing DC-SIGN. Upon entry, the particle is delivered to the acidic endosome, where the virus needs to fuse with the endosomal membrane to infect the cell. The extent to which prM cleavage is required for membrane fusion is unknown [53]. For WNV as little as two adjacent trimers were found to be able to mediate viral fusion [54]. Therefore, I propose that a relative small “hot spot” with sufficient E monomers being available to associate into two simultaneously acting trimers would be enough to mediate fusion of a partially immature DENV particle. Alternatively, if the virion does not reach the critical threshold of maturation to mediate fusion, maturation upon entry can render partially immature particles infectious.

Despite infection upon DC-SIGN binding, fully immature DENV preparations are by far not as infectious as std DENV preparations. This might be related to the positioning of the sugar groups on immature particles. Perhaps glycosylation of both prM and E is less favorable for efficient DC-SIGN binding. Another possibility is that furin-mediated cleavage upon entry is a rather inefficient process in imDCs and most internalized particles will be degraded. Alternatively, immature DENV may induce a higher antiviral response in imDCs thereby limiting the production of virus progeny. Indeed, a recent report showed that in particular immature DENV particles stimulate the IFN response of plasmacytoid DCs. The
authors proposed that the prolonged retention time in the endosome leads to this enhanced immune activation [55]. It will be of interest to determine whether this also holds true for imDCs and can explain why immature DENV is less infectious than std DENV.

To summarize, during a primary DENV infection, imDCs, monocytes and macrophages are considered of high importance with regard to the development of viremia and virus dissemination [42,56-58]. Fully immature DENV displayed low infectivity in imDCs and was found essentially non-infectious in macrophage-like and monocytic cell lines. Therefore, I conclude that the contribution of fully immature particles to the total viral load can be described as marginal. Viremia and virus dissemination are predominantly caused by partially immature particles that do not require furin cleavage and/or mature DENV particles. Even though immature DENV particles do not play an active role in virus production in primary infection their role in disease pathogenesis needs further attention. On the one hand, sensing of immature particles might be important in the establishment of an antiviral state of the cell without initiating the production of a large number of progeny virus. On the other hand, immature particles play an important role in priming the adaptive immune system for following infections. Importantly, they trigger the generation of cross-reactive B and T cells targeting the prM protein, which are mainly re-activated during secondary infection.

Secondary infection: Antibody response and the role of immature particles and partially immature particles in infection

During secondary infection, the incoming virus particles encounter pre-existing antibodies produced by long-lived plasma cells that are generated during primary infection. Moreover, DENV-specific memory B cells are rapidly expanded and secrete high levels of antibodies [16]. Many studies have shown that antibodies have a dual role in controlling disease outcome. During homotypic re-infection, the type-specific antibody response has highly neutralizing capacity and individuals are protected from disease [18,59]. Upon heterotypic re-infection and in infants born to DENV-immune mothers, a higher incidence of severe disease is seen. This led to the hypothesis that antibodies can contribute to disease severity, called antibody-dependent enhancement of disease (ADE) [14,60]. Several factors define whether DENV infectivity is neutralized or enhanced. Besides maturation state of the virion, and therefore availability of epitopes, also the antibody titer, binding specificity and antibody affinity play an important role in neutralization vs. enhancement. To better understand these factors, I will first take a closer look at the human antibody response against DENV.

The vast majority (≥80%) of antibodies that are generated during primary DENV infection target the E protein (reviewed in [61]). Most E antibodies target...
domain I/II and the highly conserved fusion loop (FL) located on domain II. Many of these antibodies were found broadly cross-reactive and had low neutralizing capacity [19,62,63]. Furthermore, given the large structural differences between immature and mature particles, differential epitopes are exposed and depending on the maturation status of particles neutralization or enhancement may occur. For example, it has been shown that an anti-WNV antibody targeting the FL preferentially binds to the spike of immature particles and renders both immature WNV and DENV infectious [34,64]. Thus, considering the high occurrence of FL antibodies in combination with the observed enhancing capacities towards immature particles, it is conceivable that these antibodies can contribute to ADE. Yet, cross-reactive FL antibodies were also found to contribute to the neutralizing capacity of human polyclonal sera against heterotypic infection [65]. In my opinion, these at first glance contradictory results reflect the fact that it is likely that a higher occupancy of the virion can be obtained in a polyclonal situation compared to a monoclonal situation.

A small fraction of the human antibody response is targeting E DIII epitopes. It is not understood yet why the human antibody response against E DIII is rather low, especially as the majority of the murine antibody response against DENV is targeting E DIII. Antibodies against E DIII were shown to be serotype-specific as well as cross-reactive. Like FL antibodies, also E DIII antibodies are able to render immature DENV infectious, especially at low concentrations [66]. However, most antibodies displayed high neutralizing capacities with low enhancing potential against std DENV [19,36,67-69].

Recently, a new class of antibodies binding quaternary epitopes across several E proteins has been described. These antibodies are mainly serotype-specific and were shown to be highly neutralizing. By binding multiple E proteins, the particle is “locked” in the neutral pH conformation and the conformational changes required to mediate fusion cannot occur, thereby neutralizing the virion. It was shown that these monoclonal antibodies can bind partially immature DENV preparations as efficient as more mature preparations, and are less prone to induce ADE than for example FL antibodies. Importantly, antibodies targeting quaternary structures do not bind to fully immature particles. Therefore, immature particles cannot be neutralized, but more importantly, can also not be enhanced by antibodies targeting quaternary epitopes [30,70-73].

