Chapter 7

Summarizing discussion and future perspectives
Dengue virus and disease

Dengue virus (DENV) is an arthropod-borne RNA virus, belonging to the genus Flavivirus, family Flaviviridae. The virus has four confirmed serotypes and all four serotypes can cause disease. The clinical manifestations range from asymptomatic, to mild dengue fever, to potentially life-threatening haemorrhagic fever or shock syndrome. Currently, DENV is the most prevalent arthropod-borne viral infection worldwide. Yet, there is no licensed vaccine or therapeutic available. Hence, further understanding of the host – pathogen interactions are required to find new cues for therapeutics.

In 2010, approximately 390 million human infections occurred worldwide, and about one-quarter of the individuals developed symptomatic disease. While these numbers are already astounding, the continued expansion of both the mosquito vector and the virus into new areas will, most likely, further increase the incidence of dengue. Moreover, hyper-endemicity, i.e. concurrent circulation of multiple serotypes within the same region, will be more often seen. This is particularly troublesome in case of dengue as severe disease is most often observed in individuals experiencing a secondary DENV infection with a heterotypic serotype.

The increased chance of severe disease can be explained by ‘original antigenic sin’, a phenomenon in which the human immune system preferentially activates memory T and B cells against the previous infecting serotype, rather than instructing naive T and B cells against the current serotype. Therefore, during heterotypic re-infection, high numbers of cross-reactive antibodies and low avidity T cells are seen. These T cells are less effective in clearing infection and respond with a highly inflammatory cytokine profile. Indeed, dengue-infected patients show a sharp increase in both T cell response and inflammatory cytokines after peak viraemia, indicating that T cells are important in dengue pathogenesis. While a direct correlation between T cell responses and vascular leakage is missing in dengue, the role of T cells in vascular leakage has been studied in mice with lymphocytic choriomeningitis virus (LCMV) infection. Mice with chronic LCMV infection show pathogenic T cell responses, a cytokine storm (i.e. disproportional production of inflammatory cytokines) and subsequently shock syndrome. Therefore, cross-reactive T cells could cause dengue haemorrhagic shock. Furthermore, cross-reactive antibodies have been shown to enhance DENV infection of host cells and increase the virus production per infected cell, thereby increasing viral burden. This phenomenon is called ‘antibody-dependent enhancement’ (ADE) of
DENV infection 24, 25. The higher viral burden may subsequently trigger a cytokine storm, which on its turn induces vascular leakage and haemorrhage 25, 26. The fact that haemorrhagic fever also has been observed during primary DENV infection in infants with waning dengue immunity 27 suggests that antibodies alone can aggravate dengue disease since these children inherited antibodies from their mothers, not T cells.

The complex pathogenesis of disease severely hampered the development of anti-dengue therapeutics and vaccines. Rational design of therapeutics and vaccine requires a better understanding of the virus – host interaction. This thesis focuses on the virus cell tropism and the mechanism of ADE. The results of the thesis will be discussed on the basis of four major questions within the field of DENV research:

I) How do the individual host cell types contribute to DENV viraemia and continuation of disease?

II) How do antibodies enhance infection and increase virus production per infected cell?

III) How do macrophages (Mφ) respond to infection with DENV?

IV) What is the role of Mφ in DENV neutralization, and how is this best studied in vitro?

**Cell types important for DENV viraemia**

When a dengue-infected mosquito feeds on a human, most virus particles will be deposited into the skin, and a small fraction of the particles will be delivered directly into the blood 28. DENV virions in the skin predominantly infect dermal Mφ and skin dendritic cells (DC) like Langerhans cells 29-31. Moreover, monocytes migrate from the blood to the inflamed site in the skin and are infected there 29, 31. The infected immune cells subsequently migrate from the skin to draining lymph nodes, where progeny virus is released 32. Thereafter, systematic DENV infection is sustained within monocytes, Mφ and DC 33-37. Of note, two stages of DC can be distinguished: immature DC (iDC) and mature DC (mDC). The former are in a resting state, but respond to DENV by maturing into mDC 34, 38, 39. Both iDC and mDC are permissive to DENV 34, 35.

