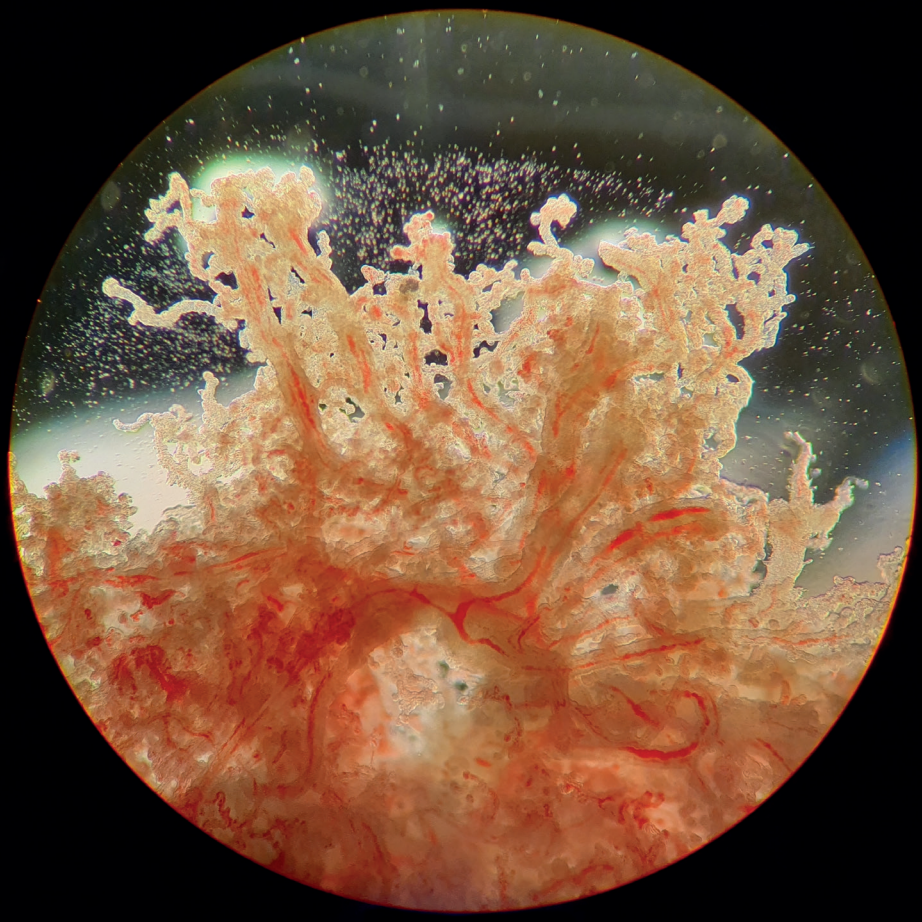
Studies on Zika Virus

Neurological Complications, Epidemiology and Antibody-Dependent Enhancement



Thomas Langerak

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Contents

CHAPTER 1

General Introduction

Arboviruses

Viruses that can be transmitted through arthropods, such as mosquitoes and ticks, are called arthropod-borne viruses, or arboviruses. Many arboviruses that can infect humans are part of the *Flaviviridae* family. These include dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) tick-borne encephalitis virus (TBEV) Usutu virus (USUV) and Zika virus (ZIKV). With an estimated incidence of 390 million infections per year, DENV is the flavivirus with the highest incidence, while the incidence of YFV, WNV, JEV and TBEV is estimated to be between several thousand and a hundred thousand annual infections worldwide (1-4). For humans, there are commercially available vaccines for YFV, JEV, TBEV and DENV (for individuals who were previously exposed to DENV), but not for WNV and ZIKV.

Zika virus

Zika virus (ZIKV) is a positive-sense single-stranded RNA virus of the *Flaviviridae* family, genus flavivirus. It was first isolated in 1947 from a sentinel rhesus monkey in the Zika forest in Uganda (5). The 10.8-kb genome of ZIKV codes for a single polyprotein which, after translation, is cleaved into three structural proteins; capsid (C), precursor membrane (prM) and envelope (E), and seven non-structural proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (6). ZIKV transmission to humans mainly occurs through the bites of infected *Aedes* mosquitoes. ZIKV transmission between humans can then occur through sexual contact, blood transfusions, and from mother to child during pregnancy (7-9). When an infected *Aedes* mosquito bites, Zika virions are inoculated into the skin of the host and, in part, directly into the bloodstream (10). Subsequently, ZIKV can replicate in host skin fibroblasts and epidermal keratinocytes (11). As with other flaviviruses, ZIKV can spread from the skin to the lymph nodes by infecting immature dendritic cells. Once in the lymph nodes, ZIKV can infect monocytes and macrophages, from where it can reach the bloodstream and spread throughout the body (12-15). In addition to myeloid cells, the cellular tropism of ZIKV consists of neuronal progenitor cells and cells in immune-privileged sites, such as the eye, the reproductive tract and the placenta (16, 17). ZIKV can enter host cells via receptor-mediated endocytosis and multiple-entry cofactors have been identified for ZIKV, including AXL, DC-SIGN, L-SIGN and TIM1 (11, 18, 19). After entering the cell, the low pH in the early endosome induces a conformational change in the ZIKV E protein, allowing fusion of the viral membrane with the endosome membrane. Afterwards, the viral genome is released in the cytoplasm of the infected cell and viral replication commences (20). ZIKV is assembled at the endoplasmic reticulum as spiked immature particles (21). In the trans-Golgi network, low pH induces a conformation of ZIKV particles from a spikey particle into a smooth

particle (22). After this conformational change, host furin-like protease can cleave prM into a membrane and precursor fragment, the latter of which dissociates from the particle upon cell release (22, 23). As with other flaviviruses, inefficient prM cleavage can lead to partly mature mosaic particles, some of which can still be infectious (22, 24).

Natural history of Zika virus

Between the initial identification of ZIKV in 1947 and 2007, there were no reports of large-scale ZIKV outbreaks. The small number of ZIKV seroprevalence studies indicates that this virus circulated in Africa and some countries in Asia, resulting in African and Asian lineages of ZIKV that differ around 10% at the nucleotide level (16, 25). However, this changed in 2007 when there was an outbreak of an illness causing skin rash, conjunctivitis, and arthralgia. The outbreak was on the island of Yap in the federal states of Micronesia. It turned out that the virus responsible for these symptoms was ZIKV and that a large proportion of the population on this island became infected (26). Later, in 2013, a similar event occurred in French Polynesia, where an outbreak of a dengue-like illness turned out to be a ZIKV outbreak that infected approximately 11.5% of the inhabitants (27). In May 2015, an outbreak of a disease causing skin rash and fever led, for the first time, to the detection of a local transmission of ZIKV in the north-eastern Brazilian region of Bahia (25).

There are several theories on how and when ZIKV reached the mainland of Brazil. Early phylogenetic analysis revealed that there was a single introduction of the Asian lineage of ZIKV in Brazil around December 2013, which shared a common ancestor with the ZIKV strain that circulated in French Polynesia earlier that year (28). Furthermore, it was found that from mid-2014, ZIKV spread undetected from the north-eastern part of Brazil to different regions of the country and from there to other countries in the Americas (29, 30). By December 2015, there had been between 440,000 and 1,300,000 suspected cases of ZIKV in Brazil (31). Around this time, other countries in the Americas and the Caribbean started to report local ZIKV transmission. At the end of 2015, an increase in the incidence of microcephaly in newborns was reported in the north-eastern region of Brazil and it was suggested that this could be caused by ZIKV infections during pregnancy (25). The rapid spread of ZIKV throughout the Americas and mounting evidence of ZIKV-associated birth defects, led the World Health Organization to declare on 1 February 2016 that the complications of the ZIKV outbreak were a Public Health Emergency of International Concern. The incidence of suspected and confirmed ZIKV cases in the Americas peaked at the beginning of 2016 and then rapidly declined in mid-2016. In the Caribbean, ZIKV cases peaked slightly later; from mid- to late-2016 (32). In 2017, there were significantly less suspected and confirmed ZIKV cases in the

Americas and the Caribbean (33). The total number of ZIKV infections that occurred during the 2015-2016 outbreak is estimated to be around 130 million (34).

Zika virus compared to other flaviviruses

Symptoms of infections with flaviviruses are often non-specific, such as fever, headache, skin rash, conjunctivitis, myalgia, and arthralgia. This makes it difficult to clinically distinguish a ZIKV infection from a DENV infection, for example. Flaviviruses can be classified into neurotropic and visceral flaviviruses (35). WNV, JEV and TBEV are flaviviruses that are neurotropic; infection with these viruses can result in meningitis and encephalitis (36). DENV and YFV, on the other hand, are significantly less neurotrophic and they mainly infect visceral organs, such as the liver, as well as endothelial cells, which can result in haemorrhage and plasma leakage (37, 38). Interestingly, ZIKV has features of both groups of flaviviruses; it can infect visceral organs and foetal endothelial cells but can also infect neural progenitor cells in the developing foetus (17, 35, 39, 40). Furthermore, ZIKV can infect cells of the reproductive tract and the human placenta (41). To date, the exact mechanism behind the difference in tropism of several flaviviruses is unclear. Non-structural protein 1 (NS1) of different flaviviruses can trigger tissue-specific endothelial dysfunction, which, in part, correlates with the tissue tropism of these flaviviruses. For ZIKV, it has been demonstrated that NS1 can cause increased vascular permeability in umbilical vein endothelial cells, as well as in brain endothelial cells (42).

Of the above-mentioned medically significant flaviviruses, ZIKV is most closely related to DENV. DENV consists of four serotypes (DENV 1-4) that share 60-70% amino-acid sequence identities, while ZIKV and DENV share around a 56% amino-acid sequence identity (43, 44). This relatedness between ZIKV and DENV causes cross-reactivity of antibodies against these viruses. Due to this cross-reactivity, serologically distinguishing ZIKV from DENV can be challenging (45, 46). In addition to diagnostic challenges, the cross-reactivity between ZIKV and DENV antibodies can also have clinical implications. It has been shown that cross-reactive DENV antibodies can reduce the risk of getting a symptomatic ZIKV infection (47, 48).

Complications of ZIKV infections

Guillain-Barré syndrome

Before the increased incidence of birth defects was flagged up, an increased incidence of the Guillain-Barré syndrome (GBS) was noted in several Brazilian regions at the beginning of

the ZIKV outbreak in 2015 (49). GBS is an immune-mediated syndrome in which damage to peripheral nerve fibres leads to symmetrical muscle weakness, decreased or absent deep-tendon reflexes and sometimes sensory cranial nerve involvement and autonomic dysfunction (50, 51). GBS can be triggered by an infection with several pathogens, such as *Campylobacter jejuni (C. jejuni)*, cytomegalovirus (CMV), Epstein-Barr virus, *Mycoplasma pneumoniae* (*M. pneumoniae*), hepatitis E virus and SARS-CoV-2 (52, 53). The exact immunopathogenic mechanism behind GBS has not yet been fully elucidated, but it is thought that the molecular mimicry between microbial antigens and the host nerves plays a role (54). Due to this molecular mimicry, autoantibodies against ganglioside can occur after an infection with, among others, *C. jejuni*, CMV, *M. pneumoniae* and ZIKV (55, 56). These anti-ganglioside antibodies can be neuropathogenic by binding to peripheral nerves. This can cause macrophage attack and antibody-mediated complement-dependent attack, as is illustrated in Figure 1 (57, 58).

GBS is a clinical diagnosis. Other diseases that have similar symptoms to GBS must be ruled out before making a GBS diagnosis (59). The case definitions of the Brighton Collaboration can be used for determining the level of diagnostic certainty of GBS, ranging from 1 (highest diagnostic certainty) to 4 (lowest diagnostic certainty) (59). Patients with GBS develop rapidly progressive symmetrical muscle weakness, which is often preceded by an infection 1-2 weeks prior to the start of the symptoms (51, 60). The progressive muscle weakness typically peaks after 2-4 weeks, after which a plateau phase occurs that can last for several weeks, or even months, before patients start to slowly recover (61). Recovery can be accelerated by administering intravenous immunoglobulins, or with plasma exchange (51). Most GBS patients recover completely, but some of them suffer from long-term disability (62). In approximately one third of GBS patients, respiratory failure occurs due to the weakness of respiratory muscles (63). Even though GBS often has a good prognosis, the mortality of this syndrome is around 3-7% (61). In 2014, the link between ZIKV and GBS was first described in a case report of a ZIKV-infected patient in French-Polynesia (64). Further evidence of an association between ZIKV and GBS was provided by case reports during the ZIKV outbreak, and a case-control study performed in French Polynesia during the ZIKV outbreak in 2013-2014 (65-67). It is estimated that GBS occurs in 0.06-0.32 per 1,000 symptomatic ZIKV infections (67-69), compared with *C. jejuni*, which occurs in 0.25-0.65 per 1,000 infections (67, 70).

Because of the time-lag between the initial infection that might trigger GBS and the onset of GBS symptoms, the pathogen that triggered the onset of GBS is often no longer present at the time of onset of the GBS symptoms. Therefore, finding the pathogen that caused the onset of GBS is often challenging and mainly dependent on serology, instead of molecular tests such as polymerase chain reaction (PCR).

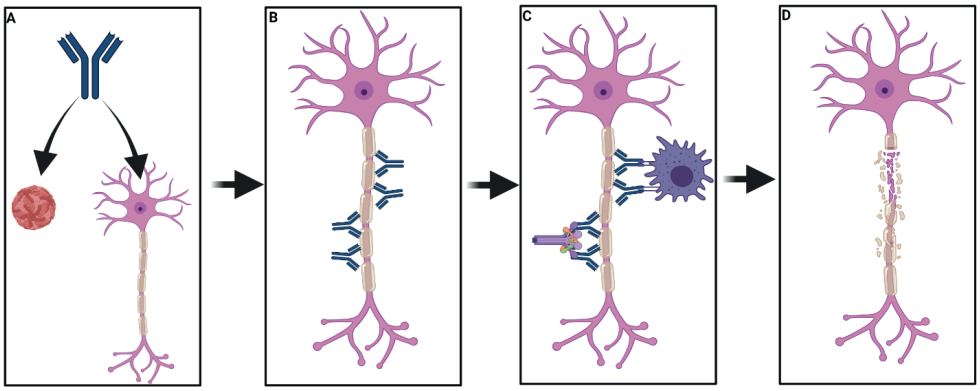


Figure 1. Proposed mechanism of GBS pathogenesis after a ZIKV infection due to molecular mimicry. In some cases, antibodies produced against ZIKV can cross-react with gangliosides present on human nerves (**A**). These auto-antibodies can bind human nerve gangliosides (**B**) which triggers the activation of macrophages and complement (**C**). The activated complement, resulting in membrane attack complexes, and macrophages induce damage to the neuronal cell, resulting in axonal degeneration and/or demyelination of the nerves (**D**).

Birth defects

The first reports of an increase in microcephaly incidence in Brazil came at the end of 2015 from the north-eastern region that also reported ZIKV circulation in Brazil for the first time (71). Subsequently, in other regions of Brazil that previously reported ZIKV transmission, the microcephaly incidence significantly exceeded the microcephaly incidence of regions that did not report ZIKV transmission (71). Evidence of causality between an *in-utero* ZIKV infection and microcephaly increased at the beginning of 2016. This led the WHO to declare the complications of the ZIKV outbreak a Public Health Emergency of International Concern (9, 72). Congenital microcephaly occurs when there is an impaired brain growth *in-utero* and it can lead to mental retardation. The aetiology of congenital microcephaly is heterogeneous, and it includes infections with rubella virus and CMV, genetic mutations, metabolic disorders and teratogens like alcohol and drugs (73-75).