In addition to antibodies targeting the E protein, a small fraction of the antibody response is targeting the prM protein and the non-structural protein 1 (NS1). Interestingly, during secondary infection, especially the prM and NS1 response is boosted [16,31,63,74]. It was shown in several studies that after primary infection, 20-40% of the patients have antibodies against prM, which increased to 61-100% after secondary infection [16,63,74]. The NS1 protein is secreted and expressed on the surface of infected cells. As NS1 is not a component of the virions, antibodies against NS1 can neither enhance nor directly neutralize viral infectivity [75]. prM antibodies recognize immature particles and functional studies revealed that they are poorly
neutralizing, cross-reactive and highly enhancing [19,20,31,32,62]. These antibodies cannot bind to mature virions or the mature patches on partially immature virions, which explains why these antibodies exhibit a flat neutralization curve with a fraction resistant to neutralization. This fraction, which is depending antibody specificity and tested serotype ranging from 10%-60%, thus resembles the mature and nearly mature virions of the virus population [20,31]. Antibodies binding to prM on immature and partially immature virions are probably removed with the pr peptide during furin cleavage. Subsequently, post-entry maturation can take place followed by fusion with the endosomal membrane.

Taken together, no antibodies have been identified to date that solely neutralize DENV infectivity. So the question remains: what are critical hallmarks of neutralization and ADE and what role do immature and partially immature virions play in both scenarios? Neutralization of flaviviruses requires binding of a critical number of antibodies, the so called “multiple hit” phenomenon [53,76,77]. To achieve sufficient binding for neutralization, antibodies must be present in high concentrations, and/or the affinity of the antibodies must be high [78]. Even a neutralizing antibody can therefore enhance infection if the concentration is so low that large areas on the virion remain un-occupied and can mediate viral fusion.

During homotypic re-infection, serotype-specific antibodies can bind with high affinity, which efficiently neutralizes the infecting virus particles [78,79]. Moreover, highly-specific memory B and T cells are quickly reactivated, and a large number of cross-reactive and serotype-specific antibodies is secreted [16]. Due to high antibody titers and therefore high polyclonal occupancy, most DENV particles will be neutralized, even fully immature and partially particles. This because only few highly neutralizing, high affinity antibodies are required to inhibit virus infectivity. Indeed, for WNV, a theoretical occupancy of 30 antibodies was found sufficient to block membrane fusion [80]. This hypothesis is further supported by our observation that low dilutions of homotypic anti-DENV serum (taken 28 days after convalescence) neutralize immature DENV infectivity in imDCs (chapter 3). Likewise, if re-infection with a heterotypic serotype occurs within a few months after the primary infection, antibody titers are high enough to prevent infection, even if cross-reactive antibodies display rather low affinity [81,82]. As the majority of virions will be neutralized at an early stage and T cells quickly clear the few infected cells, individuals will be protected from systemic infection and disease [18].

However, individuals with waning antibody titers (starting after approximately 6 months to 3 years after primary infection or in children born to DENV immune mothers) are at risk of ADE [81,82]. The pre-existing the antibody-response is cross-reactive and often weakly neutralizing towards the newly infecting serotype. This results in a low degree of opsonization and enhanced virus production early in infection. Furthermore, the production of serotype-specific antibodies is hampered, as preferentially pre-existing cross-reactive memory B cells are expanded as part of the “original antigenic sin” phenomenon [16]. Generally, cross-reactive
DENV antibodies have weaker neutralizing properties than serotype-specific antibodies and tend to facilitate ADE. Low affinity cross-reactive antibodies may dissociate from the virus after Fc-receptor mediated uptake thereby allowing the virus to initiate a productive infection. Furthermore, cross-reactive antibodies that bind to cryptic sites on the particle may not reach the opsonization grade necessary for neutralization [78]. In addition, high numbers of cross-reactive prM antibodies are secreted. prM antibodies likely dissociate from the particle upon furin-mediated cleavage of prM thereby rendering the particle infectious. Therefore, prM antibodies are more prone to facilitate ADE than E antibodies. Although prM and E antibodies expand the number of infectious circulating virus particles, immature particles and the antibodies recognizing them are not the sole factor in severe disease development [83]. In my opinion, severe disease development is triggered by cross-reactive E and prM antibodies that facilitate ADE of mature, partially immature and fully immature particles; and the ability of an antibody to set the stage to neutralization or enhancement of infection is determined at the epitope level.

Which target cells are important during ADE? It was demonstrated before that monocytes, macrophages, and mature DCs are susceptible for ADE by std DENV, but imDCs are not [41,42,84,85]. In line with these studies, we found that human anti-DENV serum did not enhance infectivity of std DENV or immature DENV (chapter 3). It has been proposed that DC-SIGN levels negatively correlate with levels of ADE [84]. Immature DCs express high levels of both DC-SIGN and Fc-receptors and therefore, there is no net increase in productive infection when antibodies are present. Yet, earlier studies and the work described in this thesis were obtained using mosquito-derived virus. This mimics the first round of infection after a mosquito bite, which is important to establish the infection. Interestingly, std DENV produced in imDCs does not bind to DC-SIGN and supports ADE in imDCs [86]. This finding strengthens the “competition” theory between DC-SIGN mediated entry and Fc-receptor mediated entry. Thus, during sequential rounds of infection imDCs may also contribute ADE. Although infectivity of mosquito-derived immature particles is not enhanced in imDCs, these particles were found to facilitate ADE in monocytes and macrophages, thereby increasing viral load (chapter 3, [31,32,66]). Hence, I propose that early in infection, monocytes, macrophages and mature dendritic cells are the key contributors to ADE, with a possible role of imDCs later in infection.