**Cell tropism in the absence of DENV-antibodies**

Within chapter 3, we evaluated the infectivity of mosquito-derived and human-derived DENV in Mφ, iDC and mDC. This approach allowed us to study DENV tropism during the initial round of infection and subsequent rounds of infection. The role of monocytes and monocyte-derived DENV could not be evaluated here since their culture proved to be quite challenging. In line with previous literature 34, we observed that iDC are highly susceptible to mosquito-derived DENV particles. Immature DC are more susceptible to DENV than mDC and Mφ. The relative susceptibility of mDC and Mφ is dependent on the multiplicity-of-infection (MOI) used to infect the cells. At MOI 1, mDC were more susceptible than Mφ. Yet, at an MOI of 10, both Mφ and mDC were equally susceptible, indicating that the local virus titre determines the observed susceptibility.

We also quantified the number of virus particles secreted from DENV-infected Mφ, iDC and mDC. In line with literature 35, we noted that iDC produced most DENV particles. Yet, when we assessed the infectious properties of the DENV2 virions derived from Mφ, iDC and mDC, the infectious quality of iDC-derived DENV was 10-100 fold lower compared to DENV derived from Mφ or mDC. In fact, at MOI 10, Mφ and iDC secrete an equal number of infectious particles. This implies that the lower susceptibility of Mφ and mDC, when compared to iDC, is (partly) compensated for by the higher infectivity of progeny virions. For virus dissemination, Mφ and DC may therefore be equally important. However, due to the high susceptibility of iDC to mosquito-derived DENV, iDC likely represent the initial target cell.

Intriguingly, DENV particles secreted by infected iDC are severely hampered in re-infecting new iDC [chapter 3, 40]. In contrast, Mφ-derived DENV can infect iDC, albeit at lower efficiency than mosquito-derived DENV. The susceptibility of Mφ to both Mφ-derived and iDC-derived DENV is quite low, and at the same level as iDC infected with iDC-derived DENV. These results imply that, during primary infection, mosquito-derived DENV will initially target iDC and thereafter may cycle between cells to sustain infection.

Future research should address how monocytes contribute to viraemia and to the potential cycling of the virus between the various host cells.

**Cell tropism in the presence of DENV-antibodies**

Upon secondary infection, pre-existing antibodies will bind to the newly infecting virus and facilitate uptake of virions via antibody receptors (FcR’s) 41, 42. These receptors are expressed on the cell surface of monocytes, Mφ and DC 35; DENV thus targets the same cells in the presence as in the absence of antibodies.

Whether antibodies enhance or neutralize DENV infection depends on several parameters as the characteristics of the antibody and the FcR involved 43, 44. Enhancement of infection also depends on the susceptibility of the cells in absence...
of antibodies. For example, iDC do not support ADE. In DCs, expression of the receptor molecule DC-SIGN is positively correlated with susceptibility to DENV, yet negatively with enhancement of infection. Thus, in cells highly susceptible to DENV the efficiency of infection cannot be further enhanced through FcR’s.

In chapter 3, and in agreement with Boonnak et al., we showed that mosquito-derived DENV facilitates ADE of infection in Mϕ and mDC. The highest fold enhancement of infection was seen for DENV-infected Mϕ. Furthermore, Mϕ-derived DENV is more prone to ADE than iDC-derived DENV. Thus, our results suggest a vicious circle of enhanced infection of Mϕ through ADE. Subsequently, ADE with Mϕ–derived DENV2 results in enhanced productive infection of Mϕ. In line with these observations, infected Mϕ are often seen in dengue patients, and become more pronounced during ADE. Although it is clear that mosquito-derived DENV does not support ADE, a recent study showed that iDC-derived DENV can facilitate ADE in iDC. Therefore, it is tempting to speculate that iDC-derived DENV does not interact efficiently with DC-SIGN and that antibodies rescue the susceptibility of these cells via interaction with FcR’s. Further research is required to investigate the relative contribution of iDC during secondary infection and the ability of human-derived DENV to facilitate ADE on iDC. Moreover, the role of monocytes and their relative contribution to viraemia should be studied in more detail.