In addition to microcephaly, it is now known that a congenital ZIKV infection can also result in several other birth defects (72). Collectively, these neurological congenital abnormalities are clustered in the congenital ZIKV syndrome (CZS) (76). CZS can have severe neurological complications, such as microcephaly, hydrocephalus, congenital contractures, hypertonia, and seizures, as well as eye abnormalities, such as macular scarring and optic-nerve hypoplasia and sensorineural hearing loss and arthrogryposis (77). Apart from congenital birth defects, other severe complications of an *in-utero* ZIKV infection are spontaneous abortion and foetal demise (78-80). It is estimated that of all the ZIKV infections during pregnancy, 4-7%

result in foetal demise or spontaneous abortion, while between 2% and 20% result in CZS (81-83). Little is currently known about the potentially negative long-term effects of an *in-utero* ZIKV infection in infants born with and without ZIKV-associated birth defects. There are indications that ZIKV-exposed infants can suffer delayed neurodevelopment, mainly in language development but also in motor- and cognitive-development (84, 85).

Many of the complications that accompany CZS are thought to be the result of direct infection of the foetal brain by ZIKV. In foetuses of mothers who had a ZIKV infection during pregnancy, ZIKV has been observed in neural cells, neurons and degenerating glial cells within the cerebral cortex (86).

In-vivo studies with animal and *in-vitro* studies with neuronal cells and brain slices have demonstrated that ZIKV infects and replicates efficiently in neuronal progenitor cells (87, 88). Infection of the neuro-progenitor cells results in cell death and impaired neurogenesis, which causes many of the neurological birth defects that are observed in children with CZS (88-90).

Transplacental Zika virus transmission

For vertical ZIKV transmission to occur during pregnancy, the virus must first cross the placenta. The human placenta is a temporary foetal organ that supports normal growth and the development of the foetus through, among other things, gas and nutrient exchange between mother and foetus. Furthermore, the placenta forms an important physical and immunological barrier to protect the foetus from pathogens that might be present in maternal blood (91). Humans have a haemochorial placenta, which means that there is direct contact between maternal blood and the placental chorionic villi. The placenta consists of many of these chorionic villi that are either bathed in maternal blood in the intervillous space, or are anchored to the maternal part of the placenta (decidua) through extravillous trophoblasts. The chorionic villi are lined by two layers of trophoblasts; an outer layer of multinucleated trophoblasts (syncytiotrophoblasts) and an underlying layer of cytotrophoblasts that can differentiate in syncytiotrophoblasts and extravillous trophoblasts. The stroma of the chorionic villus core consists of foetal macrophages (Hofbauer cells), fibroblasts and endothelial cells (Figure 2). From the second trimester of pregnancy onwards, maternal blood reaches the intervillous space through the uterine spiral arteries, which are remodelled by invasive trophoblasts into large, dilated arteries in order to increase the blood flow into the intervillous space (92, 93). From this period, maternal IgG is also actively transported across the placenta through the neonatal Fc-receptor (FcRn) that is expressed by syncytiotrophoblasts. There are several pathogens that can cross the placenta and infect the foetus. These pathogens are called 'TORCH pathogens', TORCH being an acronym for *Toxoplasma gondii*, Other (e.g.,

HIV, *Listeria monocytogenes, Treponema pallidum*, parvovirus B19), Rubella, CMV and Herpes simplex virus 1 and 2. Different mechanisms of transplacental transmission have been identified although, for many TORCH pathogens, the mechanism of transplacental transmission has not yet been elucidated. *Listeria monocytogenes* and *Toxoplasma gondii* that can infect extravillous trophoblasts could possibly cross the placenta by infecting these cells, while CMV might be transported across syncytiotrophoblasts as a virus-antibody immune complex through FcRn-mediated transcytosis (93-95). The syncytiotrophoblasts form a physical barrier against pathogens and have proved to be resistant to infection with many pathogens, in part through the production of type III interferons (96). Since the intervillous space is only filled with maternal blood from the second trimester of pregnancy onwards, it is likely that the mechanism of vertical transmission differs between the first, and the second and third trimesters of pregnancy. For ZIKV, the risk of vertical transmission is greatest when the infection occurs in the first trimester of pregnancy and it decreases in the second and third trimesters, although vertical transmission can still occur during these trimesters (97). The mechanism behind vertical ZIKV transmission has not yet been fully elucidated. In the placentas of women with a ZIKV infection during pregnancy, ZIKV was mainly detected in Hofbauer cells and, to a lesser extent, in cytotrophoblasts. Infection in syncytiotrophoblasts has not been observed (86, 98-100). Histopathological changes that were observed in placentas infected with ZIKV *in-utero* are, among others, villitis, fibrin deposition, an enhanced number of Hofbauer cells and syncytial sprouts, which can indicate a maturation disorder of the villous (98, 100). Unravelling how ZIKV can cross the human placenta, which might include multiple mechanisms that differ during the different stages of pregnancy, might be an essential first step in the development of prevention strategies for transplacental transmission of ZIKV and possibly other pathogens.

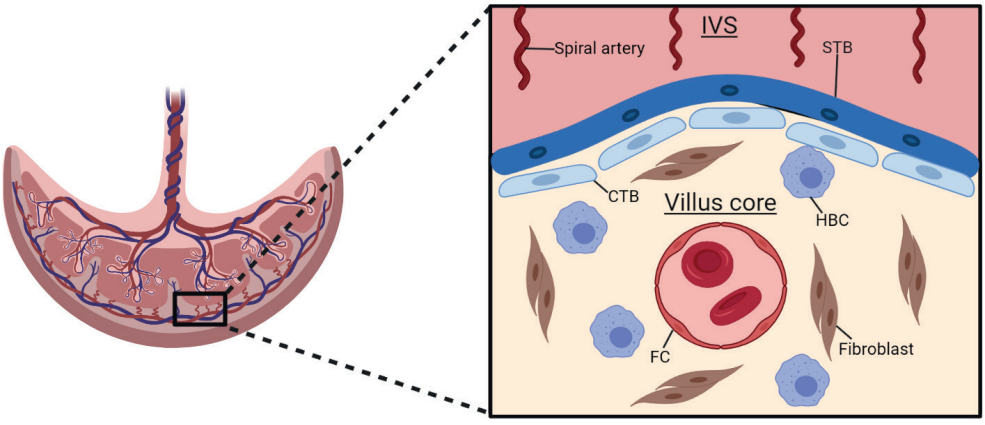


Figure 2. Schematic representation of the human placenta and a chorionic villus (inset). IVS; intervillous space, STB; syncytiotrophoblasts, HBC; Hofbauer cell, CTB; cytotrophoblast, FC; foetal capillary.

Antibody dependent enhancement of ZIKV infection

Apart from protection against symptomatic ZIKV infection, DENV antibodies could theoretically also have a negative (exacerbating) effect on a ZIKV infection through antibody-dependent enhancement (ADE). ADE is a phenomenon mainly known for DENV infections, in which the presence of specific immunoglobulins can enhance pathogen infection, or disease severity. This is a paradoxical phenomenon since immunoglobulins are normally important protective effectors of the adaptive immune system. *In-vitro* ADE of infection was first described by Hawkes et al. in 1964 (101). Subsequently, it was observed for a DENV infection that more severe disease mainly occurred in adults who had a secondary DENV infection with another DENV serotype and in children whose maternal antibody concentrations waned below protective concentrations (102).

Studying the role of ADE in disease severity in clinical studies is challenging. This is partially because ADE is a rare phenomenon, but mainly because to be able to correlate disease severity to the presence of pre-existing antibodies, one needs a recent pre-sample from before the patient fell ill. Therefore it took until 2017 before sound evidence of the clinical significance of ADE in severe DENV was presented. In that study, which was carried out among a paediatric cohort in Managua, Nicaragua, serum was collected yearly from all participants. This made it possible to correlate pre-existing DENV antibody titres to the risk of getting severe DENV during the following year (103). Here, it was found that a specific range of pre-existing DENV antibody titres – not too low and not too high – significantly increased the risk of developing severe DENV. Soon after this study was published, results from a phase III vaccine trial with a tetravalent dengue vaccine (Dengvaxia®) showed that when this vaccine is administered to DENV naïve children, they have an increased risk of developing a severe dengue infection, which is probably caused by ADE (104). ADE has been reported for many viruses other than DENV, such as Chikungunya virus, SARS-CoV, Ebola virus and influenza A virus (105-109). However, these are often observations from *in-vitro* experiments in which a sub-neutralising concentration of a monoclonal antibody or convalescent serum can enhance infection in the Fc-γ receptor (FcγR) bearing cell (lines). For many of these viruses, except for DENV, ADE of the disease has not been observed in *in-vivo* studies and especially not regularly in humans (110).

Different mechanisms of FcγR-mediated ADE are described; extrinsic- and intrinsic-ADE (111). Extrinsic ADE leads to more infected cells, when compared with cross-reactive antibodies not being present (Figure 3 A&B). In extrinsic ADE, cross-reactive IgG antibodies that bind a virus but cannot neutralise it, are phagocytosed by cells such as monocytes and macrophages via their FcγRs. Given that the virion is not neutralised by the antibodies that are

bound to it, the virions can escape the lysosomal pathway and replicate in these cells. Intrinsic ADE is a phenomenon that leads to more virion production per infected cell. The Infection of FcγR-bearing cells through the uptake of IgG-virion complexes by leads to a virus-friendly state in the cell, which results in the production of more viral progeny in this cell (112, 113). For DENV, it has been demonstrated that intrinsic ADE can shift the immune response from a T-helper cell 1 response (production of pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-8 and TNF-α) to a T-helper cell 2 response (production of eosinophilic and anti-inflammatory cytokines such as IL-10, IL-6 and IL-4) (111, 112). Extrinsic- and intrinsic-ADE are separate mechanisms that can lead to the enhancement (increased severity) of an infection and disease. However, they are not mutually exclusive; both mechanisms can occur at the same time *in-vivo* and can cause significant release of virus and inflammatory cytokines (110).

During the 2015-2016 ZIKV outbreak, it was speculated that ADE of ZIKV infection, caused by cross-reactive flavivirus antibodies, could explain the emergence of ZIKV-infection complications that were previously unseen (114). The reasons for this speculation were

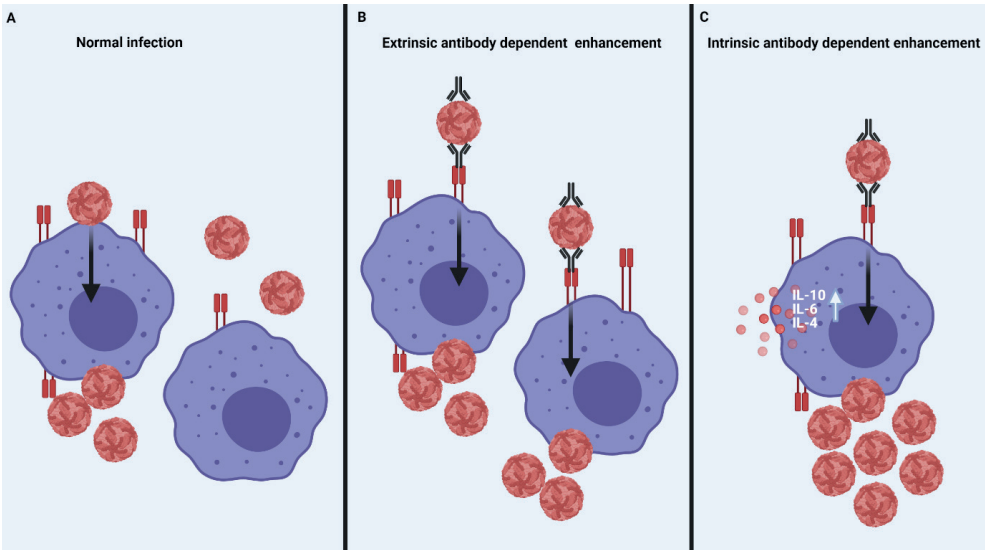


Figure 3. Schematic representation of ZIKV infection of FcγR-bearing cells in the absence and presence of cross-reactive flavivirus antibodies. **A:** In the absence of antibodies, ZIKV can attach to a cell via one of its entry cofactors and subsequently enter this cell and replicate. **B:** In the presence of cross-reactive, but not cross-neutralising, anti-flavivirus antibodies, ZIKV-IgG complexes are efficiently taken up by FcγR-bearing cells through their FcγR. ZIKV can subsequently escape from the early endosome and replicate in these cells. **C:** The uptake of ZIKV-IgG complexes through FcγR changes the antiviral response in this cell when compared with the normal infection in **A**. This results in a more virus-friendly state and higher viral progeny production per cell. IL; interleukin.

the strong antibody cross-reactivity between ZIKV and mainly DENV, the introduction of ZIKV in DENV (hyper)endemic areas in the Americas and the observation that ADE plays an important role in causing severe DENV disease. ADE of ZIKV infection and disease has been, and still needs to be, studied extensively. And while ADE of ZIKV infection has been observed in many of these studies in experimental settings, it seems that ADE of ZIKV infection by DENV antibodies does not play an important role in the clinical disease severity of a ZIKV infection (115). The role of cross-reactive flavivirus antibodies of a ZIKV infection at the maternal-foetal interface has yet to be fully elucidated and is an important topic of study (116).