Pre-existing antibodies are clearly associated with severe disease development [14,59]. Patients suffering from severe dengue often have high viral loads early in infection, and in my opinion, ADE contributes to the higher amount of viral progeny and infected cell mass in these patients [87]. It is important to note however that upon secondary infection the majority of individuals experience no or only mild disease. It is as yet unknown why some individuals are more prone to develop severe disease than others. Unknown host genetic factors as well as virulence and sequence of the
infecting DENV have been proposed to further influence disease outcome (reviewed in [88]). To conclude, the factors mentioned above make dengue pathogenesis very complex. Hence, further research is needed to define the group at risk for severe disease and optimize the treatment of these patients.

**Perspectives and concluding remarks**

In the work presented in this thesis, we addressed the infectivity of DENV virions of different serotypes and maturation status in varying target cells types, including DC-SIGN expressing imDCs and macrophage-like cells. We found that like immature DENV-2 – also immature DENV-1, 3, and 4 is essentially non-infectious in epithelial cells. Moreover, we showed that immature DENV-1 and DENV-4 can be rendered infectious in presence of heterotypic serum. We also observed notable differences in viral infectivity between different serotypes. For example, DC-SIGN dependency is strongly dependent on the virus serotype, with DENV-1 infectivity being most enhanced by DC-SIGN. These differences should remind us that conclusions derived from research on the prototype DENV serotype (DENV-2) do not always apply on all DENV serotypes or strains. It would be interesting to investigate whether DC-SIGN dependency impacts disease outcome, as it is known that clinical manifestation and severity of disease are correlated with the infecting serotype [89-94]. One could imagine two opposite consequences of increased DC-SIGN dependency. On the one hand, the increased infection of imDCs by a virus strain could lead to higher viral loads early in infection, and therefore increase the chance to develop severe disease. On the other hand, imDCs are also important connectors between the innate and the adaptive immune system. The increased infection of imDCs could therefore lead to a quicker and more efficient antiviral response. However, given the complexity of DENV infection that goes beyond the sole usage of DC-SIGN, it is questionable if such a linear correlation exists or can be identified.

An important factor that contributes to the complexity of DENV is the inefficient maturation of the virions. Though only approximately 3% of mosquito-grown DENV particles are completely immature, at least 40%, but probably a higher proportion of virions is partially immature. The inefficient furin cleavage is conserved within Dengue viruses, but not other flaviviruses [28,95]. The particle heterogeneity found in DENV was proposed to reflect an evolutionary advantageous immune evasion strategy, in which occupancy by highly neutralizing antibodies is reduced due to residual prM proteins on the viral surface [96]. Indeed, it has been shown that partial maturation influences the accessibility of certain antibodies on WNV particles [97]. Furthermore, the observation that flaviviruses cannot be completely neutralized by monoclonal antibodies is likely due to partial maturation [98]. It would be of interest to determine the infectious properties of partially immature DENV in primary, but especially during secondary infection. It is as yet not possible
to isolate or investigate partially immature virions in detail. For example, the degree of maturity can range from none to 180 cleaved prM proteins, and it will be difficult – if not impossible – to distinguish the maturation status on a per particle basis. However, research on fully immature particles like the study presented in this thesis can give us indications on how partially immature virions enter a cell. In addition, comparing results obtained from particles derived from different cell types and therefore different degrees of maturation can assist in characterizing the role of partially immature virions in infection [30].

The development of an antibody-based therapy or vaccine has been challenging as preventing vaccine-induced ADE is of paramount importance. To further lead vaccine development, several groups are trying to identify which antibody properties are important for neutralization of DENV. Recently, a new class of cross-reactive, highly neutralizing antibodies against quaternary epitopes on the E protein has been discovered [30,70-73]. These antibodies efficiently “lock” adjacent E dimers by binding to epitopes across several E proteins, and therefore presumably prevent fusion, an important feature of post-attachment neutralization [72]. Importantly, these antibodies cannot bind to immature DENV and bind partially immature virus preparations as efficient as more mature preparations, which reduces the risk of ADE [30]. Therefore, quaternary epitopes represent a valuable antigenic determinant that should be considered in future vaccine research.