**iDC and DENV; low infective particles**

The observation that iDC produce low-infectious virus particles compared to Mϕ and mDC sparks curiosity. Two questions arise: (i) How do iDC render their progeny virions less infectious than the progeny virions from Mϕ? (ii) What could be the biological importance of these low-infectious virions? These questions will be discussed below.

**(i) the mechanism of low-infectivity**

Initially, we hypothesized that the low infectivity of iDC-derived DENV2 was caused by a high concentration of the human antiviral cytokine interferon alpha (IFNα) in the cell supernatant. Surprisingly, higher levels of IFNα were found in Mϕ cultures compared to iDC cultures, and none in mosquito cell cultures. Hence, the IFNα levels did not explain the different infectivities. Furthermore, the antiviral activity of Mϕ-derived and iDC-derived culture supernatants was found to be comparable in a BHK15-based bio-assay, indicating that the reduced infectivity of iDC-derived DENV2 is not caused by soluble factors in the medium. Next, we hypothesized that iDC-derived DENV2 possesses infection-inhibiting sugar groups on its viral structure. Deglycosylation of the viral spike proteins, however, reduced the viral infectivity of iDC-derived DENV, indicating that glycans possess beneficial rather than inhibitory properties.

Then, what causes the low infectivity of iDC-derived DENV2? Below, I will discuss several hypotheses: First, host proteins from iDC could be incorporated into progeny virions, as seen for e.g. vesicular stomatitis virus (VSV) and herpes simplex virus 1, and influenza virus. These incorporated proteins can induce an antiviral response in the cells-to-be-infected and thus impair infection. However, the similar infectivity of both iDC-derived and Mϕ-derived DENV on Mϕ argues against such a hypothesis.

Second, insufficient processing of the viral glycoprotein prM can occur in iDC-infected cells. Previous studies showed that DENV requires furin-mediated cleavage of prM to become infectious. However, a recent report found that iDC secretes less prM-containing particles than mosquito cells. Third, the low-infectious virions of iDC may derive from defective interfering particles, as seen in e.g. VSV and Sindbis virus. Defective interfering particles are virions with deletions within the genomes, hence these viruses lack one or more of their viral proteins and therefore require complimentary genomes encoding for these proteins. Yet, the shorter genomes of defective interfering particles allow for faster replication and consequently higher titres. Indeed, defective interfering particles have been reported in the sera of dengue patients. Typically, these defective particles arise when cells are infected at high MOI. In line with this, we noticed an MOI-dependent specific infectivity of DENV derived from iDC; the higher the MOI the lower the infectivity. Therefore, defective interfering particles may explain the MOI-effect in our experiments.

**(ii) Biological impact of low-infectivity; ‘Winkelried strategy’**

The combination of high susceptibility with low-quality virus production is reminiscent of the ‘Winkelried strategy’: a phenomenon named after the Swiss Arnold von Winkelried who threw himself into the spears of the Austrian army to enforce a breach in their ranks and thus brought victory for the Swiss. Similarly, a cell type can sacrifice itself in order to produce antigens and to facilitate adaptive immune responses. Splenic Mϕ fulfill this function in VSV-infected mice. For DENV, such a cell type has not yet been noted. The results in chapter 3 suggest that for DENV iDC may act to produce the antigens and trigger adaptive immunity. Indeed, the hallmarks of the ‘Winkelried strategy’ are found in skin-resident iDC.
(Langerhans cells); high susceptibility to DENV, large production of progeny virus, low sensitivity to IFNβ, and efficient induction of T cell proliferation. Thus, I propose that IDC may employ the ‘Winkelried’ strategy in the context of dengue infection. More research is needed to prove the role of IDC as promoters of both antigen production and adaptive immune responses against DENV infection in vivo.