Outline of this thesis

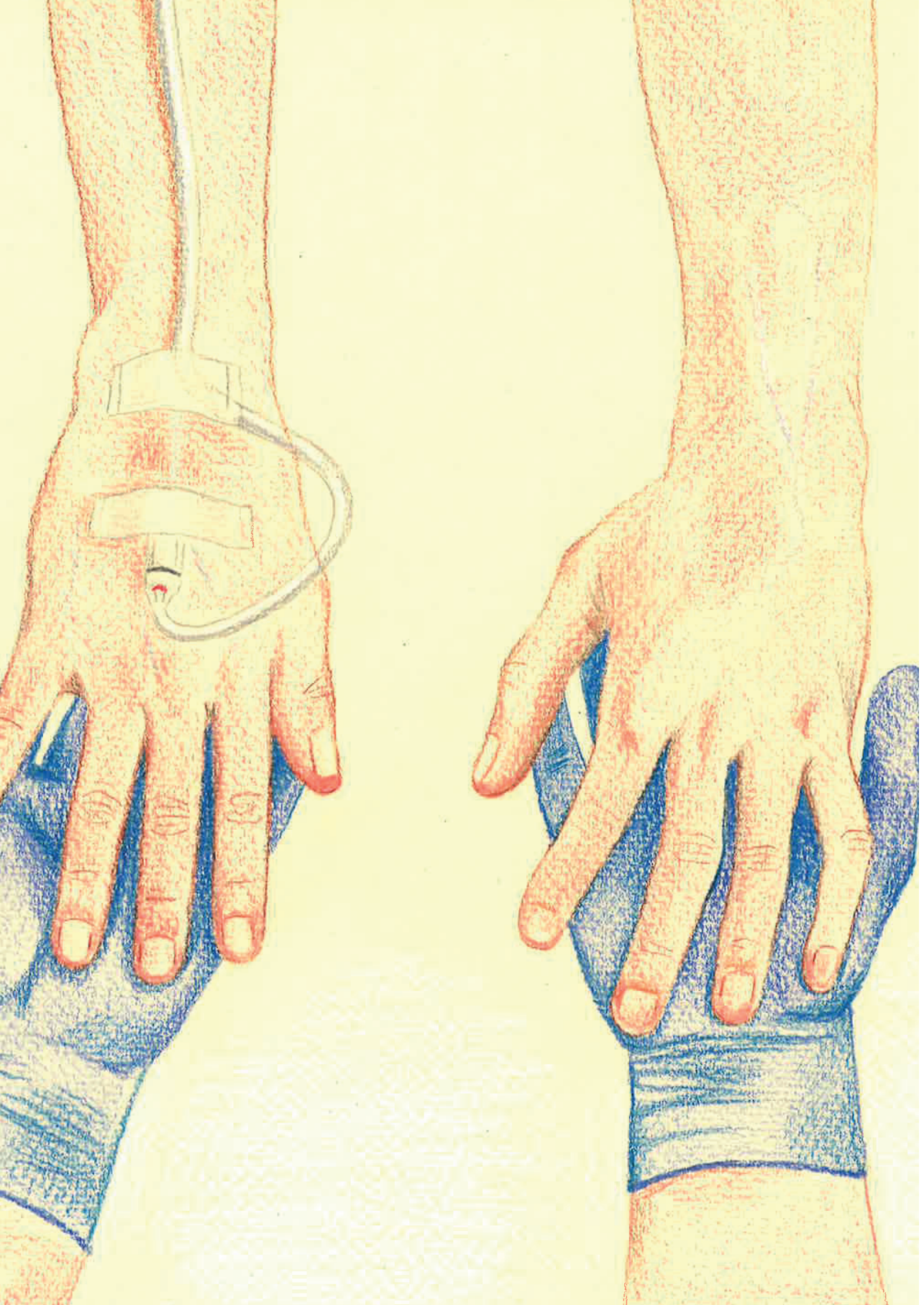
Even though ZIKV had already been discovered in 1947, because of the presumed mild and uncomplicated infection presentation in humans, limited research on ZIKV was carried out prior to the 2015-2016 outbreak in the Americas. Therefore, the emergence of ZIKV and its associated complications came as a surprise to many healthcare providers, scientists, and public-health workers worldwide. A better understanding of the pathogenicity of ZIKV is essential for the prevention of future ZIKV outbreaks and the prevention of ZIKV-associated complications and their possible treatment. In this thesis, we aim to study multiple facets of ZIKV, such as its epidemiology, the complications of a ZIKV infection and the possible interactions between ZIKV and other flaviviruses.

In **Part I**, we describe the results of a prospective study on GBS in Paramaribo, Suriname. In **Chapter 2** we describe three patients with GBS that might have been triggered by ZIKV. In **Chapter 3** we describe the clinical presentation and aetiology of the previous infection of 12 GBS patients in Suriname.

In **Part II** we investigate the epidemiology of ZIKV and the antibody response after a ZIKV infection. We carried out a ZIKV seroprevalence study in both rural and urban regions of Suriname a year after the peak of the ZIKV outbreak in this county and we describe the results in **Chapter 4**. With this study we aim to get an indication of the magnitude of the ZIKV outbreak in Suriname and the possible risks of new ZIKV outbreaks. In **Chapter 5** we demonstrate that, three years after a confirmed infection, ZIKV antibodies can often not be detected with two commonly used serological assays. Furthermore, by measuring the presence of DENV antibodies in this cohort, we try to get a better understanding of the immunological interplay of these two related viruses.

In **Part III** we focus on ADE of ZIKV infection and the possible implications that it can have on ZIKV infections. In **Chapter 6** we review the possible risks and evidence for ADE of ZIKV infection caused by DENV antibodies, and vice versa. In **Chapter 7**, we test which primary myeloid cells and cell lines can become infected with ZIKV and in which cells ADE of an *in-vitro* ZIKV infection can be induced. Furthermore, we test whether myeloid cells from pregnant women are more susceptible to ZIKV infection and ADE of ZIKV infection, compared with those of non-pregnant women. In **Chapter 8** we study the role played by cross-reactive DENV antibodies on ZIKV infection at the maternal-foetal interface. In so doing, we use placental explants, primary placental cells, and a highly relevant *ex-vivo* placental perfusion model to study how ZIKV crosses the placenta and to investigate whether DENV antibodies can increase the risk of transplacental ZIKV transmission.

The key findings of this thesis and their possible implications are discussed in **Chapter 9.**



CHAPTER 2

Zika Virus Infection and Guillain–Barré Syndrome in Three Patients from Suriname

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PART I
Zika virus and Guillain-Barré syndrome

Abstract

We present three patients from Suriname who were diagnosed with Guillain-Barré syndrome (GBS) during the Zika virus (ZIKV) outbreak in this country. One patient had a positive ZIKV urine real-time RT-PCR (qRT-PCR) result. The other two patients had a negative ZIKV urine qRT-PCR but a positive virus neutralization test and presence of IgG antibodies against ZIKV in the serum. Considering the evidence of a past ZIKV infection and absence of evidence for recent infections with the most common preceding infections of GBS, it is very likely that these GBS cases were triggered by ZIKV.

Introduction

Since the Zika virus (ZIKV) outbreak in South America, an increased incidence of microcephaly and the Guillain-Barré syndrome (GBS) is reported in several affected countries in South America (117, 118). Guillain-Barré syndrome is a heterogeneous postinfectious immune-mediated syndrome characterized by rapidly progressive symmetrical muscle weakness and decreased or absent deep tendon reflexes (51). GBS is a clinical diagnosis, but supported by investigation of cerebrospinal fluid (CSF) for an elevated protein level with normal cell count (albuminocytologic dissociation) and by nerve conduction studies. Various types of preceding infection are associated with GBS such as Campylobacter jejuni, Cytomegalovirus (CMV), Epstein–Barr virus (EBV), Mycoplasma pneumoniae, hepatitis E virus, and now ZIKV (52, 67, 119). In addition to supportive care, the treatment of GBS includes intravenous immunoglobulins (IVIg) or plasma exchange (PE). Since the start of the ZIKV outbreak in Suriname in October 2015, clinicians in Suriname have noted a 5- to 10-fold increase in GBS incidence until March 2016 [personal communication with Dr. Harvey Yang, Neurologist, Academic Hospital Paramaribo, Suriname, March 2016 and Ref. (118)]. This case series consists of three consecutive, well-documented patients that were diagnosed with GBS in 2016 and who had a preceding infection with ZIKV.

Clinical description

Case 1

Early 2016, a male in his forties was admitted to a local hospital in Paramaribo, Suriname, because of muscle weakness in the legs and paresthesias in the hands and feet. He reported development of skin rash, red eyes, and arthralgia 7 days before the start of the paresthesias and muscle weakness. Physical examination upon admission showed symmetrical muscle weakness in the arms and legs; Medical Research Council (MRC) score 4 (ranging from 0; paralysis to 5; normal strength), absent deep tendon reflexes in the legs and decreased deep

tendon reflexes in the arms. During admission, the patient developed a unilateral peripheral facial nerve paralysis and areflexia in both legs and arms. A lumbar puncture was performed 3 days after hospital admission, and an elevated protein level was found (1.98 g/L, $N < 0.5$ g/L) in the CSF in absence of cells. Due to the typical presentation of symmetrical flaccid muscle weakness with decreased or absent deep tendon reflexes, an albuminocytologic dissociation in the CSF and absence of a sensory level, fever, encephalopathy, or other clinical signs of myelum involvement, the diagnosis of GBS was considered the most probable. ZIKV real-time RT-PCR (qRT-PCR) was performed locally on urine taken at day five after admission and was negative (120). A motor nerve conduction study (NCS) was performed 13 days after the start of muscle weakness. The NCS showed a decreased nerve conduction velocity (NCV) and compound muscle action potential (CMAP) and an increased distal motor latency (DML) (see Table S1 in Supplementary Material) (121). The results of the NCS were consistent with the criteria for the primary demyelinating form of GBS according to the Hadden's criteria (122) . A needle EMG was also performed and showed no abnormalities. The patient was still able to walk independently and, as symptoms remained relatively mild, was not treated with IVIg or PE. During admission, the muscle strength of the patient improved. He was discharged from the hospital after 11 days and followed-up at the neurology outpatient clinic until 12 weeks after hospital discharge, at which time he was fully recovered.

Case 2

In February 2016, a male in his 40s was admitted to the neurology department of a hospital in Paramaribo, Suriname, because of muscle weakness in the legs, which started 7 days prior to admission. At presentation in the hospital, the patient was unable to walk without help. Two weeks before muscle weakness started, he visited the hospital because of urinary retention. Acute myelitis and a cauda syndrome were excluded with an MRI-scan, which showed no abnormalities. The week before muscle weakness started, he had an influenza-like syndrome and had a short episode of diplopia. At first physical examination after admission, muscle weakness was observed in predominantly the legs (MRC score 4). Areflexia in both legs and arms was noted. The patient complained of paresthesia and pain in the legs but did not have a sensory level or other signs of myelum involvement. A lumbar puncture was performed 2 days after admission, and a high protein level was found in the CSF (4.4 g/L) while the cell count was slightly increased (32.7×10^6/L). A urine sample, taken at day two after admission, was tested positive for ZIKV RNA with a Ct-value of 35.7 (120). Four days after admission his muscle power improved (MCR score arms 5, legs; proximal 5, distal 4), while the areflexia in arms and legs persisted. Because of the little availability of IVIg in Suriname and because PE is not possible in Suriname, the patient did not received immunotherapy. A motor NCS,

performed 15 days after the start of muscle weakness, showed severe decreased NCV and a prolonged DML in all tested nerves. The CMAP was decreased in predominantly the legs (see Table S2 in Supplementary Material). These results were consistent with the demyelinating subtype of GBS (123). At discharge, 7 days after admission, he was still unable to walk without help due to muscle weakness in the legs. Four months after hospital discharge he was able to walk a few steps without assistance, but he still could not live independently or return to work.

Case 3

In March 2016, a male in his 60s visited a hospital in Paramaribo, Suriname, because of right-sided peripheral facial nerve paralysis since 1 day. The patient did not report having had ZIKV-like symptoms prior to admission. Neurological examination revealed absent deep tendon reflexes in the lower extremities but no muscle weakness. The patient was admitted to the neurology department for observation. At day three of admission he developed a bilateral peripheral facial nerve paralysis. Four days after admission, the patient was unable to walk or stand due to muscle weakness in the legs (MRC score 4) and areflexia in all limbs. An elevated protein level with a mild increased cell count was found in the CSF (protein 2.93 g/L, cells 15.7 × 106/L) taken 5 days after admission. The progressive phase of the muscle weakness lasted for 6 days after which the patient started to recover. Motor NCS showed a decreased dCMAP and NCV and increased DML in predominantly the legs but also the arms. The tibial nerve was inexcitable in both legs (see Table S3 Supplementary Material). Features of demyelination were only found in one nerve, and as such the NCS results were classified as equivocal (122). ZIKV qRT-PCR was performed locally on urine taken at day four after admission and was negative (120). The patient received no PE or IVIg because of the limited availability of IVIg. At discharge on day 18, the patient was able to walk a small distance with assistance. The patient was followed-up at the neurology outpatient clinic until 4 months after hospital discharge, at this time he did not have residual deficits anymore.

Laboratory analysis

Real-time RT-PCR for ZIKV in urine was performed in the Academic Hospital Paramaribo, Suriname. Plasma, serum, and CSF samples were collected from the three patients and sent to the WHO collaborating centre for arbovirus reference and research at Erasmus Medical Centre in Rotterdam, the Netherlands for further testing. The samples were collected at days 8 (case 1), 5 and 7 (case 2), and 3 (case 3) after hospital admission. Zika virus qRT-PCR in plasma and CSF was negative in all three patients (see Table 1) (120). A ZIKV ELISA (Euroimmun, Lübeck, Germany) was used according to the manufacturer's recommendation

to detect anti ZIKV IgM and IgG antibodies in serum samples (124). Anti ZIKV IgM ELISA was negative in all patients, while anti ZIKV IgG antibodies were present in all patients. A ZIKV neutralization test (VNT) based on a Suriname ZIKV isolate (GenBank: KU937936.1) was performed to detect ZIKV neutralizing antibodies. All three patients had neutralizing antibodies against ZIKV with titers ranging from 25 (case 1) to 256 (case 3). Serology in all patients was negative for recent infections with *C. jejuni* and showed an infection in the past with CMV (high avidity IgG anti CMV) and EBV. All serum samples tested negative for dengue virus (DENV) IgM antibodies and non-structural protein 1 (NS1) antigen and positive for DENV IgG antibodies. ELISA was used to detect the presence of IgM and IgG antibodies against GM1, GM2, GD1a, GD1b, GT1b, and GQ1b, and paired complexes of all these gangliosides in the acute phase serum of the patients (125). These anti-ganglioside antibodies were negative in all patients.

Table 1. Results of diagnostic tests

Assay	Material	Case 1	Case 2	Case 3
ZIKV qRT-PCR	Urine	NEG	POS	NEG
	Plasma CSF	NEG NEG	NEG NEG	NEG NEG
Anti ZIKV antibodies (ELISA)	Serum	IgM NEG IgG POS	IgM NEG IgG POS	IgM NEG IgG POS
ZIKA VNT (titer + result)	Serum	25, POS	40, POS 81, POS	256, POS
DENV	Serum	IgM NEG IgG POS NS1 NEG	IgM NEG IgG POS NS1 NEG	IgM NEG IgG POS NS1 NEG
Campylobacter jejuni	Serum	IgM NEG IgG POS	IgM NEG IgG POS	IgM NEG IgG POS
CMV	Serum	IgM NEG IgG POS	IgM NEG IgG POS	IgM NEG IgG POS
EBV	Serum	IgM NEG IgG VCA POS EBNA POS	IgM NEG IgG VCA POS EBNA POS	IgM NEG IgG VCA POS EBNA NEG

VNT: virus neutralization test, NS1: non-structural protein 1, VCA: viral capsid antigen, EBNA: Epstein-Barr virus nuclear antigen.