**Part B**

**Early events in Chikungunya virus infection – viral entry mechanisms and neutralization by antibodies**

Since its re-emergence in 2004, Chikungunya virus (CHIKV) has spread across 44 countries in Southern Europe, Africa, South-East Asia and the tropical regions of the Americas, causing millions of infections [99]. CHIKV is a plus-sensed enveloped RNA virus that belongs to the genus of the alphaviruses and is transmitted by mosquitoes of the *Aedes (Ae.*) species [100]. Infection with CHIKV leads in the majority of the cases to Chikungunya fever, which is characterized by high fever, myalgia, headache, rash, and debilitating joint pain [101]. The latter can be persistent for months to years, posing a serious health-economic problem in the affected areas [102,103]

The first major epidemic of CHIKV – which spread from the west coast of Africa – was facilitated by adaptation of the virus to transmission by *Ae. albopictus* without seriously compromising fitness in the original vector *Ae. aegypti*. It has been shown that mutations in the envelope glycoproteins E1 and E2 were pivotal for this adaptation. The envelope glycoproteins mediate the cell entry process of CHIKV, and the altered requirements for viral entry are likely responsible for the increased fitness
of the virus in *Ae. albopictus* [104-106]. As CHIKV outbreaks had been small and spatiotemporal confined before 2004, research on the biology of CHIKV was scarce. Therefore, the majority of knowledge on the infection mechanism of the virus was relying on research on closely related alphaviruses [107]. Fortunately, during the last decade numerous studies (including the work presented in this thesis) have greatly improved our specific understanding of how CHIKV enters the host cell ([6,108-111], *chapter 5, chapter 6, chapter 8*). Here, I will focus on the infection mechanism of CHIKV, including virus binding, internalization, and membrane fusion. Interference with these early stages of infection represents an attractive approach for antiviral therapy. We and others investigated the potential of monoclonal antibody therapy against CHIKV ([9,112-117], *chapter 7*), which will be discussed in the last part of this chapter.

The first step in infection: Virus binding & uptake

In *chapter 4*, we summarize the current state of knowledge on the cell entry pathway of CHIKV. CHIKV infection begins with binding of the virion to the cell. Receptor binding is mediated by binding motifs located on the E2 protein [118-120]. Several molecules that can serve as a receptor or attachment factor have been identified so far like for example prohibitin, phosphatidylserine-mediated virus entry-enhancing receptors, glycosaminoglycans, and ATPsynthase β subunit [121-124]. Interestingly, none of these molecules were found to be essential for CHIKV entry, suggesting that the virus may not need a true receptor for cell entry. For other alphaviruses, comparable results and overlap in receptor usage has been found [107]. Most alphaviruses, including CHIKV, are using a mosquito vector for transmission to vertebrates, and the usage of multiple attachment factors or receptors has been postulated to facilitate the ability of crossing the species barrier [125].

Alphaviruses often enter cells via clathrin-mediated endocytosis (CME) [126-129]. Previous studies using biochemical inhibitors and dominant negative mutants showed that CHIKV is dependent on Eps15, a component of clathrin-coated pits [6] and dynamin, which is important for the pinching of endocytic vesicles [108]. Though these findings suggest that CHIKV indeed enters the cell via CME, another study showed that inhibition of the clathrin heavy chain via siRNA only partially inhibited infection [109]. To gain more insight into the cell entry process of CHIKV we used live-cell microscopy and cell-based assays to study virus entry (*chapter 5*). This approach allowed us to study the route of cell entry during natural infection without the use of inhibitors that might perturb additional cellular processes. We found that CHIKV rapidly enters cells, with half of the fusion events occurring within 9 minutes post-infection, and 95% of fusion events taking place within 22 minutes after infection. Furthermore, by simultaneously tracking yellow-fluorescent protein (YFP)-clathrin and DiD-labeled virions, we found that 89% of all virions first co-localized with clathrin. Furthermore, we showed the CME inhibitors reduced viral
infectivity and prevented viral fusion. These findings support the hypothesis that the major entry pathway of CHIKV is via CME. In addition, I believe that CHIKV is also able to use other cell entry pathways. It is likely that these alternative pathways are especially enhanced when CME is inhibited, thereby explaining why CME inhibitors do not completely abrogate viral infectivity.

**Endocytic trafficking and membrane fusion**

Following virus internalization, the clathrin molecules dissociate from the endocytic vesicle, and the vesicle fuses with Rab5-positive early endosomes. These endosomes further mature into Rab7-positive late endosomes via an intermediate called “maturing endosome” which contains both Rab5 and Rab7 [130]. It is known that most alphaviruses fuse from early endosomes, though VEEV fuses from within late endosomes [127]. In chapter 5, we revealed by live-cell microscopy that 95% of all CHIKV particles fuse from within Rab5-positive early endosomes. This is in agreement with the study of Bernard and colleagues, which showed that infection of CHIKV is dependent on the integrity of the Rab5 endosomal compartment [6]. Membrane fusion is triggered by exposure of the virus to low pH. In chapter 5, chapter 6 and chapter 8, we investigated the pH dependency of fusion for different CHIKV strains in a liposomal model system. We found the optimal fusion was reached at pH values below pH 5.6. The threshold of fusion was situated between pH 6.2 and 5.9 (depending on strain), which indeed resembles the pH range in early endosomes [131]. Interestingly, 40% of the virus particles fused almost immediately (<10 seconds) after co-localization with the endosome. In the bulk fusion assay, also a subset of viruses fused around the pH threshold fusion. Here, the total fusion extent of the same strain was around 48% at pH 5.8, 30% at pH 5.9, and around 5% at pH 6.0. This indicates that the pH in the fused organelle is just below the threshold of fusion, allowing immediate fusion for a subset of particles. The observation that only a fraction of particles immediately fuses suggests that there is certain heterogeneity within the virus population regarding the pH dependency. This theory is supported by our observation that upon exposure to low pH in the absence of target membranes, two populations with different susceptibility for acid-induced inactivation were identified (chapter 6). From an evolutionary point of view, it can be advantageous for CHIKV to exhibit variation in pH dependency. CHIKV has to alternate between hosts and infects different target cell types, and it is known that cell types vary in their endosomal pH [131]. Also, it has been found that CHIKV infectivity in mosquito cells is dependent on the integrity of both Rab5 and Rab7 [111]. The heterogeneity might be explained by the presence of several quasispecies with slightly different genomes, a phenomenon that is common in RNA viruses [132]. By producing particles fusing at different proton concentrations, the virus can ensure that fusion can take place at an optimal place in various cell types for at least a subset of particles.
Requirements for CHIKV membrane fusion