**ADE in Mφ: the molecular mechanism**

Although the first descriptions of ADE were in the 1960’s, its molecular mechanism is largely unknown. The overall dogma is that DENV binding/entry into host cells is improved in the presence of antibodies. Furthermore, activation of the antibody receptor (FcR) induces immune suppressive signaling within the cell. Hence, more cells are infected (extrinsic ADE), and the virus production per infected cell (burst size) increases (intrinsic ADE) which together give raise to strongly increased virus titres and more severe disease. Yet, in the past, most studies into the mechanism of ADE were conducted in cell lines.

To get as close as possible to the situation in vivo, we studied ADE in primary human Mφ, a cell type highly relevant during secondary infection. In chapter 4, we studied each step of the viral life cycle to determine how antibodies stimulate viral infectivity in primary Mφ.

Optimal ADE-conditions resulted in a 2-fold increase in the infected cell mass and a 7-fold increase in virus particle production. This suggests that both extrinsic and intrinsic ADE exist in primary Mφ. Strikingly, and in contrast to literature, antibodies did not enhance binding or entry of DENV2 into primary human Mφ under conditions of ADE. A finding which was confirmed in human Mφ-/monocyte-like cell lines. This indicates that the interaction of DENV-immune complexes with the FcR’s is as efficient as the interaction of DENV with its native receptors on Mφ.

Interestingly, ADE enhanced the fusion potential of DENV within human Mφ. The membrane fusion potential was enhanced in two ways: (i) more fusion events per cell were seen, (ii) more cells were positive for fusion. While the enhancement of fusion activity varied among the donors, the enhancement of fusion activity correlated with the enhancement of DENV production. Thus, the enhanced fusion potential of the virus is likely responsible for the increased number of infected cells.

**intrinsic ADE and enhanced burst size**

The hypothesis of intrinsic ADE stipulates that the higher virus production is due to an immunosuppressive state within the host cells. To understand the molecular mechanism of intrinsic ADE in more detail, we analyzed the transcriptional profiles of the Mφ during early (2h post infection) and late (24h post infection) stages of infection. Surprisingly, during the early stages of infection, the presence of DENV antibodies did not alter the transcriptional profiles of DENV-infected Mφ. This result is in line with the similar binding/entry characteristics that we observed. Furthermore, these findings contradict the hypothesis of intrinsic ADE and its immunosuppressive action. Indeed, addition of IFN-α during the early stages of infection inhibited DENV infection in Mφ, irrespective of the infection mechanism. This has also been observed in DENV-ADE in primary monocytes and monocyte-like cell lines. Taken together, I believe that antibodies indeed do not trigger an immunosuppressive state within the Mφ.

If intracellular suppression of antiviral responses is not required for the higher burst size, then how is this facilitated? Recently, Medina et al. found that a higher degree of infection within a culture correlated with altered IFN signaling within infected cells. This work suggests that either: (i) the ratio of infected cells to non-infected cells determines the antiviral responses within infected cells, or (ii) that the higher degree of infection itself may have facilitated the observed effects.

The microarray analysis did not find lower antiviral responses under conditions of ADE, hence contests the first hypothesis. With regards to the second hypothesis, it should be noted that Medina et al. had obtained their curve by titrating DENV on A549 cells. Therefore, the results can also be interpreted as if the observed effects actually correlated with the MOI used to infect the cells. Infection at higher MOI may allow for superinfection of the cells: a multitude of viral genomes entering the same cell. Superinfection would not only disprove intrinsic ADE but also provides an explanation for the observed enhancement of the burst sizes. Indeed, under conditions of ADE, the enhancement of fusion activity was larger than the enhancement of fusion-positive cells (i.e. +65% and +40%, respectively), suggesting that per-cell the fusion activity was higher and more genomes were delivered into the cytosol of the same cell.