Discussion

Here, we presented three patients from Suriname with acute flaccid paralysis during the height of the ZIKV outbreak in this country. Differential diagnostic considerations were GBS and acute (*Flavivirus*) myelitis. None of the patients had a sensory level, increased or pathological

reflexes, fever, encephalopathy, or other symptoms suggesting an acute myelitis, and there was no marked pleocytosis in the CSF. Only motor and no sensory NCS were performed in these patients. The results of the motor NCS were consistent with GBS in all three patients. Furthermore, all patients had progressive symmetrical muscle weakness with areflexia, a monophasic disease course, an albuminocytologic dissociation in the CSF, and absence of an identified alternative diagnosis for muscle weakness. All patients thus scored the highest level for diagnostic certainty for GBS (level I) according to the diagnostic criteria for GBS of the Brighton collaboration and were therefore diagnosed with GBS (59). No anti-ganglioside antibodies were found in the serum of these patients, but as anti- ganglioside antibodies are only found in an estimated 50% of GBS cases, their absence does not rule out the diagnosis GBS (126). Typically, muscle weakness in GBS occurs 1–2 weeks after the preceding infection that triggers GBS (51). This delay makes it difficult to identify the infection that could have triggered the onset of GBS based on molecular methods, as the infection may have been cleared by the time of onset of the GBS symptoms. Especially in ZIKV suspected GBS cases, it is difficult to deter-mine if the GBS is indeed caused by a recent ZIKV infection. This is due to a relatively small diagnostic time window of qRT-PCR for ZIKV in EDTA-plasma (3–5 days), to a lesser extent in urine (up to 30 days) and semen (up to 92 days), although the sensitivity of testing at these later time points remains to be validated (127-130). Confirmation of an infection based on serology will have a broader window of detection. However, in case of past *Flavi*virus exposure, ZIKV IgM response may be delayed, limited, or even absent, further complicating etiological diagnosis (131). The diagnostic work-up presented for this case series reflects these challenges of identifying possible triggers for the onset of GBS in these patients. Definitive evidence for recent ZIKV infection was obtained in one patient who tested positive by qRT-PCR for ZIKV in the urine. In cases 1 and 3, it is possible that the diagnostic window for reliable ZIKV RNA detection had already expired once the GBS symptoms occurred and the samples were collected. No IgM antibodies to ZIKV were detected, including the patient with confirmed ZIKV shedding by RT-PCR, but all three patients had neutralizing antibodies indicating a past ZIKV infection. The absence of IgM antibodies is remarkable, although the assays that currently are available have demonstrated short-lived IgM responses and as mentioned above, IgM response can be absent in case of prior *Flavivirus* exposure (e.g. DENV in these patients) (130). A recent infection with the most prevalent preceding infections of GBS—*C. jejuni*, CMV, and EBV—were excluded in the patients, which makes it more plausible that the GBS was triggered by a recent ZIKV infection. The exact pathophysiological mechanism behind ZIKV- associated GBS remains to be elucidated, but antibody-dependent enhancement (ADE) of ZIKV infection might play a role. It has recently been described that DENV antibodies that are able to bind ZIKV, but cannot neutralize ZIKV, can promote ADE

that can result in greater ZIKV replication (114). The three patients in this case series all had IgG antibodies against DENV. It remains to be seen if the combined effects of past and recent *Flavivirus* exposures plays a role in the pathogenesis of ZIKV- associated GBS.

Conclusion

Taken together, we can conclude that in the three presented cases, the relation between ZIKV infection and GBS was confirmed in one patient, and was plausible in the two others, given the increased GBS incidence during the ZIKV outbreak in Suriname, the ruling out of most prevalent preceding infections of GBS and the presence of ZIKV neutralizing antibodies in these patients. For definitive proof of this association, case–control studies are needed, using stringent and standardized diagnostic criteria, both for GBS and for the laboratory diagnosis.

Ethics statement

This study was approved by the Ministry of Health of Suriname. All participants signed informed consent prior to participation in this study.

Supplementary material

Supplementary Table 1. Motor nerve conduction study results from case 1.

Nerve	DML (ms)	dCMAP (mV)	NCV (m/s)
Left ulnar	4.22 (<4.5)	2.54 (>7)	32.41 (>49)
Right ulnar	6.25 (<4.5)	0.15 (>7)	67.07 (>49)
Left median	7.73 (<4.4)	3.33 (>4)	49.04 (>49)
Right median	7.03 (<4.4)	2.52 (>4)	49.89 (>49)
Left peroneal	NP	NP	NP
Right peroneal	6.56 (<6.5)	2.55 (>2)	25.13 (>44)
Left tibial	8.52 (<5.8)	2.07 (>4)	27.38 (>41)
Right tibial	NP	NP	NP

Normal adult values for NCS according to (132) are presented between brackets. DML, distal motor latency; dCMAP, distal compound muscle action potential; NCV, nerve conduction velocity; NP, not performed

Supplementary Table 2. Motor nerve conduction study results from case 2.

Nerve	DML (ms)	dCMAP (mV)	NCV (m/s)
Left ulnar	6.33 (<4.5)	4.48 (>7)	27.0 (>49)
Right ulnar	5.43 (<4.5)	4.78 (>7)	20.3 (>49)
Left median	10.1 (<4.4)	4.50 (>4)	21.8 (>49)
Right median	8.64 (<4.4)	4.97 (>4)	17.8 (>49)
Left peroneal	12.4 (<6.5)	0.54 (>2)	12.4 (>44)
Right peroneal	6.56 (<6.5)	0.79 (>2)	10.6 (>44)
Left tibial	8.52 (<5.8)	1.85 (>4)	14.7 (>41)
Right tibial	15.8 (<5.8)	1.51 (>4)	15.8 (>41)

Normal adult values for NCS according to (132) are presented between brackets. DML, distal motor latency; dCMAP, distal compound muscle action potential; NCV, nerve conduction velocity; NP, not performed

Supplementary Table 3. Motor nerve conduction study results from case 3.

Nerve	DML (ms)	dCMAP (mV)	NCV (m/s)
Left ulnar	NP	NP	NP
Right ulnar	NP	NP	NP
Left median	4.83(<4.4)	2.32 (>4)	46.5 (>49)
Right median	4.71 (<4.4)	7.20 (>4)	41.7 (>49)
Left peroneal	7.83 (<6.5)	0.12 (>2)	34.1 (>44)
Right peroneal	7.44 (<6.5)	0.21 (>2)	30.0 (>44)
Left tibial	Inexcitable	Inexcitable	Inexcitable
Right tibial	Inexcitable	Inexcitable	Inexcitable

Normal adult values for NCS according to (132) are presented between brackets. DML, distal motor latency; dCMAP, distal compound muscle action potential; NCV, nerve conduction velocity; NP, not performed.

3

CHAPTER

Guillain-Barré Syndrome in Suriname; Clinical Presentation and Identification of Preceding Infections

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Abstract

Guillain-Barré syndrome (GBS) is associated with various types of preceding infections including Campylobacter jejuni and cytomegalovirus, but there is also an association with arthropod borne viruses (arboviruses), such as Zika virus, that are endemic in tropical regions. Here we present the clinical characteristics of 12 GBS patients from Suriname that were hospitalized between the beginning of 2016 and half 2018. Extensive diagnostic testing was performed for pathogens that are commonly associated with GBS, but also for arboviruses, in order to identify the preceding infection that might have led to GBS. With this extensive testing algorithm, we could identify a recent infection in six patients of which four of them had evidence of a recent Zika virus or dengue virus infection. These results suggest that arboviruses, specifically Zika virus but possibly also dengue virus, might be important causative agents of GBS in Suriname. Furthermore, we found that more accessibility of intravenous immunoglobulins or plasma exchange could improve the treatment of GBS in Suriname.

Introduction

Guillain-Barré syndrome (GBS) is an immune-mediated polyradiculoneuropathy, characterized by a rapidly progressive symmetrical limb weakness and decreased or absent deep tendon reflexes (51). GBS can be a life-threatening disease because of respiratory and autonomic failure, and has an estimated mortality of 3–7% (59). The exact pathogenesis of GBS is unknown, but it is thought that preceding infections or vaccinations may trigger the production of autoantibodies to components of peripheral nerves due to molecular mimicry, leading to peripheral nerve injury (51). There are multiple clinical variants and electrophysiological GBS subtypes, such as acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor and sensory axonal neuropathy (AMSAN) and Miller Fisher syndrome (51). Diagnosis of GBS is based on clinical characteristics but can be supported by investigation of cerebrospinal fluid (CSF) and nerve conduction studies (50). Diagnosis and classification of GBS can be challenging because of the heterogeneity of the syndrome and the extensive differential diagnosis. Proven effective treatments of GBS are intravenous immunoglobulins and plasma exchange (50, 51) . Multiple pathogens are associated with GBS such as *Campylobacter jejuni* (*C. jejuni*), Epstein-Barr virus (EBV), cytomegalovirus (CMV), *Mycoplasma pneumoniae* (*M. pneumoniae*) and hepatitis E virus (HEV) (52). The type of preceding infection is related to the clinical presentation and course of GBS, and the variety of preceding infections contributes to the diversity in clinical variants and prognosis. Besides the above mentioned pathogens that are

associated with GBS, during the 2015–2016 Zika virus (ZIKV) outbreak in the Americas, it became clear that this virus is also associated with GBS (67, 133). More recently, an association between the newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and GBS was suggested (134, 135). Diagnosis of the preceding infection that might have triggered GBS can be difficult. Firstly, direct pathogen detection, for example, with polymerase chain reaction (PCR), is quite often not possible when GBS is diagnosed because of the time lag of 1–4 weeks between the initial infection and onset of GBS symptoms. Furthermore, diagnosis of preceding infections of GBS with serology can be challenging due to cross-reactivity of antibodies between certain pathogens and reactivation of nonrelated antibodies during infection. Here we describe 12 patients from Suriname with GBS who were recruited in a prospective observational study taking place from February 2016 to June 2018. Suriname is a country in South America which has, due to the tropical climate, a relative high burden of arthropod borne viruses (arboviruses) like ZIKV and dengue virus (DENV) (136, 137). The aim of this study was to describe the clinical presentation, treatment and outcomes of GBS patients in Suriname and to diagnose the preceding infections that lead to GBS in these patients. We were specifically interested in the role of arboviruses as potential preceding infections that can lead to GBS. The results of this study may result in more knowledge about the clinical presentation and the etiology of preceding infections of GBS in Suriname which could help to improve diagnostic preparedness and treatment of GBS in Suriname, and possibly other countries in South America.

Materials and Methods

Participants

Patients with suspected GBS were recruited for this prospective study as soon as possible after clinical GBS suspicion. Participants were recruited in all three hospitals in Paramaribo, Suriname, from February 2016 until July 2018. The case definitions of the Brighton Collaboration were used to determine the level of diagnostic certainty of GBS ranging from 1 (most certainty for GBS diagnosis) to 4 (least certainty for GBS diagnosis) (59).If during admission another diagnosis than GBS was made, or of if insufficient clinical information was available to verify the GBS diagnosis, these participants were excluded, as were participants from whom no blood was collected. One participant was initially suspected of a paraparetic form of GBS with paralysis of the legs and normal strength of the arms. This diagnosis was changed during admission to transverse myelitis because of a sensory level at Th5 and CSF pleiocytosis of 206 cell/µl and no further suspicion of GBS. Even though concomitant GBS and transverse myelitis can occur, this patient did not meet the Brighton diagnostic criteria

for GBS and was therefore excluded from this study (138-140). Three patients (patient 1, 2, and 6) were previously described in a smaller case series on possible ZIKV associated GBS in Suriname (141).

Data and Sample Collection

Serum and plasma were collected at enrolment in this study and 10–14 days later, or at the day of hospital discharge if this was before day 10. Neurological examination was performed by one of the researchers every seven days until hospital discharge. Additional information regarding medical history, (onset of) symptoms, and antecedent events like infections or vaccinations was collected with a questionnaire. Data from nerve conduction studies and analysis of cerebrospinal fluid were, when available, collected from all participants.

Ethics

This study protocol was approved by the ethical board of the Ministry of Health in Suriname. Informed consent was obtained from all patients and in case the patient was younger than 16 years old, from their

parents or representatives. The study was carried out in accordance with the Declaration of Helsinki.

Diagnostic Tests

Serum samples were tested for presence of antibodies against pathogens commonly associated with GBS; *C. jejuni*, EBV, CMV, HEV and *M. pneumoniae*. Furthermore, serological tests were performed to diagnose (recent) infections with the arboviruses ZIKV, DENV and chikungunya (CHIKV). All the serological tests -except serology for C. jejuni- were performed at the department of Viroscience at Erasmus MC, Rotterdam, the Netherlands. Presence of IgM antibodies against ZIKV and DENV and IgM and IgG antibodies against HEV and *M. pneumoniae* were assessed with use of commercial ELISA kits (ZIKV and DENV; Euroimmun, HEV; Wantai Biological, *M. pneumoniae*; Serion Diagnostics). EBV and CMV serology was performed using a chemiluminescent immunoassay (DiaSorin LIAISONR). Chikungunya antibodies were detected with an indirect immunofluorescence test (Euroimmun). All these assays were performed according to the manufacturer's instructions. In case of a positive IgM response for EBV or CMV, additional

testing, detection of Epstein-Barr virus Nuclear Antigen (EBNA) antibodies and a CMV avidity test respectively, was performed to determine if this was likely to be a primary

infection or not. C. jejuni serology was performed with an indirect IgG ELISA and antibody class capture ELISAs for IgM and IgA antibodies at the Department of Medical Microbiology, Reinier de Graaf Gasthuis, Delft, the Netherlands.

Neutralizing antibodies against ZIKV and DENV-2 [used as a representation of total DENV immunity (46)], were determined with an in-house micro-neutralization test (VNT) as previously described (136). Sera were tested in triplicates and the geometric mean of the highest final serum dilution was reported as titer. For both ZIKV and DENV-2, the cut-off of a positive VNT was a final serum dilution >1:32. For ZIKV diagnosis, a reverse transcriptase polymerase chain reaction (RT-PCR) was performed on plasma or, when available, urine using the primer/probe set described by Lanciotti et al. (120).

Case Definitions for Preceding Infections

For the interpretation of the serological and molecular tests performed in these patients, a distinction was made between confirmed recent infections, probable recent infections and possible recent infections. A recent infection was considered confirmed if one serum or urine sample was RT-PCR positive, or if IgM antibodies against the specific pathogens were present and there was a more than four-fold increase of IgG- or neutralization titer in paired samples. Presence of IgM antibodies with an increasing IgG- or neutralization titer in paired samples was considered a probable recent infection. Presence of IgM but with no increase in IgG or neutralization titer in paired samples was considered a possible recent infection. When paired blood samples were not available, presence of IgM in a single blood sample was considered a possible recent infection. Specifically, for CMV presence of anti-CMV IgM in combination with a rising IgG titer with low avidity was considered a confirmed recent CMV infection. The combination of CMV IgM with IgG with high avidity was considered as reactivation of CMV antibodies and not a recent infection (142). Presence of IgM against EBV in combination with IgG against EBV nuclear antigen (EBNA) was considered a non-recent infection (143).

Results

Participant Enrolment

In total, 27 patients with suspected GBS were enrolled in this study. Of these 27 patients, 15 were excluded because during admission another diagnosis than GBS was made or because the availability of clinical data or blood samples was insufficient, illustrated by the flowchart in Figure 1A. The number of GBS patients recruited per quarter of a year is displayed in Figure 1B. The highest peak of GBS patients, in the first quarter of 2016, coincided with the peak of the ZIKV outbreak in Suriname and South America in the first months of 2016 (144, 145).