The results in chapter 5 and 6 clearly show that CHIKV membrane fusion is critically dependent on exposure to low pH. Upon lowering the pH below a critical threshold, CHIKV fuses rapidly with membranes lacking a protein receptor. This finding is in agreement with the observations that CHIKV uses a variety of attachment factors, but that no specific receptor interaction is required to activate the fusion machinery (described above and in chapter 4).

For the alphaviruses Semliki Forest virus (SFV) and Sindbis virus (SINV), it is known that cholesterol and sphingomyelin are promoting low-pH triggered fusion [133-135]. Using target membranes with varying concentrations of these lipids, we revealed in chapter 6 that CHIKV fusion is also strongly dependent on target membrane cholesterol and sphingomyelin. CHIKV fusion is dose-dependent on cholesterol. Increased cholesterol contents lead to higher fusion extents, with a maximum fusion extent reached at ~ 40 mol%. In contrast, already limited amounts (~6 mol%) of sphingomyelin were sufficient for optimal fusion efficiency. In the absence of either lipid, only residual fusion activity was measured. Such a specific lipid requirement can influence the place of viral fusion within the endocytic pathway. For example, DENV fusion requires negatively charged lipids, and it is postulated that DENV ensures fusion from within the late endosome by using these lipids [136]. In contrast, the lipid requirements for alphavirus fusion are in line with membrane fusion from within early endosomes. Previous studies showed that the composition of the membrane lipids changes as the endosome matures, with decreasing levels of cholesterol and sphingomyelin; and enrichment of negatively charged lipids in late endosomal compartments (reviewed in [137]). In the early endosome, the membrane composition largely resembles the plasma membrane, with cholesterol and phospholipids in a ratio of 1:1. The cholesterol content gradually decreases in the endosomal pathway, with a 2:1 phospholipid ratio in the late endosome towards nearly cholesterol-free lysosomes [138,139]. Furthermore, sphingomyelin is rapidly degraded to ceramide by sphingomyelinase as the endosome matures. The occurrence of sphingomyelin is likely reduced from around 25% to below 10% in the late endosome [137,138,140]. However, SFV was found to also fuse with target membranes that do not contain sphingomyelin, but ceramide [141,142]. Hence, the influence of cholesterol on the place of fusion is likely greater than the influence of sphingomyelin. Taken together, the changes in lipid composition in the endosome support the conclusion that CHIKV fuses from within early endosomes.

Structural transitions and kinetics of membrane fusion

Chikungunya virus membrane fusion (described in detail in chapter 4) involves a series of major re-arrangements of the envelope proteins E1 and E2. E1 forms a heterodimer with the E2 glycoprotein, and three of these heterodimers
are assembled as one spike at the viral surface. Each virion has 60 spikes. The E1 glycoprotein is structurally divided in the three domains DI, DII and DIII and contains the hydrophobic fusion loop located at the tip of DII. The E2 protein also contains three functional domains, termed A, B, C; with domain B shielding the fusion loop at neutral pH [120]. The fusion process has been thoroughly characterized for SFV and SINV [107,135,143,144]. Recent publications, including the work presented in this thesis, indicate that CHIKV follows a comparable mechanism (chapter 6, chapter 8, [120,145]). The structural transitions that mediate fusion include destabilization of the E2/E1 heterodimer, insertion of the E1 fusion loop into the target membrane, E1 trimerization, hemifusion and finally fusion pore formation. Both the E1 and E2 proteins possess a series of highly conserved histidines, which serve as a “pH switch” that initiate the fusion process [145]. Protonation of histidines causes disordering of domain B and the acid-sensitive region of the E2 protein. This leads to displacement of domain B, exposure of the fusion loop of E1 and loosening of the E1-E2 interaction [120,146]. Subsequently, the fusion loop inserts into the target membrane. For SFV, this process requires low pH and cholesterol and is supported by sphingomyelin [147-149]. In chapter 5 and chapter 6, we show that fusion of CHIKV is not strictly dependent on cholesterol, indicating that fusion loop insertion is not completely reliant on the presence of this lipid. Yet, cholesterol and sphingomyelin likely promote fusion loop insertion of CHIKV, as fusion efficiency is strongly promoted by these lipids.

Several residues of the alphavirus E1 protein are involved in the “sensing” of specific lipids in the target membrane. One of these, E1-226, is located in close proximity to the fusion loop, and was suggested to directly interact with the target membrane or to change the conformation of the fusion loop via a yet unknown mechanism [133,150,151]. Indeed, we and others showed that CHIKV strains with an E1-A226V mutation are more dependent on cholesterol for viral fusion (chapter 5, [104,150,152]). Two other residues that play a role in “lipid sensing” are E1-44 and E1-178 [133,152]. These residues are located near a flexible region in the E1 protein, and it has been suggested that changes in the amino acids at this region probably alter the angle between the fusion loop and the target membrane, thereby affecting lipid dependency [153].