As a retrospective and explorative approach to (preliminary) validate this hypothesis, all our paired values of the infected human Mφ (%) and the resulting burst sizes (PFU/cell) were plotted in a correlative plot. Because of the donor variations, a wide range of infected cell masses were found between independent experiments. Interestingly, the infected cell mass correlates with the burst size. This strengthens the hypothesis that the phenomenon of intrinsic ADE is due to superinfection.
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Fig. 1: An explorative figure with correlation between burst size (PFU/cell) and infection (%). Paired values of burst size (PFU/cell) and degree of infection (%) were collected from all experiments throughout this thesis (N=51), and plotted in a correlative plot. Each dot represents a value, the black line denotes the linear correlation while the dashed lines show the 95% confidence interval of the correlation. The Pearson’s R of the correlative line is 0.41, which has a 2-tailed p-value of 0.003.

Hypothetically, each incoming viral genome may independently replicate and translate within the cell. During superinfection, increased translation favours the production of viral non-structural proteins, which are known to inhibit cellular IFN production and signaling 77-79. Superinfection would thus explain the results obtained by Medina and colleagues, since more non-structural proteins within the cell likely results in stronger inhibition of the IFN signaling pathway. Concurrently, the higher replication and production would explain the increased burst size typically observed under conditions of ADE.

The possibility and mechanisms of superinfection remain to be investigated further. In contrast with this hypothesis, DENVs have been reported to resist superinfection 80, 81 yet these reports are mostly based on attempts to superinfect the same cell with a time difference between the first infection and the second infection. Superinfection under conditions of ADE or high MOI does not have this time difference and thus could be essentially different.

What also remains to be elucidated is how antibodies enhance the fusion potency of internalized DENV virions. Antibodies may enhance the intrinsic fusion properties of the DENV particle, e.g. through stabilization of the viral structure. This hypothesis implies that antibodies stabilize the viral structure and thus may slow-down the inactivation of DENV particles. Yet, this is not very likely since antibodies against various epitopes of the closely related West Nile virus were tested, and none of the antibodies possessed such stabilizing properties 82.

Instead, I believe that DENV infection in the presence of antibodies follows a different trafficking pathway than DENV in the absence of antibodies. Indeed, Ayala-Núñez et al. recently found that the entry of antibody-opsonized virus particles into P338D1 cells is PI3K-dependent, contrary to entry in absence of antibodies 83. Moreover, antibody opsonized dengue particles were found to fuse more rapidly than those entering in absence of antibodies 83. Taken together, it is clear that opsonized DENV particles employ an alternative port of entry and trafficking pathway than DENV particles in absence of antibodies 83. Similar to DENV, West Nile virus also employs different entry pathways in the absence and in the presence of antibodies 84.

The benefit of this alternative, antibody-dependent trafficking pathway is that it may bring the virus into an organelle where the environment is more beneficial for fusion (i.e. higher chance of fusion). Alternatively, the trafficking pathway leads to a site within the cell where the viral RNA has a higher chance to initiate productive infection.

Inflammatory response in DENV-infected Mφ

In chapter 4, the Mφ response to DENV infection was studied by microarray technology and annotation of the genes differentially regulated in response to DENV. The primary goal was to gain insight in the molecular mechanism of intrinsic ADE in DENV2-infected human Mφ. Surprisingly, no immunosuppressive program was identified in the gene profiles of human Mφ infected with DENV2 in the presence of antibodies. Rather, the microarray indicated that ADE of Mφ resulted in an ‘inflammatory response’ at both 2h and 24h post infection. By 24h post infection, this inflammatory response conferred antiviral properties to the culture supernatants of the infected cells [chapter 4, 85]. Moreover, the antiviral response in the culture was related to the degree of infection and/or virus production [chapter 4, 86]. Given the strong inflammatory response in both the microarray and the culture medium, we calculated the expression of several cytokines known to be involved in dengue disease; i.e. interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), IL10, and type I IFN (α/β). At conditions of high infection in Mφ, only IFNβ, IL6 and TNFα expression were upregulated [chapter 4]. Below, I will discuss the potential role of these factors on DENV infectivity.