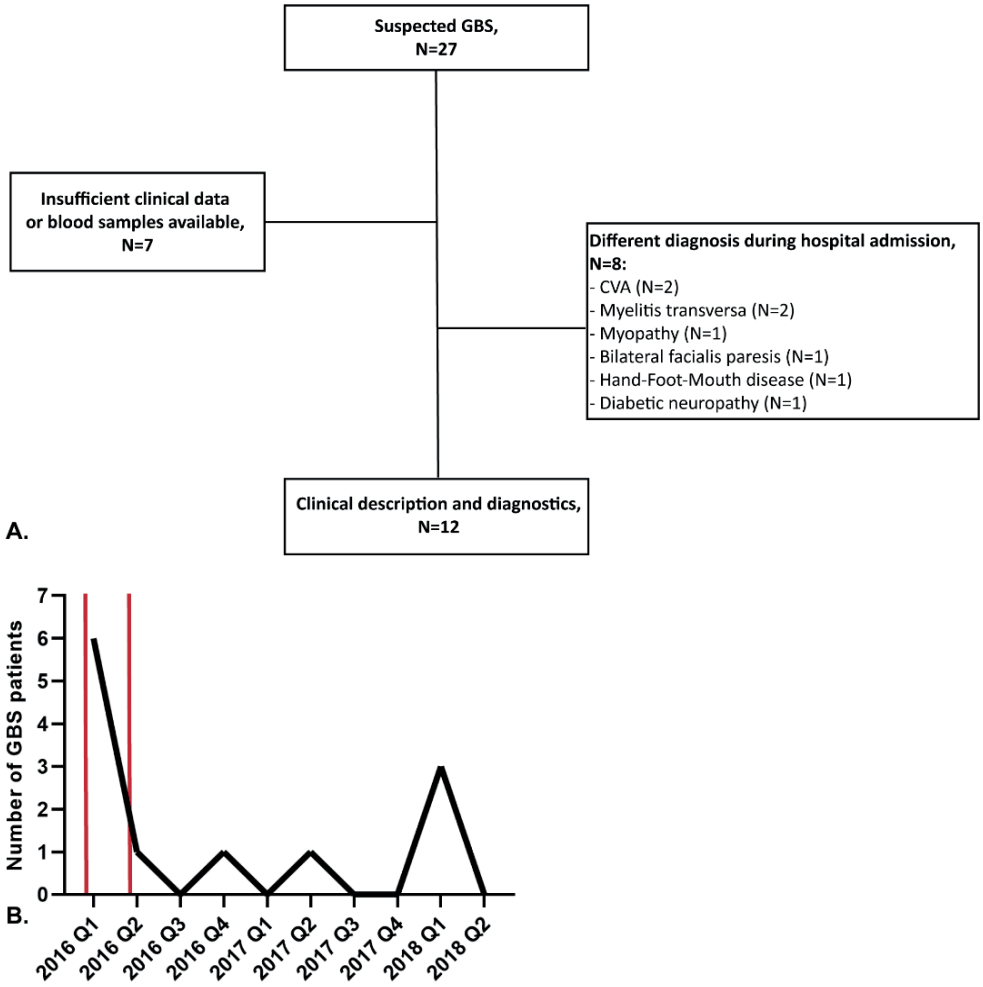

Figure 1. (A) Flowchart of patient recruitment and exclusion. (B) Number of GBS patients recruited in this study per quarter of a year (Q). The time period of the peak of the ZIKV outbreak in Suriname is marked with two red bars. CVA, cerebrovascular accident.

Clinical Characteristics

Seven out of 12 patients (58.3%) had a pure motor form of GBS (without sensory deficits, **Table 1**). Cranial nerves were affected in five patients (41.7%), and a facial palsy was present in four of these patients (80.0%). At nadir, six patients (50.0%) were confined to bed or chair of which three patients (50.0%) required assisted mechanical ventilation. Five patients (41.7%) received treatment with intravenous immunoglobulins (IVIg) while the other seven patients (58.3%) only received supportive care. None of the patients were treated with plasma exchange. Eight

out of 12 patients (66.7%) underwent nerve conduction studies and electromyography during admission of which five (62.5%) showed a demyelinating type of GBS, one (12.5%) showed an axonal GBS type, one (12.5%) was classified as equivocal and in one (12.5%) the potentials were absent. Brighton criteria 1 (highest level of diagnostic certainty) for GBS diagnosis was met in six patients (50.0%), level 2 in five patients (41.7%) and level 3 in one patient (8.3%). Median hospital stay was 14 days (IQR 11–34 days).

Table 1: Clinical characteristics of GBS patients.

Patient	**1**	**2**	**3**	**4**	**5**	**6**
A						
Sex, Age (yr)	M, 46	M, 44	F, 67	F, 54	M, 12	M, 64
Start of GBS symptoms	09-Feb-2016	10-Feb-2016	19-Feb-2016	09-Feb-2016	22-Feb-2016	30-Jan-2016
Antecedent symptoms	Fever, rash, arthralgia, conjunctivitis	Influenza-like symptoms, double vision	Fever, myalgia	None	Influenza-like symptoms	Myalgia and arthralgia
Clinical subtype	PM	SM	PM	PM	SM	SM
Cranial nerve involvement	Facial nerve, glossopharyngeal nerve	No	Facial nerve	No	No	Facial nerve
CSF cell count	0/µl	33/µl	3/µl	56/µl	0/µl	16/µl
Protein in CSF	1.98 g/L	4.4 g/L	0.5 g/L	2.33 g/L	0.18 g/L	2.93 g/L
NCS result	Demyelinating	Demyelinating	-	-	-	Equivocal
GBS disability score[1] at nadir	2	3	4	4	3	4
Diagnostic certainty GBS diagnosis (59)	1	1	2	3	2	1
IVIg / PE treatment	No	No	No	No	No	No
Preceding infection	Unknown	Confirmed ZIKV	Probable ZIKV, possible DENV	Unknown	Confirmed DENV	Unknown

Patient	7	8	9	10	11	12
B						
Sex, Age (yr)	F, 39	F, 20	F, 10	M, 48	M, 58	F, 58
Start of GBS-symptoms	19-Apr-2016	14-Nov-2016	23-May-2017	14-Jan-2018	23-Jan-2018	24-Feb-2018
Antecedent symptoms	None	Fever, diarrhea, vomiting	Conjunctivitis	Influenza-like symptoms	Diarrhea, vomiting	Fever, myalgia
Clinical subtype	SM	PM	PM	SM	PM	PM
Cranial nerve involvement	No	No	N. Oculomotorius, N. Facialis	No	No	No
Cells in CSF	0/µl	4/µl	2/µl	3/µl	2/µl	3/µl
Protein in CSF	2.09 g/L	2.56 g/L	0.16 g/L	0.30 g/L	0.90 g/L	0.66 g/L
NCS result	Demyelinating	Demyelinating	-	Axonal	Absent potentials	Demyelinating
GBS disability score[1] at nadir	4	5	5	4	5	4
Diagnostic certainty GBS diagnosis (59)	1	1	2	2	2	1
IVIg / PE treatment	Yes, IVIg	Yes, IVIg	Yes, IVIg	Yes, IVIg	Yes, IVIg	No
Preceding infection	Unknown	Confirmed CMV, possible ZIKV/DENV	Probable *M. pneumoniae,* possible *C. jejuni*	Unknown	Possible *C. jejuni*/*M. pneumoniae.*	Unknown

A; patient 1-6. B; patient 7-12.
M; male, F; female, PM; Pure Motor, SM; Sensory-Motor, -: not performed, NCS; nerve conduction studies, IVIg; intravenous immunoglobulins, PE; plasma exchange
[1]GBS disability score: 0: Healthy. 1: Minor symptoms or signs of neuropathy but capable of manual work/capable of running. 2: Able to walk without support of a stick but incapable of manual work/running. 3: Able to walk with a stick, appliance or support. 4: Confined to bed or chair bound. 5: Requiring assisted ventilation (for any part of the day or night). 6: Death

Preceding infections

Nine patients (75%) reported symptoms of a possible preceding infection and the average time between these symptoms and the first neurological symptoms was 9 days (range 5–22 days). Most of the patients (N = 5, 41.6%) reported non-specific, flulike symptoms such as fever and myalgia. Two patients (16.7%) reported symptoms of a gastrointestinal infection while two other patients reported symptoms associated with an arbovirus infection, such as conjunctivitis and skin rash. The interpretation of the diagnostic test results is shown in Table 1 and the results of the diagnostic tests are shown in Table 2. In three patients (25%) we could confirm the preceding infection based on the criteria described in the methods. Of these three, patient 2 had a confirmed recent infection with ZIKV, patient 5 had a confirmed recent infection with DENV and patient 8 had a confirmed recent infection with CMV. Two patients (16.7%) had a probable recent infection, one with *M. pneumoniae* (patient 9) and one with ZIKV (patient 3) although a recent DENV infection could not be ruled out in this last patient. In one patient (8.3%, patient 11) we found evidence of a possible recent infection with *M. Pneumoniae* and/or *C. jejuni*. Finally, in six patients (50.0%) we could not find sufficient evidence of a recent infection based on our diagnostic criteria. Four of these six patients (patient 1, 6, 10, and 12) did have non-specific symptoms of an infection, such as fever and myalgia, prior to the start of GBS symptoms. None of the patients reported to have recently received a vaccination.

Table 2: Results of diagnostic tests for GBS preceding infections.

Patient		Days since onset of preceding symptoms	*Campylobacter jejuni*			Cytomegalovirus			Epstein-barr virus			Hepatitis E virus		*Mycoplasma pneumoniae*	
			IgM	IgA	IgG	IgM	IgG	Avidity	IgM	IgG VCA	IgG EBNA	IgM	IgG	IgM	IgG
A															
1		15	N	N	P (1.5)	N	P (92.5)	-	N	P (>750)		-	-	N	P (21.6)
2	**1st**	24	N	N	P (0.7)	N	P (93.3)	-	N	P (249)		N	N	-	-
	2nd	26	-	-	-	-	-	-	-	-		-	-	-	-
3	**1st**	11	N	N	P (0.9)	P (31.0)	P (79.1)	High (0.64)	P (50.0)	P (505)	P (68.0)	N	N	N	P (15.1)
	2nd	17	N	N	P (0.8)	P (29.9)	P (69.5)	-	P (73.9)	P (>750)	P (45.4)	N	N	N	B (10.6)
4	**1st**	-	N	N	P (0.5)	N	P (158)	-	N	P (22.6)		N	N	N	P (19.6)
	2nd	- (12 days between 1st and 2nd sample)	N	N	P (0.4)	N	P (173)	-	N	N		N	N	N	B (12.3)
5	**1st**	11	N	N	P (1.2)	N	P (103)	-	N	P (203)		N	P	N	B (12.2)
	2nd	20	N	N	P (0.7)	-	-	-	-	-		-	-	P (49.1)	N
6	**1st**	39	N	N	P (1.7)	N	P (90.5)	-	N	P (>750)		N	N	N	P (15.9)
	2nd	53	N	N	P (1.2)	N	P (118)	-	N	P (710)		N	N	N	P (27.0)
7	**1st**	-	N	N	P (1.8)	N	P (>180)	-	N	P (>750)		N	P (2.1)	N	P (24.6)
	2nd	- (10 days between 1st and 2nd sample	-	-	-	N	P (>180)	-	N	P (>750)		-	-	N	P (33.8)
8	**1st**	20	N	N	P (2.4)	**P (123)**	P (69)	**Low (0.09)**	B (36.2)	P (314)		N	N	B (13.4)	P (19.7)
	2nd	32	N	N	P (2.2)	**P (85.8)**	P (115)	-	N	P (313)		N	N	N	B (12.1)
9	**1st**	7	**P (.25)**	P (1.9)	**P (2.7)**	N	P (80.5)	High (0.77)	N	P (218)		N	N	**P (46.6)**	**B (14.5)**
	2nd	17	N	P (.25)	**P (2.6)**	P (37.8)	P (112)	-	N	P (455)		N	P (5.6)	**P (51.6)**	**P (38.2)**
10		31	N	N	P (1.7)	N	P (126)	-	N	P (>750)		N	P (1.4)	N	P (41.0)
11	**1st**	9	**P (.44)**	P (2.4)	**P (2.6)**	N	P (177)	-	N	P (>750)		N	P (1.1)	-	**P (42.5)**
	2nd	20	N	P (.81)	**P (2.6)**	N	P (157)	-	N	P (>750)		N	P (1.8)	**P (40.3)**	**P (37.6)**
12		17	N	N	P (2.3)	N	P (148)	-	N	P (>750)		N	P (9.5)	N	P (51.6)

Patient		Days since onset of preceding symptoms	Zika virus				Dengue virus		Chikungunya virus	
			IgM	VNT	PCR plasma	PCR urine	IgM	VNT	IgM	IgG
B										
1		15	N	P (1:161)	N	N	N	P (1:64)	N	N
2	**1st**	24	N	P (1:80)	-	**P**	N	P (1:162)	N	P (>100)
	2nd	26	N	P (1:102)	-	-	N	P (1:204)	N	P (>100)
3	**1st**	11	**P (5.4)**	**P (1:101)**	N	N	**P (12.2)**	**P (1:812)**	N	P (10)
	2nd	17	**P (2.8)**	**P (1:203)**			**B (0.9)**	**P (1:646)**	N	P (10)
4	**1st**	-	N	N	N	-	N	N	N	N
	2nd	- (12 days between 1st and 2nd sample)	N	N	-	-	N	N	N	N
5	**1st**	11	N	N	N	N	N	**P (1:64)**	N	P (>100)
	2nd	20	N	N			**P (1.33)**	**P (1:408)**	N	P (>100)
6	**1st**	39	N	P (1:323)	-	N	N	P (1:812)	N	P (>100)
	2nd	53	N	P (1:323)	-	-	N	P (1:646)	N	P (>100)
7	**1st**	-	N	N	N	-	N	P (1:64)	N	N
	2nd	- (10 days between 1st and 2nd sample)	N	N			N	P (1:102)	N	N
8	**1st**	20	**P (2.4)**	**N**	N	-	**P (2.35)**	**P (1:162)**	N	N
	2nd	32	**B (1.0)**	**N**	-	-	**P (1.25)**	**P (1:102)**	N	N
9	**1st**	7	N	N	N	-	N	P (1:40)	N	P (>100)
	2nd	17	N	N	-	-	N	P (1:80)	N	P (>100)
10		31	N	N	-	-	N	-	N	P (10)
11	**1st**	9	N	P (1:203)	N	-	-	-	-	-
	2nd	20	N	P (1:128)	-	-	N	-	N	N
12		17	N	N	N	-	N	P (1:162)	N	N

A: diagnostic results of pathogens commonly associated with GBS; **B:** diagnostic results of arthropod borne viruses that are possibly associated with GBS. Results on which the diagnosis was based are marked bold and in red. 1st , first sample; 2nd, second sample; VCA, viral capsid antigen; EBNA, Epstein Barr nuclear antigen; VNT, virus neutralization test; P, positive; N, negative; B, Borderline; -, not performed.

Discussion

Diagnosis and Clinical Presentation GBS

In this study, we recruited patients as soon as the treating neurologist suspected GBS. In a relatively large amount of the patients, another diagnosis than GBS was subsequently made by the treating physician and these patients were excluded from this study. This demonstrates that diagnosing GBS, especially in low-and middle-income countries with sometimes limited diagnostic facilities, can be challenging and that other conditions need to be ruled out before the diagnosis of GBS can be made. Because of the limited sample size of this study it is difficult to draw conclusions from the clinical data of these patients. In general, the diversity in clinical presentation of the described patients corresponds with what is known about GBS (1). One observation that can be made is that only five GBS patients were treated with IVIg while 11 patients were unable to walk or wheelchair bound and had an indication

for treatment (1, 3). This undertreatment is explained by the limited availability of IVIg or plasma exchange facilities in Suriname.