Upon insertion of the fusion loop and after further increase in proton concentration, the E2 protein dissociates from E1, and a core trimer is formed by the DI and DII domains of three E1 proteins. Then, the E1 DIII domain re-folds against the core trimer, forcing the opposing membranes together. This leads to hemifusion followed by pore formation [146,154,155]. Assembly of the SFV core trimer is dependent on pH and likely cholesterol and sphingomyelin as well [142,148]. Using sedimentation analysis, we found low-pH induced the E2/E1 heterodimer dissociated even in absence of liposomes, but that the formation of an E1 trimer is strictly dependent on the presence of target membranes (chapter 8). Hence, I propose that also formation of the core trimer of CHIKV is supported by
cholesterol and sphingomyelin. Interestingly, we found that the extent of E1 trimer formation was with ~14% surprisingly low (chapter 8). Under the same conditions and in accordance with literature, the extent of E1 trimerization of SFV was three times higher [134]. The reported low-pH induced trimer formation of SINV (which is more distant related to CHIKV than SFV) was with 76% even higher [156]. Yet, despite these differences comparable extents of membrane fusion were measured for all three viruses.

To understand the membrane fusion process in more detail, we employed high resolution Total Internal-Reflection Fluorescence (TIRF) microscopy (chapter 6). This technique allowed us to investigate the kinetics of the membrane fusion process of single CHIKV particles. By analyzing the distribution of lag time of individual particles fusing after acidification, we show that fusion is preceded by several intermediate states. The number of these intermediate steps lies around \( N=2-3 \) for the three different pH values we investigated. If this number would represent sequential steps, at high pH the proton-dependent step would become rate-limiting and reduce \( N \) to 1 [157]. As the value for \( N \) remains comparable across the different pH values, I suggest that the rate-limiting steps represent parallel steps. For Influenza virus, using the same analysis, it has been proposed that the three rate-limiting steps of fusion represent the simultaneous collapse of three activated hemagglutinin trimers [157-159]. In line with this finding, it is likely that the 2-3 rate-limiting steps described here represent the simultaneous re-folding of two to three CHIKV E1 trimers. This theory is further supported by the observation that CHIKV pH dependency is very sharp, as we measured an eightfold reduction in fusion extent over only 0.2 pH units. This also suggests that the fusion process involves a form of cooperativity between neighboring fusion trimers [160,161], which could represent the interactions of several E1 trimers mediating fusion. In line with such a mechanism, assembly of 5-6 SFV E1 trimers in a ring-like structure was visualized by cryo-electron microscopy [160,162]. In theory, up to 80 E1 trimers can be formed, but apparently only a fraction is needed to mediate fusion. This observation might explain why high total fusion extents despite low extents of E1 trimerization were observed for CHIKV fusion.

What do we know about fusion stoichiometry of other viruses? In all cases known, a relatively small number of fusogenic trimers is sufficient to mediate fusion. Depending on viral strain, 3-4 or 8-9 hemagglutinin trimers are needed for Influenza fusion [157,159,163], at least 2 E homotrimers are needed for WNV fusion [54], and, depending on viral strain, 1-7 envelope glycoprotein trimers are needed for HIV fusion [164]. Interestingly, for HIV low entry stoichiometry was correlated with high fitness, which might explain why many viruses, including CHIKV, only need a few activated trimers for entry.
Influence of fusion requirements on vector competence and viral fitness

The first major outbreak of CHIKV that spread from the West coast of Africa was at least partially caused by adaptation of the virus to a new mosquito vector [106]. A single amino acid mutation from alanine to valine at position 226 in the E1 protein (discussed in detail in chapter 4) was identified to be responsible for the switch of the main vector of CHIKV IOL strains from *Ae. aegypti* to *Ae. albopictus*. This adaptation facilitated spread of the virus to new areas [104,106]. The E1-A226V mutation has also been connected to viral entry, as E1-226A CHIKV strains display higher dependency on low pH and cholesterol for fusion than E1-226V strains (as shown in chapter 5, and in accordance with [104,150,152]). The CHIKV IOL strains adapted further to *Ae. albopictus* by amino acid changes to glutamic acids or glutamine in the acid-sensitive region of E2. It was proposed that the combination of mutations influences the CHIKV life cycle at different stages, including probable change of virus-receptor binding and stabilization of the heterodimer during virus assembly. Moreover, and importantly, the mutations were proposed to increase viral fitness by changing pH sensitivity and ensuring fusion at the optimal endosomal stage in mosquito midgut cells [105,165,166]. Additional research will be needed to proof this hypothesis and to investigate the causal relationship of vector adaptation and changes in viral entry requirements. Furthermore, a detailed understanding of how CHIKV adapts is important as the recent outbreak in the Americas is caused by the Asian lineage of the virus, which is predominantly transmitted by *Ae. aegypti* [106]. When this strain also adapts to *Ae. albopictus*, an even larger epidemic is suspected, as this mosquito species is more widespread in North America and Europe than *Ae. aegypti* [167,168].