TNFα and IL6 in DENV infection of Mφ

High levels of TNFα and IL6 are also found in patients with severe dengue 87, 88, and both cytokines are associated with subsequent vascular damage 89, 90.
Moreover, IL6 was recently described as an immunosuppressive factor in ADE-infected Mφ. The latter study highlights IL6 as a factor contributing to severe disease by promoting intrinsic ADE in Mφ.

Within chapter 5, however, we found that application of exogenous TNFα or IL6 to Mφ did not alter DENV2 infectivity or production by primary human Mφ. While the results for TNFα are in line with previous reports \(^{73, 93}\), the lack of effect of IL6 is in contrast with its putative role in inducing a pro-viral state within human Mφ \(^{92}\). It should be noted that the annotation of IL6 as an immunosuppressive cytokine is based on the lower expression of IFNβ, and higher expression of SOCS3, a suppressor of the IFN-signaling pathway \(^{92}\). However, the authors did not quantify the biological outcome hereof (i.e. efficiency of infection or virus production). We did not observe a pro-viral effect of IL6 in Mφ. It is, however, still possible that IL6 enhances SOCS3 expression and subsequently reduces IFNβ, yet without affecting viral infectivity.

Since both IL6 and TNFα are associated with vascular leakage, these cytokines could thus be targets for therapeutic intervention with e.g. Mφ-specific siRNAs \(^{94}\). This hypothesis is strengthened by results obtained in mice with LCMV-induced shock. In these mice, Mφ express high levels of IL6 and TNFα and the survival of these mice significantly improved when both IL6- and TNFα-signaling were blocked\(^{22}\). Future research is required to see if dengue-induced shock can be ameliorated by interfering with TNFα- and/or IL6-signaling pathways \(^{79}\). Yet, care should be taken since both IL6 and TNFα also have beneficial functions which are essential to clear DENV from the system: e.g. clearance of DENV-infected cells by natural killer cells \(^{96}\), promoting DC-maturation and subsequent T cell activation \(^{39}\), and supporting B cell proliferation \(^{97}\).

The role of IL10 in DENV2-ADE infection of Mφ

Previously, IL10 was thought to be an immunosuppressive factor whose expression is characteristic for intrinsic ADE \(^{25, 68}\). However, two observations argue against a putative role of IL10 in intrinsic ADE in Mφ: (i) Mφ do not produce IL10 under conditions of ADE [chapter 4, \(\sim 2\%\)] and (ii) IL10-primed Mφ become highly susceptible for DENV but do not produce progeny virus \(^{98}\). Hence, IL10 alone is not responsible for the enhanced burst size seen in DENV-ADE-infected Mφ.

IL10 is predominantly expressed by monocytes \(^{35, 99}\) or monocytic cell lines \(^{80, 99}\). In these cells, expression of IL10 is enhanced under conditions of ADE \(^{35, 99}\). The relevance of IL10 expression to DENV-infectivity of monocytes was elucidated by Boonnak and colleagues using the natural polymorphisms in the IL10 promoters to stratify donors into low, medium and high producers \(^{35}\). No differences were found in DENV infectivity. This strengthens the notion that IL10 does not cause intrinsic ADE but is a response to the virus particles in the culture. Indeed, the surface receptor CLEC5A can bind extracellular DENV particles and subsequently initiate both inflammatory responses and IL10 expression \(^{99, 100}\).

**DENV and interferons**

High infection and viraemia also triggered higher expression of type I IFNs and IFN-stimulated genes in Mφ cultures. IFNα, a type I IFN, possess antiviral activity to DENV \(^{73, 81, 101}\), chapter 4]. Furthermore, IFNα can ameliorate disease in humans with dengue fever \(^{102}\) and therefore has therapeutic value.

Yet, in chapter 4 and chapter 5, two major limitations can be discerned against the therapeutic use of IFNα: the temporal constrain \(^{73, 101}\), chapter 4; chapter 5] and the apparent inefficiency on serotypes/genotypes other than the DENV2, s16681 [chapter 5]. Especially DENV3 strain H87 and DENV4 strain 1036 produced near identical total virus particles, irrespective of IFNα treatment. Because of these constrains, IFNα treatment might not be effective against all DENV serotypes and genotypes. Further research could explore the efficacy of IFNγ since IFNγ was efficacious against both de novo and established DENV infection in primary monocytes \(^{101, 103}\).