Preceding Infections of GBS

We performed extensive diagnostic testing for preceding infections that are commonly associated with GBS and arboviruses that are endemic in Suriname and are possibly associated with GBS. From 9 out of 12 patients we could collect paired serum samples during hospital admission. As a result, we found indications of a preceding infection in 6 of the 12 GBS patients (50.0%). Four of these six patients (66.7%)

in which we could diagnose a recent infection had evidence of a recent ZIKV or DENV infection. It is possible that this relatively high percentage of possible ZIKV associated GBS cases is because recruitment of patients for this study started during the peak of the ZIKV outbreak in Suriname at the beginning of 2016. As is indicated in Figure 1A, during this period there was a peak in GBS patients that were recruited

for this study. From the results of the serological tests in Table 2, it can be concluded that it is difficult to distinguish a recent ZIKV infected from a recent DENV infection based on serology because of cross-reactivity of anti-flavivirus antibodies. In this study we used neutralization assays for DENV-2 and ZIKV serology which is the gold standard for flavivirus serology and has shown to give less cross-reactivity than, for example, ELISA tests (45, 46, 136). Another diagnostic challenge is that asymptomatic reactivation of CMV and EBV can occur during acute infections with other pathogens which can lead to the presence of CMV or EBV IgM

antibodies (142, 146). In order to differentiate between a primary CMV or EBV infection or reactivation of these viruses, it is possible to measure the avidity of IgG antibodies against CMV, which are low after a recent infection, and to test for presence EBNA IgG antibodies against EBV which are not present after recent EBV infection (142, 143). Interestingly, in patient 5 we found IgM antibodies against DENV and a more than six-fold increase in the neutralizing antibody titer in paired serum samples taken nine days apart from each other. No IgM or neutralizing antibodies against ZIKV were detected. Based on these results and the fact that this 12-year-old patient did not recently receive a yellow fever vaccination, he was classified as having a confirmed recent DENV infection. The patient presented with pain in the backside of both legs, bilateral foot drop and difficulty walking. During physical examination, symmetrical areflexia and pain in the lower extremities was found, the Lasègue sign was positive and no neck stiffness was observed. The CSF did not contain white blood cells or elevated albumin levels and the CSF culture was negative. An EMG was not performed in this patient. DENV has previously sporadically been associated with GBS and other neurological complications (147-149). It might be worthwhile to study this

possible association in more detail with specific attention to the clinical presentation of suspected GBS or a GBS-like syndrome caused by DENV. Besides ZIKV and DENV, we also found evidence of preceding infections with pathogens that are commonly associated with GBS; CMV, *M. pneumoniae* and *C. jejuni*. In two patients (9 and 11) we found evidence of a possible recent infection with both *M. pneumoniae* and *C. jejuni*. For *C. jejuni*, IgM was only positive in the first collected sample from both patients,

this can indicate that the possible *C. jejuni* infection was not very recent. However, it has been shown that IgM antibodies against *C. jejuni* can be short lived or even absent, especially in asymptomatic infections (150). A recent *M. pneumoniae* infection was probable in patient 9 because of the presence of *M. pneumoniae* IgM antibodies and a rise in the IgG titer. In patient 11, a recent infection with *M. pneumoniae* was less likely since, even though IgM antibodies were present in the first sample, no kinetics were observed in the *M. pneumoniae* IgG titers. We did not find evidence of recent infections with HEV, EBV or CHIKV in any of these patients. In patient 1 and 6 we could not make a diagnosis of a preceding infection based on the diagnostic criteria described above. However, both patients already had a high titer of neutralizing antibodies against ZIKV early in the ZIKV outbreak (145). This could be indicative of a recent ZIKV infection since ZIKV did not circulate in Suriname before the end of 2015 and both patients reported to have had symptoms that are associated with arboviral infection such as myalgia and arthralgia. However, since it has been shown that after a recent DENV infection, cross-neutralization

of ZIKV can occur in some cases, it is also possible that these two patients had a recent DENV infection (45, 46).

Strengths and Limitations

A strength of this study is that we performed extensive and state of the art, diagnostic testing to try to identify preceding infection that might have triggered GBS. Because of this extensive testing we were able to demonstrate the challenges that arise with the interpretation of serological diagnostic results. A limitation is that the small sample size of this study and the period of recruitment of the patients (partially during the ZIKV outbreak) does not allow us to generalize the results found in this study with respect to amongst others the incidence of GBS in Suriname and the exact contribution of the different pathogens in causing GBS. Furthermore, because of insufficient clinical data, we had to exclude a relatively large amount of study participants.

Conclusion

In conclusion, we found that—apart from infections with pathogens that are commonly associated with GBS—infections with ZIKV and possibly DENV might play an important role in causing GBS in Suriname. Furthermore, more accessibility to IVIg or plasma exchange could improve the treatment of GBS in Suriname.

CHAPTER 4

Zika Virus Seroprevalence in Urban and Rural Areas of Suriname, 2017

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PART II

Zika virus epidemiology and immunity

Summary

In 2015–2016, a Zika virus (ZIKV) outbreak occurred in the Americas. In 2017, we conducted a ZIKV serosurvey in Suriname in which 770 participants were recruited from one urban area and two rural villages in the tropical rainforest. All collected samples were tested for presence of ZIKV antibodies using a ZIKV immunoglobulin G enzyme-linked immunosorbent assay and a virus neutralization assay. We found that 35.1% of the participants had neutralizing antibodies against ZIKV. In one remote village in the rainforest, 24.5% of the participants had neutralizing antibodies against ZIKV, suggesting that ZIKV was widely spread across Suriname.

Introduction

In 2015–2016, an outbreak of the arthropod-borne flavivirus, Zika virus (ZIKV), occurred in the Americas. This was the first time ZIKV was detected in this region (29, 151). Early in this outbreak, it became clear that a ZIKV infection can have severe complications such as Guillain-Barré syndrome and congenital malformations in offspring of mothers who were infected with ZIKV during pregnancy (16). In many areas, ZIKV circulation decreased rapidly after the initial wave of infections (47, 152). The quick decline of ZIKV circulation in these regions can have multiple causes, one of which is rapid development of population immunity due to a high number of infections during the peak of the outbreak. Following the first ZIKV outbreaks in other regions, ZIKV seroprevalence studies performed in Micronesia in 2009 and French Polynesia in 2014–2015 reported a seroprevalence of 73% and 49%, respectively (26, 68). However, these studies did not use a virus neutralization assay, which is of importance for flavivirus serology as recent studies have shown that, in samples from dengue virus (DENV)–endemic areas, commonly used nonstructural protein 1 (NS1)–based ZIKV antibody enzyme linked immunosorbent assays (ELISAs) can give a high amount of false-positive results due to cross-reactivity with other flavivirus antibodies (153-155). Although cross-neutralization can also be observed when using a viral neutralization assay, this assay measures functional antibodies that are capable of blocking ZIKV infection of cells, and therefore is a better measure of immunity than binding antibodies detected by most ELISAs (45). Three ZIKV seroprevalence studies using a micro–virus neutralization test (VNT) or plaque reduction neutralization test were recently performed in the Americas and reported a ZIKV seroprevalence ranging from 0% in the highlands of Bolivia to 63.3% in Salvador, Brazil (153, 155, 156). More knowledge about the level of population immunity to ZIKV is important for risk estimation of future ZIKV outbreaks in these areas and to determine whether implementation of a ZIKV vaccine would be beneficial in increasing the population

immunity rate for ZIKV. In this study, we collected samples from Surinamese inhabitants in the capital, Paramaribo, and in 2 remote villages in the tropical rainforest of Suriname to assess the spread of ZIKV in these areas.

Methods

Study Ethics

This study was approved by the national medical ethical board of Suriname. Informed consent was signed after provision of information on paper or verbally in the local language with the help of local health workers.

Study Population

Participants were recruited from 3 different locations in Suriname (Supplementary Figure 1) in January and February 2017, which was 1 year after the peak of the ZIKV outbreak in Suriname when there was almost no reported ZIKV circulation in Suriname (157). In the capital of Suriname, Paramaribo, recruitment was performed at the emergency department of the Academic Hospital Paramaribo to ensure an even distribution of the area of residence in Paramaribo as it is the only emergency department in town. All patients visiting the emergency department were asked to participate in this study except those who were marked with triage code 1 (life threatening) and those who were not living in Suriname for at least 2 years. The second location from where participants were recruited was at the healthcare center of Laduani where a Maroon community of around 1300 persons lives in the rainforest. Last, participants were recruited in Kwamalasamutu, a very remote Trio Amerindian community consisting of around 1100 persons, located in the rainforest near the Brazilian border. In both Laduani and Kwamalasamutu, 1 member of all households was asked for participation in this study to ensure an even distribution of residence area of the participants in the villages. To test the serological cross-reactivity of the different diagnostic tests used in this study, 44 serum samples from patients with fever, which were sent to the Bureau of Public Health in Suriname, were also tested. These samples, henceforth called the pre-ZIKV cohort, were collected between 2012 and the beginning of 2014, well before ZIKV was reported to circulate in Suriname (29, 151, 157).

Sample and Data Collection

Blood samples were collected from each participant via venipuncture. Participants in Paramaribo were asked to fill out a short questionnaire asking about their yellow fever virus

(YFV) vaccination status and if they had experienced any clinical symptoms of a ZIKV infection (e.g., skin rash, arthralgia, conjunctivitis, fever) in the past 2 years. Because of a language barrier, this questionnaire was not conducted in the 2 inland villages.

Serological Assays

At least 30 minutes after collection, blood samples were centrifuged at 3500 rpm for 8 minutes and serum was stored at -20°C upon shipment to the Netherlands for analysis in the World Health Organization (WHO) Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research at Erasmus Medical Centre in Rotterdam, the Netherlands. The 44 samples of the pre-ZIKV cohort were tested with a DENV-2 virus particle-based commercial DENV ELISA kit (Euroimmun) according to the manufacturer's instructions. For ZIKV immunoglobulin G (IgG) antibody detection, all of the collected samples were tested with a commercial NS1-based ZIKV IgG ELISA kit (Euroimmun) according to the manufacturer's instructions. All the collected samples were also tested with a ZIKV VNT. For the VNT, 2-fold dilutions of serum were incubated with 100 50% Tissue culture Infective Dose ($TCID_{50}$) of ZIKV Suriname strain 2016 (GenBank reference KU937936, EVAg Ref-SKU: 011V01621) and transferred to a confluent monolayer of Vero cells. After 1 hour of incubation, the serum-virus mix was removed from the Vero cells, 2% fetal calf serum containing Dulbecco's modified Eagle's medium was added and plates were incubated for 5 days at 37°C and 5% carbon dioxide. Readout of the VNT was done through cytopathogenic effect (CPE) detection via light microscopy. Samples were tested in triplicate and the geometric mean of the highest final serum dilution that completely prevented infection (i.e., no CPE) was calculated per sample and reported as reciprocal titer. For quality control, a standard positive and negative control serum was included for each VNT run. The cutoff for a positive ZIKV VNT result was set at a final serum dilution >1:32 according to the results from an internal validation process at the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research at Erasmus Medical Centre, in which sera from confirmed ZIKV infected patients were compared with sera that were seropositive for other flaviviruses such as DENV, YFV, and West Nile virus.

Statistical Analysis

Correlations between age and ZIKV VNT titers were analyzed using Spearman correlation analysis. The Mann–Whitney U test was used to compare ZIKV VNT titers between sexes and sampling locations. Pearson $\chi 2$ test was used to compare VNT seroprevalence between the 3 different sampling locations, sexes, participants with or without reported ZIKV symptoms,

and YFV-vaccinated and -unvaccinated participants. All statistical analyses were performed with IBM SPSS for Windows, version 24. A P value <.05 was considered to be a statistically significant difference.

Results

In total, the 2017 cohort consisted of 770 participants with a mean age of 44.8 years (standard deviation, 17.8 years). The pre-ZIKV cohort consisted of samples from 44 patients from Suriname that were collected between 2012 and 2014. All samples were tested with both the ZIKV IgG ELISA and ZIKV VNT (see Supplementary Table 1 for comparison of results). In the pre-ZIKV cohort, 39 samples (88.6%) tested positive for DENV IgG with ELISA. In this cohort, 23 samples (52.3%) tested positive for ZIKV IgG with ELISA, whereas none of the 44 pre-ZIKV samples tested positive with the ZIKV VNT (Table 1). In the 2017 cohort, 530 samples (68.8%) tested positive for ZIKV IgG with ELISA, whereas 270 (35.1%) samples tested positive with ZIKV VNT. The ZIKV VNT seroprevalence was comparable between Paramaribo and Laduani (38.2% vs 36.7%; P = .71), but significantly lower in the remote village Kwamalasamutu compared to Paramaribo (24.5% vs 38.2%; P = .002). ZIKV VNT titers were comparable between Paramaribo and Laduani (median titer, 20 vs 26; P = .72) but were significantly higher in Paramaribo compared to Kwamalasamutu (median titer, 20 vs 0; P <.001). All the tested ZIKV VNT titers are represented in Figure 1. There was no difference in ZIKV VNT seroprevalence between the different age groups (Table 1; P = .49). Additionally, there was no correlation between age and ZIKV VNT titer (Spearman correlation, r = 0.02; P = .52). The seroprevalence of ZIKV neutralizing antibodies did not differ between males and females (33.8% vs 36.0%; P = .51), nor did the ZIKV VNT titer (median titer, 16 vs 16; P = .77). ZIKV VNT seroprevalence between participants who reported 1 or more symptoms of ZIKV infection in the past 2 years did not differ compared to asymptomatic participants (34.6% vs 40.4%; P = .24). Last, the ZIKV VNT seroprevalence did also not differ between participants reported to be vaccinated against YFV and participants who were not YFV vaccinated or did not know if they were YFV vaccinated (41.6% vs 36.0% vs 36.1%; P = .43). Assuming that participants from the pre-ZIKV cohort were indeed ZIKV naive, as there was no ZIKV circulating in the Americas at the time of sampling, the specificity of the ZIKV IgG ELISA was 47.7% (21/44).