Neutralization of CHIKV by monoclonal antibodies

The antibody response against CHIKV has been shown to control viral infection and limit tissue injury [169-173]. A promising antiviral strategy is therefore the deployment of CHIKV-specific antibodies for both prophylactic and therapeutic protection [112,113]. In the past years, several murine (chapter 7, [114,174-176]) and human [9,115,116] neutralizing monoclonal antibodies have been identified. In chapter 7, we describe the generation of 230 murine monoclonal antibodies (MAbs). Of these, 36 exhibited broadly neutralizing activity against all CHIKV genotypes. In an in vivo mouse model, several of these antibodies were shown to be protective as prophylaxis and limit disease if given as a combination therapy in a therapeutic setting.

How is antibody-mediated neutralization of CHIKV infectivity achieved? For alphaviruses, it has been shown that antibody opsonization can prevent binding of the virion to the host cell receptor [118], interfere with the pivotal conformational changes needed to mediate fusion [177], and inhibit egress of newly formed
CHIKV particles [176]. In this thesis, we focused on deciphering the neutralization mechanism of a selection of monoclonal antibodies (chapter 7, 8). One of the most potent antibodies studied by us and others is the E2 antibody CHK-152. We show that this MAb prevents CHIKV infection and inflammatory arthritis in a murine mouse model, both alone and in combination with an additional antibody. Besides direct effects by neutralizing infectivity of the virion, also Fc effector interactions of CHK-152 contributed to protection in vivo. In vitro, CHK-152 prevents CHIKV infection both in pre- as well as in a post-binding setting. This indicates that neutralization presumably takes place both at the level of virus-cell binding and at the level of membrane fusion. Indeed, we demonstrated that CHK-152 efficiently inhibits CHIKV fusion in a dose-dependent way. In line with this, we showed that E1 trimerization is partially inhibited by CHK-152. Yet, as this antibody binds the E2 glycoprotein and not E1, it is likely that the membrane fusion process is already affected before E1 trimerization. Cryo-electron microscopy analysis revealed that CHK-152 binds several residues spanning domain A, domain B, and the linker peptide between both domains. It has been proposed that CHK-152 stabilizes domain B and prevents exposure of the E1 fusion loop [11]. This result is in line with our finding that CHK-152 almost completely blocks low-pH induced binding of CHIKV to liposomes (chapter 8), a process that requires insertion of the fusion loop in the target membrane [149].

Upon screening of 18 neutralizing antibodies for their fusion inhibitory capacities, we found that about one third of the antibodies strongly inhibited fusion, one third showed mediocre fusion inhibitory activity and the last third did not or only slightly inhibit fusion (chapter 8). Interestingly, antibodies binding to the E1 fusion protein belonged to the group that did not or only partially inhibit viral fusion. Antibody binding is likely impaired as epitopes on E1 are less accessible, since E1 is largely shielded by E2 on the viral surface [120]. Two E2 antibodies and one E1 antibody were selected for further analysis to gain insight on how antibodies can interfere with membrane fusion. These antibodies were highly neutralizing but less potent than CHK-152. We found that also these antibodies inhibited viral fusion, E1 trimerization and binding of CHIKV to liposomes, albeit to a lower extent than CHK-152. It is therefore likely that other mechanisms are involved in fusion inhibition for these antibodies, like for example cross linking of several spike proteins or prevention of fusion by steric hindrance.

In addition to blocking infection in an early stage, we demonstrated that CHK-152 and other anti-CHIKV-MAbs are able to inhibit viral egress (chapter 8). Progeny alphaviruses are assembled at the cellular plasma membrane. In the infected cell, newly produced E2/E1 heterodimers accumulate at the plasma membrane, where they associate with the nucleocapsid to form viral particles. It has been shown that correct assembly and budding of alphaviruses is dependent on interaction of the cytoplasmic domain of E2 with the nucleocapsid [11,178,179]. Antibody-binding to E2/E1 heterodimers on the cell surface has been proposed to re-positing of E2,
thereby preventing interaction with the nucleocapsid. Alternatively, it is possible that budding is blocked through steric hindrance with the antibody [176].

When looking at the general properties of neutralizing antibodies of our and other studies, it emerges that the majority of neutralizing CHIKV antibodies is generated against highly accessible sites on domain A, domain B, and connecting areas and ribbons (including the acid-sensitive region) of the E2 protein. Many of these antibodies inhibit membrane fusion ([9,116,172,174,180], chapter 7, chapter 8). For example, in a recent study potent human MAbs were analyzed for their binding properties and mechanism of action. The group identified several ultra-potent neutralizing antibodies which blocked viral fusion, likely through a comparable mechanism as CHK-152 [9]. Also neutralization through blockage of receptor binding, egress inhibition [176] and most commonly, a combination of these three mechanisms were reported. However, antibodies that also inhibit fusion inhibition seem to be the most efficient in neutralization of viral infectivity.