**Mφ and antibody-mediated neutralization of DENV2 infection**

While working with primary Mφ, we did not observe (complete) neutralization of infection despite using antibody concentrations up to 1000ng/mL, i.e. 200 antibodies per epitope. The absence of neutralization was also observed by others \(^{35, 72}\) and in the Mφ-like cell line P38D1 [chapter 3]. Contrary to Mφ, neutralization was observed in human iDC and mDC [chapter 3, \(\sim 2\%)\]. Moreover, the same condition which resulted in ADE on Mφ and cell lines with an antibody receptor (FcR) resulted in antibody-mediated neutralization on FcR-negative cell lines [chapter 3, \(\sim 104, 105\)]. This raises the question whether DENV2 infection of Mφ is resistant to antibody-mediated neutralization.

It is important to note, however, that the number of Mφ that ingest DENV particles (\(\sim 30\%)\) is much larger than the number of infected Mφ (\(\sim 2\%)\) [chapter 4], indicating that DENV infection of Mφ is quite inefficient. This suggests that most of the internalized DENV particles are in fact neutralized. In line with this, Mφ are considered a key cell type to control DENV viraemia \(^{106}\), by clearing antibody opsonized particles through FcR-mediated phagocytosis \(^{107}\). Therefore, the FcRs...
have a paradoxal role in being pivotal for both the disease-augmenting effect of antibodies (i.e. ADE) as well as the in vivo protective efficacy of antibodies against viruses.

A better understanding is required of how Mϕ aid in promoting DENV infection, as well as why only few of the internalized particles initiate productive infection. In this perspective, it would be interesting to study the characteristics of DENV binding/entry/fusion under conditions of high antibody-concentrations (i.e. non-enhancing conditions) relative to antibody concentrations that show ADE. Three hypothetical mechanisms of antibody-mediated neutralization in Mϕ may occur: (i) Binding/entry of virus particles is inhibited, (ii) FcγRIIB-mediated entry leads to another trafficking pathway with a highly degradative character, thus particles are degraded before they can initiate infection, or (iii) activation of FcγRIIB triggers STAT1-mediated antiviral state within the cell. The latter is reminiscent of our 0hpi IFNα add-on [chapter 4, figure 6B]. A deeper understanding of the factors that allow incoming virus particles to initiate a productive infection is required for the development of effective therapeutics, as well as the functioning of Mϕ in vivo.

**Final remarks**

I believe that ADE might be the consequence of the ‘butterfly effect’; starting with a subtle effect and ending in strongly altered gene profiles and high viraemia. Antibodies enhance infection of Mϕ by increasing the fusion activity of the virions while maintaining the same levels of antiviral responses early in infection. The combination of higher fusion potency and unperturbed antiviral response allows for enhanced infection and higher burst sizes.

The severity of dengue disease is determined by a combination of factors like cytokine responses, antibody epitopes, virus fitness, and virus titres reviewed in chapter 6 and the recent discovery of a fifth serotype. The development of a complete and protective vaccine may be more complicated than assumed before. Hence, understanding the molecular mechanisms of DENV-infection within primary cells will open new possibilities for rational design of therapeutics.

or NK cells, this could result in release of a large amount of viral antigens, which subsequently can trigger excessive T cell responses. Hence, further research is required into the mechanism of IFN-mediated suppression as well as the T cell responses.

Primary cells are challenging to work with, but provide better insight into the molecular mechanisms of virus clearance and enhancement of infection as seen in vivo. Moreover, given the incomplete protection of the tetravalent vaccines reviewed in chapter 6 and the recent discovery of a fifth serotype, the development of a complete and protective vaccine may be more complicated than assumed before. Hence, understanding the molecular mechanisms of DENV-infection within primary cells will open new possibilities for rational design of therapeutics.
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