Table 1. ZIKV IgG ELISA and VNT results in 2017 cohort and pre-ZIKV cohort

	Participants (% of total)	Positive ZIKV IgG ELISA result, No. (% [95% CI])	Positive VNT result, No. (% [95% CI])
2017 cohort			
Total cohort	770 (100)	530 (68.8 [65.5 – 72.0])	270 (35.1 [31.8 – 38.5])
Paramaribo	424 (55.1)	290 (68.4 [63.8 – 72.6])	162 (38.2 [33.7 – 42.9])
Laduani	191 (24.8)	159 (83.2 [77.3 – 87.9])	70 (36.7 [30.1 – 43.7])
Kwamalasamutu	155 (20.1)	77 (49.7 [41.9 – 57.5])	38 (24.5 [18.4 – 31.9])
Sex			
Male	314 (40.8)	219 (69.7 [64.5 – 74.6])	106 (33.8 [28.8 – 39.2])
Female	455 (59.1)	311 (68.4 [63.9 – 72.5])	164 (36.0 [31.8 – 40.6])
Unknown	1 (0.1)	0	0
Age			
18-31	203 (26.4)	132 (65.0 [58.2 – 71.3])	74 (36.5 [30.1 – 43.3])
32-45	188 (24.4)	123 (65.4 [58.4 – 71.6])	55 (29.3 [23.2 – 36.1])
46-59	210 (27.3)	149 (71.0 [64.5 – 76.7])	71 (33.8 [27.8 – 40.5])
≥ 60	169 (21.9)	126 (74.6 [67.5 – 80.5])	70 (41.4 [34.3 – 49.0])
YFV vaccination status (Paramaribo only)			
Vaccinated	166 (39.2)	114 (68.7 [61.3 – 75.2])	69 (41.6 [34.3 – 49.2])
Not vaccinated	114 (26.9)	84 (73.7 [64.9 – 80.9])	41 (36.0 [27.7 – 45.1])
Unknown	144 (33.9)	96 (66.7 [58.6 – 73.8])	52 (36.1 [28.7 – 44.2])
Experienced ZIKV related symptoms the past two years (Paramaribo only)			
One or more symptoms reported	159 (37.5)	111 (69.8 [62.3 – 76.4])	55 (34.6 [27.6 – 42.3])
No symptoms reported	265 (62.5)	183 (69.1 [63.3 – 74.3])	107 (40.4 [34.6 – 46.4])
Pre-ZIKV cohort			
Total cohort	44 (100)	23 (52.3 [37.9 – 66.2])	0

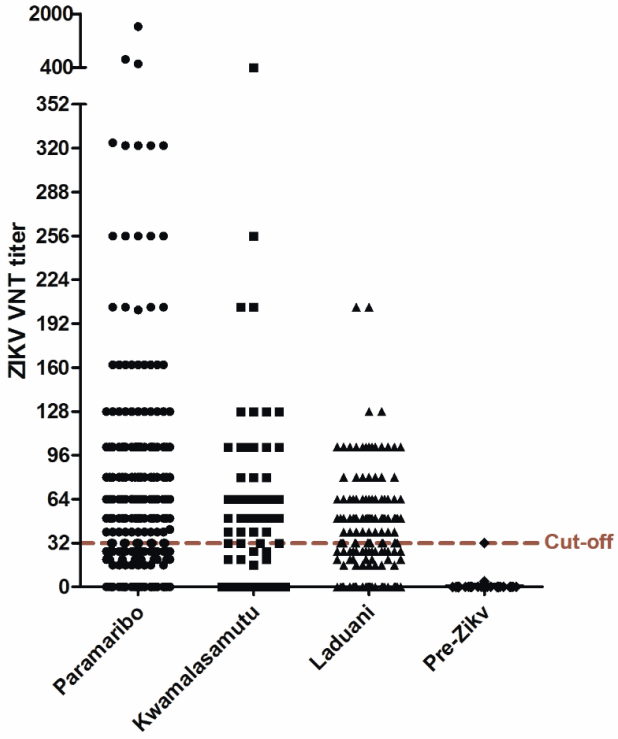

Figure 1. Distribution of all individual titers from Zika virus (ZIKV) neutralization test from the different locations of sampling. The cutoff line for a positive test is displayed at a reciprocal titer of 32; sera with titers above this cutoff were considered positive. Abbreviations: VNT, virus neutralization test; ZIKV, Zika virus.

Discussion

Our study found that approximately one-third of the recruited persons in the 2017 cohort had neutralizing antibodies against ZIKV and thus evidence for a previous ZIKV infection. These results are similar to previously performed seroprevalence studies in the Americas that demonstrated a prevalence of ZIKV neutralizing antibodies of 21.5%–33% in tropical areas in Bolivia and 42.2% in Martinique, but lower than the seroprevalence observed in Salvador, Brazil (66.2%) (153, 155, 156). Even in the very remote village Kwamalasamutu, the seroprevalence of neutralizing ZIKV antibodies was still 24.5%. This indicates that ZIKV was widely spread across Suriname, not only in urban areas but also in rural areas. The observation that two-thirds of the population was seronegative suggests that implementation of a ZIKV vaccine, when available, might be worthwhile in these regions. All of the samples of the 2017 cohort were tested with the ZIKV VNT and a ZIKV IgG ELISA; 270 samples tested positive with the ZIKV VNT while, interestingly, with ELISA, 530 samples tested positive. Due to the low specificity the ZIKV IgG ELISA (47.7%) found in this study, this higher number of positive tests with the ZIKV IgG ELISA most likely indicates false-positive results due to cross-reactivity with other flavivirus antibodies, notably against DENV. A low specificity of the ZIKV IgG ELISA used in this study was recently also reported in a study testing a pre-ZIKV cohort from Martinique that found a specificity of 62.7% (154). All of the 44 pre-ZIKV cohort samples from Suriname tested negative on the ZIKV VNT, which indicates that false-positive results due to cross-neutralization by, for example, DENV IgG, of which 88.6% of the pre-ZIKV cohort samples tested positive with DENV IgG ELISA, are not common with this test. However, it has been demonstrated that in some samples from secondary DENV–infected individuals, cross-neutralization of ZIKV can occur, mainly in samples collected during the acute and early convalescent phases of DENV infection (45, 46). False-negative results of the ZIKV VNT, on the other hand, due to waning of neutralizing antibodies below the cutoff titer that was based on samples from patients with a recent ZIKV infection, could also be possible. This would create an underestimation of the real seroprevalence of ZIKV. An indication for this is that the VNT titers of the participants in this study are relatively low and are somewhat clustered around the cutoff titer as is illustrated in Figure 1. In conclusion, the ZIKV seroprevalence found in this study is in line with previously performed ZIKV seroprevalence studies in the Americas and indicates that a significant amount of this population can still be infected with ZIKV (153-156). The results of this study can be useful for risk estimations of new ZIKV outbreaks in urban and rural areas in the Americas and for future ZIKV vaccine implementation in these regions.

Supplementary data

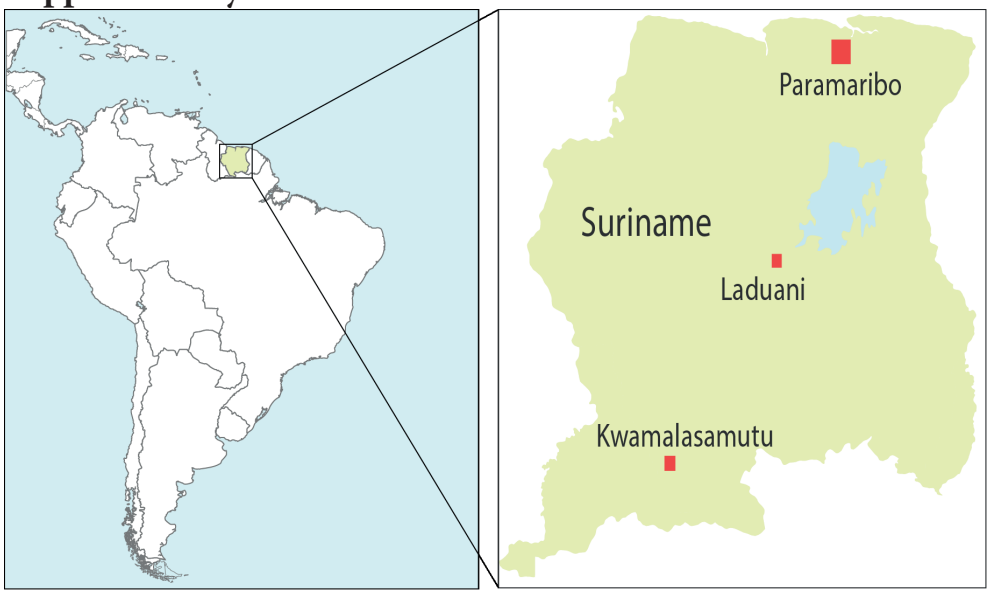

Figure S1: Schematic map of South America (left) and Suriname (right) with an indication of the three locations where participants were recruited for this study.

Table S1: Results of ZIKV IgG ELISA compared to the ZIKV neutralization test in 2017 cohort.

ELISA	ZIKV IgG ELISA POS	ZIKV IgG ELISA Equivocal	ZIKV IgG ELISA NEG	Total
Zika VNT POS	251	7	12	**270**
Zika VNT NEG	279	47	174	**500**
Total	**530**	**54**	**186**	**770**

5

CHAPTER

Zika Virus Antibody Titers Three Years after Confirmed Infection

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Abstract

Background: In 2015–2016, a large Zika virus (ZIKV) outbreak occurred in the Americas. Although the exact ZIKV antibody kinetics after infection are unknown, recent evidence indicates the rapid waning of ZIKV antibodies in humans. Therefore, we aimed to determine the levels of ZIKV antibodies more than three years after a ZIKV infection. **Methods:** We performed ZIKV virus neutralization tests (VNT) and a commercial ZIKV non-structural protein 1 (NS1) IgG ELISA in a cohort of 49 participants from Suriname who had a polymerase-chain-reaction-confirmed ZIKV infection more than three years ago. Furthermore, we determined the presence of antibodies against multiple dengue virus (DENV) antigens. **Results:** The ZIKV seroprevalence in this cohort, assessed with ZIKV VNT and ZIKV NS1 IgG ELISA, was 59.2% and 63.3%, respectively. There was, however, no correlation between these two tests. Furthermore, we did not find evidence of a potential negative influence of DENV immunity on ZIKV antibody titers. **Conclusions:** ZIKV seroprevalence, assessed with two commonly used serological tests, was lower than expected in this cohort of participants who had a confirmed previous ZIKV infection. This can have implications for future ZIKV seroprevalence studies and possibly for the duration of immunological protection after a ZIKV infection.

Introduction

Zika virus (ZIKV) is an arthropod-borne virus within the Flaviviridae family that was first identified in Uganda in 1947 and has since then circulated largely unnoticed in Africa and Asia (5, 25, 158). In 2015–2016, a large ZIKV outbreak occurred in the Americas, leading to several hundreds of thousands of confirmed ZIKV infections, although due to the high number of asymptomatic infections, estimates of the total count of infections during the outbreak are in the order of hundreds of millions (159). During this outbreak, previously unreported complications of a ZIKV infection were observed: the Guillain-Barré syndrome and congenital abnormalities in the offspring of mothers who were infected with ZIKV during pregnancy (67, 81).

Currently, little to no ZIKV circulation is reported in the countries that were affected during the 2015–2016 ZIKV outbreak. One of the reasons for this might be herd immunity; seroprevalence studies performed relatively soon after the 2015–2016 ZIKV outbreak in the Americas reported a ZIKV seroprevalence between 20–60% in the affected countries (136, 153, 155, 156). However, two recently published studies reported rapid waning of ZIKV antibodies (160, 161).

Antibody cross-reactivity between flaviviruses is a well-known problem that makes flavivirus serology extremely challenging. Virus neutralization tests (VNTs), in which neutralizing antibodies (nAbs) are detected, are considered to be the gold standard for flavivirus serology. We and others have previously shown that VNTs are well suited to distinguish dengue virus (DENV) and ZIKV nAbs and to determine that the cross-neutralization of ZIKV by DENV antibodies does not occur regularly, especially not in non-acute sera (45, 46, 136). On the contrary, we demonstrated that for the ZIKV NS1 IgG ELISA, antibody cross-reactivity between other flaviviruses and ZIKV is a significant problem (136). The cut-off for a positive ZIKV VNT result is often based on sera from patients who had a recent ZIKV infection that was compared with sera from patients who had a previous infection or vaccination with other flaviviruses such as DENV, yellow-fever virus (YFV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). However, in the case of waning ZIKV nAb titers, it could be that the ZIKV VNT is less well-suited to use as a serological test to detect ZIKV exposure several years after a ZIKV infection because of a loss of sensitivity. This potential loss of ZIKV VNT sensitivity several years after an infection can have important implications for future ZIKV seroprevalence studies because of the possible underestimation of previous ZIKV spread.

Here, we assessed the ZIKV nAb titers and antibodies against ZIKV NS1 in sera from 49 participants in Suriname who had a reverse transcriptase polymerase chain reaction (RT-PCR)-confirmed ZIKV infection more than three years ago. Furthermore, we determined nAbs against DENV-2 and binding antibodies against multiple DENV antigens in order to assess the effect of previous DENV exposure(s) on ZIKV antibody titers.

Materials and methods

Study population

People living in Suriname and aged 18 years or older who previously had a symptomatic, RT-PCR-confirmed ZIKV infection were asked by study personnel to participate in this study. Participants were asked to fill out a questionnaire about their health, YFV vaccination history and their pregnancy status during their ZIKV infection.

Ethical approval

Approval for this study was granted by the national medical ethical board of Suriname. Written informed consent was obtained from all participants. This study was performed according to the principles of the Declaration of Helsinki.

Serology

One tube of blood was collected via venipuncture for serum isolation. Serum was stored at −20 °C until shipment to the Netherlands for analysis. All serological analyses were performed at the World Health Organization Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research at Erasmus Medical Centre in Rotterdam, the Netherlands. IgG antibodies against ZIKV NS1 were assessed using a commercial ELISA kit according to the manufacturers' instructions (Euroimmun, Lubeck, Germany). The recommended cut-offs for this test are an ELISA ratio >1.1 for a positive result, an ELISA ratio between 0.8–1.1 for an equivocal result and <0.8 for a negative test result. A protein microarray was performed as previously described in detail with few exceptions, to detect IgG antibodies against several DENV and ZIKV antigens (162, 163). Slides were printed with DENV1–4 and ZIKV NS1 proteins (Sino Biological Europe GmbH and Immune Technology Corp., New York, NY) and Equad proteins (DENV envelope proteins containing four amino acid mutations in the highly conserved fusion loop domain to reduce flavivirus cross-reactivity) (164). Slides were incubated with four-fold serially diluted sera ranging from 1:20 to 1:20,480. IgG binding was detected by incubation with Alexa Fluor 647 conjugated goat anti-human IgG-Fcγ (Jackson Immunoresearch), and signals were measured using the Tecan PowerScanner at 647 nm. The median fluorescent intensity of individual spots was used to calculate the IgG titer values at which the fluorescent curve crosses 50 percent of the maximum fluorescent value. Calculations were done using R studio software. Since this assay is currently not used for routine diagnostics, a cut-off for a positive result has not yet been determined.

Virus Neutralization Tests

The presence of ZIKV- and DENV-2-specific neutralizing antibodies was determined with an in-house virus neutralization test as described before (136, 165). In short, 100 $TCID_{50}$ of dengue-2 (16681 strain (166)) or an Asian lineage ZIKV (Suriname strain 2016, GenBank reference KU937936) was incubated with two-fold serial diluted serum and transferred to a confluent monolayer of Vero cells for one hour at 37 °C and 5% CO_2. Subsequently, cells were washed three times and incubated with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for five days for ZIKV and seven days for DENV-2. Readout was performed by the detection of a cytopathic effect with a light microscope. All samples were tested in triplicate and the geometric mean of the highest final serum dilution that completely prevented the cytopathic effect was calculated for all samples. The cut-off for a positive ZIKV and DENV-2 VNT result was set at a final serum dilution above 1:32 based on an in-house validation process in which ZIKV and DENV-2 convalescent sera were compared with each

other and, for ZIKV VNT, with sera from people who had a previous infection or vaccination with other flaviviruses (YFV, TBEV, WNV and JEV).

Statistical Analyses

Statistical differences between sex, comorbidities and age within the ZIKV VNT or ZIKV NS1 IgG ELISA positive/negative groups were assessed with Pearson χ^2 test and a t-test. Differences in ZIKV and DENV-2 nAb titers were tested with the Mann–Whitney *U* test. Spearman correlation was used to test for correlations between the different serological tests used in this study. All statistical analyses were performed with IBM SPSS for Windows, version 24. A P value ≤ 0.05 was considered to be a statistically significant difference.