Why are antibodies that inhibit membrane fusion more potent than antibodies solely blocking attachment? Like for DENV, neutralization of CHIKV seems to be a multiple hit phenomenon, in which a minimum number of antibodies needs to be bound to prevent infection [76,77]. CHIKV is able to bind multiple receptors (reviewed in chapter 4) and contains at least two receptor-binding sites [120,181]. It is therefore possible that a virus opsonized by an antibody that blocks one receptor binding site can still bind to a different receptor or attachment factor. Therefore, I believe that in general, fusion inhibitory antibodies require lower the occupancy to achieve neutralization than antibodies inhibiting attachment. As mentioned before, CHIKV fusion is a cooperative process with multiple fusion proteins acting in concert to mediate fusion. Therefore, not all but only a subset of fusion proteins needs to be deactivated to prevent fusion and thus infection. This theory has been confirmed for neutralization of Influenza virus. For this virus, it has been shown that opsonization with increasing antibody concentrations lead to a prolonged lag time between acidification and viral fusion [158]. In chapter 8, we also found that the fusion lag time is increased when CHIKV is opsonized with CHK-152. The increased lag time indicates that more time is needed until the required number of trimers is available for fusion. This may be related to the fact that a subset of E1 proteins is neutralized by antibody binding. Furthermore, at high antibody concentration, unbound E1 proteins may inactivate before a sufficient number of trimers can work together to mediate fusion, and the virion is neutralized. For Influenza virus, it was shown that this condition is already met when only about half of the stoichiometrically available epitopes are occupied [158]. I propose that a comparable mechanism takes place during opsonization of CHIKV with fusion inhibitory antibodies, explaining their ultra-potent capacity. However, no detailed stoichiometric data is available yet, and more research is needed to confirm this hypothesis.
Perspectives and concluding remarks

In the research presented in the second part of this thesis, we dissected the entry pathway of CHIKV by live-cell microscopy and investigated the requirements for CHIKV fusion in a liposomal bulk assay and by single-particle TIRF microscopy. The details of the CHIKV fusion machinery have not been completely elucidated. Recent research increased our knowledge on the first steps in the fusion process, including the proton dependent disordering of the acid-sensitive region of E2 [120] and the initial dissociation of the E1/E2 heterodimer [146]. It is assumed that the subsequent steps follow the same mechanism as found for SFV and SINV. Our work on lipid dependency and envelope protein rearrangements upon acidification strongly suggests that the fusion mechanism of CHIKV is indeed similar. However, we also found that up to 20% of the viral particles can fuse in the absence of cholesterol, indicating that for at least a part of the viral particles, E1 fusion loop insertion and trimer formation is independent from cholesterol.

Interestingly, the pH threshold of fusion, but also lipid dependency varies between different strains. We found that the requirements for fusion can be influenced by only one amino acid located at E1-226. CHIKV strains of the Indian Ocean Lineage that acquired an E1-A226V mutation and subsequent mutations influencing viral fusion display enhanced viral fitness in the Ae. albopictus vector [105]. How can fusion be related to enhanced fitness? A different pH threshold could induce fusion at a different cellular location which may be beneficial for viral replication in the host cell. This might explain why the emerging CHIKV strains display enhanced infectivity in the midgut cells of Ae. albopictus [165,166]. Indeed, for other viruses like Vesicular Stomatitis Virus and DENV, fusion from a defined endosomal compartment increases fitness as it optimizes conditions for uncoating and viral replication [136,182]. Hence, it would be of interest to follow the fate of viral particles after fusion, and if fusion early in the endocytic pathway leads to enhanced infection on a cellular level.

The ongoing rapid spread of CHIKV is facilitated by the fact that the worldwide population is virtually immune-naïve for CHIKV infection and that no vaccine or treatment against CHIKV is available. However, recently a virus-like particle vaccine and a live-recombinant measles-virus based vaccine have passed phase I clinical trials. Both vaccines were shown to be safe and tolerable, yet the measles-based vaccine elicited more mild-to-moderate adverse events than the virus-like particle vaccine. Both vaccines are proposed to be sufficiently immunogenic to confer protective immunity [183,184]. In addition, several other vaccine candidates are under development, including vaccines based on live-attenuated or inactivated viruses, chimeric viruses, and virus like particles; and DNA and subunit vaccines (reviewed in [185]). In contrast to DENV, there is only one CHIKV serotype and hence no risk of antibody-dependent enhancement of disease, which facilitates vaccine development. Considering the large number of candidate vaccines, chances are high that within few years, a CHIKV vaccine will be available.
The development of a vaccine against CHIKV is especially of importance to prevent persistent disease. To date no specific treatment is available for individuals with chronical joint pain. This group of patients has high antibody titers [186], and therefore it is questionable whether antibody-based therapy or a therapeutic vaccine can alleviate long-term joint pain. The precise mechanisms of long-term disease are not completely understood, thus the development of an efficient treatment for this group of patients sets an important challenge for the future.

However, the development of an antibody-based therapy represents a promising approach to treat risk-groups like for example patients with underlying co-morbidities or newborns. If a mother is viremic during birth, mother-to-child transmission occurs in 50% of the cases. Currently, it is investigated if the administration of anti-CHIKV antibodies derived from human convalescent serum can prevent severe disease in neonates borne to CHIKV infected mothers [187]. When selecting antibodies for combination monoclonal antibody therapy, in my opinion fusion inhibiting antibodies should be considered, as these are the most potent neutralizing antibodies identified to date ([9], chapter 7). It is known that RNA viruses can quickly develop mutations that render them resistant against neutralization [117]. Selecting antibodies targeting distant epitopes likely maximizes occupancy of the virion and minimizes the emergence of therapy-resistant mutants. Therefore, a therapy based on fusion inhibiting MAbs and strongly neutralizing MAbs acting at other stages of infection might be optimal for treatment of acute CHIKV infections. Alternatively, a combination of fusion-inhibitory antibodies with inhibitors of viral replication like ribavirin of favipiravir might be a promising treatment option with low-risk of the development of viral resistance [188-190].
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