Results

In total, 49 participants were recruited for this study, of which 39 were females (79.6%) and 10 were males (20.4%). The average age of the participants was 41.3 years (Table 1). The average time between the initial RT-PCR confirmed symptomatic ZIKV infection and the blood collection for this study was 3.2 years (range 3.1–3.5 years). No considerable differences were found between ZIKV-seropositive and -seronegative participants regarding sex, age and pregnancy status during ZIKV infection (Table **1**). Yellow-fever-virus vaccination status did not differ between the ZIKV-seropositive and -seronegative groups. Comorbidities such as cardiovascular disease were more common in participants with a positive ZIKV VNT compared to participants with a negative ZIKV VNT (80% versus 20%, respectively, P = 0.05, Table **1**). Based on the ZIKV VNT results, the ZIKV seroprevalence in this cohort of participants who had a RT-PCR-confirmed ZIKV infection in the past was 59.2% (95% CI 44.2–73.0, Figure 1A). Based on the ZIKV NS1 IgG ELISA, ZIKV seroprevalence was 63.3% (95% CI 48.3–76.6, Figure 1A). Even though the ZIKV VNT and the NS1 IgG ELISA had comparable results regarding ZIKV seroprevalence, there was no correlation between ZIKV nAb titers and the ratios from the ZIKV NS1 IgG ELISA (r = 0.04, P = 0.80, Figure 2B). Additionally, of the 31 participants with a positive ZIKV NS1 IgG ELISA result, only 18 participants (58.1%) had a positive ZIKV VNT test result, further indicating the poor correlation between these tests. We subsequently performed VNTs for DENV-2 and found that DENV-2 seroprevalence in this cohort was 73.5% (95% CI 58,985.1). DENV-2 nAb titers were higher than ZIKV nAb titers (median titer 40 versus 64 P = 0.03, Figure 1C). Interestingly, five participants (10.2%, 95% CI 3.4–22.2) had no detectable ZIKV nAbs (Figure 1C). Antibody cross-reactivity, and to a lesser extent cross-neutralization, between ZIKV and DENV has been extensively reported (45, 136, 167). We did not find a correlation between the DENV-2

nAb titers and the ZIKV nAb titers, indicating that cross-neutralization did not seem to occur in our assay with these samples (r = −0.07, *P* = 0.63, Figure 1D). Contrary to ZIKV nAb titers, there was a moderate positive correlation between ZIKV NS1 IgG ELISA ratios and DENV-2 nAb titers (r = 0.58, *P* < 0.001, Figure 1E). This might indicate the detection of cross-reactive antibodies towards DENV using the ZIKV NS1 IgG ELISA.

Table 1. Baseline characteristics of the total cohort and Zika and dengue-2 VNT positive/negative participants.

	Total, *N* = 49	**ZIKV VNT Pos. 1 *N* = 29**	**ZIKV VNT Neg. 1 *N* = 20**	***P***	**ZIKV NS1 ELISA IgG Pos. 2 *N* = 31**	**ZIKV NS1 ELISA IgG Borderline/ Neg. 2 *N* = 18**	***P***
Sex, *n* (%)							
Female	39 (79.6)	23 (59.0)	16 (41.0)	0.95	26 (66.7)	13 (33.3)	0.32
Age, mean (range)	41.3 (25–59)	39.8 (25–59)	43.4 (31–58)	0.20	41.3 (25–59)	41.2 (28–58)	0.97
Comorbidities [3], *n* (%)							
Yes	15 (30.6)	12 (80.0)	3 (20.0)	.05	11 (73.3)	4 (26.7)	0.33
Yellow-fever-virus vaccinated, *n* (%)							
Yes	39 (79.6)	21 (53.8)	18 (46.2)		24 (61.5)	15 (38.5)	
Unknown	5 (9.8)	4 (80.0)	1 (20.0)		4 (80.0)	1 (20.0)	
No	5 (9.8)	4 (80.0)	1 (20.0)		3 (60.0)	2 (40.0)	
Pregnant during ZIKV infection, *n* (%)							
Yes	12 (24.5)	8 (66.7)	4 (33.3)		6 (50.0)	6 (50.0)	
Pregnancy with complications 4	2 (16.7)	0	2 (100)		0	2 (100)	

VNT; virus neutralization test, NS1; non-structural protein 1.
[1] Cut-off for positive VNT result: >1:32.
[2] Cut-off for ZIKV NS1 ELISA: <0.8 = negative, 0.8–1.1 = borderline, >1.1 = positive.
[3] Comorbidities include cardiovascular diseases such as hypertension and diabetes mellitus and autoimmune diseases such as rheumatoid arthritis.
[4] Pregnancy complications include spontaneous abortion, stillbirth and congenital abnormalities.

DENV and ZIKV are closely related, and it has been speculated that DENV exposure might negatively influence mounting a long-lasting ZIKV immune response upon ZIKV exposure (160). Therefore, we tried to assess the role of DENV immunity on ZIKV nAb titers by performing a protein microarray (PMA) to measure IgG antibodies against DENV1–4 Envelope protein (E) and NS1 antigens and ZIKV NS1 IgG antibodies measured by the protein microarray. ZIKV and DENV-2 nAb titers and ZIKV NS1 IgG ELISA ratios are displayed in the heatmap in Figure 2. Very high antibody titers for all DENV E and NS1 antigens were observed in sera from participants with high ZIKV nAb titers (e.g. participants 45 and 21, Figure 2A). However, we also observed high antibody titers against DENV in sera

from participants with low ZIKV nAb titers (e.g. participants 3, 18, 35, 47 and 12, Figure 2A). ZIKV NS1 IgG ELISA ratios correlated, in general, relatively well with DENV NS1 antibody titers determined with the PMA (e.g. in participants 12, 45 and 3, Figure 2B). As expected, ZIKV NS1 PMA titers strongly correlated with ZIKV NS1 IgG ELISA ratios ($r = 0.91$, $P < 0.0001$, Figure S1A). As seen for the ZIKV NS1 ELISA ratios, the ZIKV NS1 PMA titers correlated well with DENV-2 nAb titers ($r = 0.48$, $P < 0.001$, Figure S1B) but not with ZIKV nAb titers ($r = 0.01$, $P = 0.55$, Figure S1C).

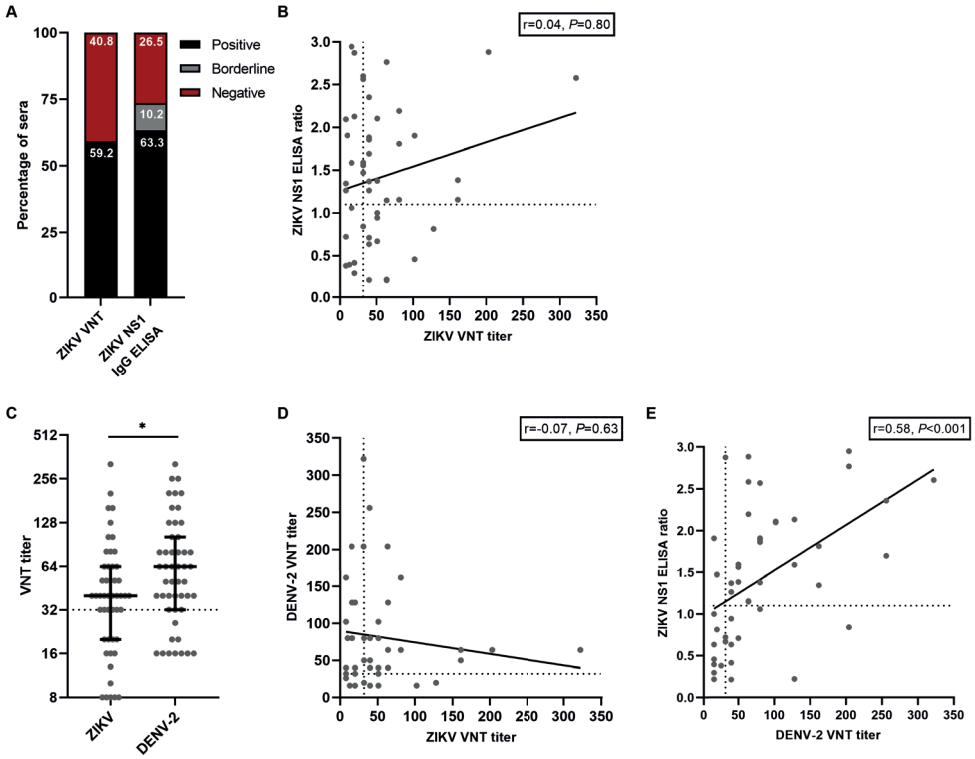


Figure 1. Results from serological assays for ZIKV and DENV antibodies.
A: Percentage of positive and negative tested sera with ZIKV VNT and ZIKV NS1 IgG ELISA. **B**: Correlation between ZIKV NS1 IgG ELISA ratios and titers from the ZIKV VNT. The dotted lines indicate cut-off values for a positive test result. **C**: ZIKV- and DENV-2 VNT titers from all participants. Lines represent median ± IQR. The dotted line indicates the cut-off value for a positive test result. Statistical differences were tested with the Mann–Whitney test. **D**: Correlation between DENV-2 VNT titers and ZIKV VNT titers. The dotted lines indicate cut-off values for a positive test result. **E**: Correlation between ZIKV NS1 IgG ELISA ratios and DENV-2 VNT titers. The dotted lines indicate cut-off values for a positive test result. * $P < 0.05$.

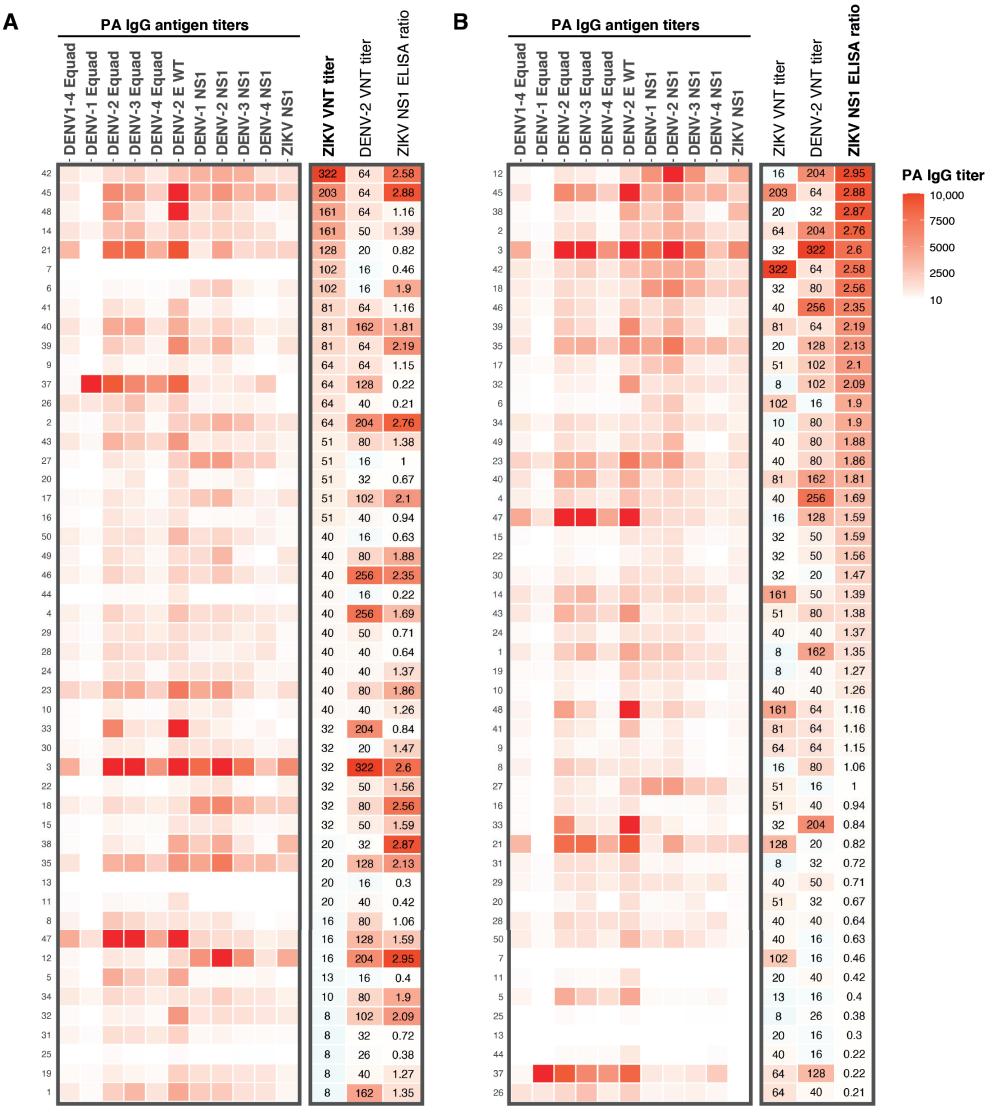


Figure 2. Heatmap of results from the different serological assays used in this study.
A: IgG antibody titers for DENV1–4 Equad and DENV1–4 and ZIKV NS1 antigens determined with a protein microarray. Corresponding ZIKV and DENV-2 VNT titers and ZIKV NS1 IgG ELISA ratios from all participants are shown on the right. Antibody patterns are ranked from highest to lowest ZIKV VNT titer. B: Protein microarray IgG antibody titer patterns for DENV1–4 Equad and DENV1–4 and ZIKV NS1, ZIKV and DENV-2 VNT titers and ZIKV NS1 IgG ELISA ratios from all participants, ranked from highest to lowest ZIKV NS1 ELISA ratio. Numbers on the left Y-axis are the study numbers of the participants in this study. PA; protein microarray, Equad; envelope proteins containing four amino acid mutations in the highly conserved fusion loop domain to reduce flavivirus cross-reactivity, VNT; virus neutralization test.

Discussion

In this study, we found a ZIKV seroprevalence of 59.2% determined by VNT and 63.3% determined by ZIKV NS1 IgG ELISA in a cohort of 49 participants who had a RT-PCR-confirmed and symptomatic ZIKV infection more than three years ago. Even though the ZIKV seroprevalence found with both tests was similar, there was no correlation between ZIKV nAb titers and ZIKV NS1 IgG ELISA ratios. This may be explained by detecting different antibody populations between these two tests, since NS1 antibodies are non-neutralizing. There was, however, a correlation between DENV-2 nAb titers and ZIKV NS1 ELISA ratios, indicating that this test might detect cross-reactive DENV NS1 IgG antibodies. The low ZIKV seroprevalence three years after infection is in line with previous studies, in which it was shown that nAbs and NS1 antibodies against ZIKV decline rapidly and indicate that ZIKV serology in flavivirus endemic populations is very challenging several years after the initial infection (160, 161). This could possibly in part explain why the reported ZIKV seroprevalence in regions in Africa and Asia, where ZIKV has already been circulating for many years, is low (168-170). One possible solution to increase the sensitivity of ZIKV serological assays in populations that did not recently suffer from a ZIKV outbreak is to set a lower cut-off for a positive test result. However, this will undoubtedly come at the cost of lower specificity due to the notorious antibody cross-reactivity between flaviviruses. Since a low degree of T cell cross-reactivity between flaviviruses has been reported, cellular assays might be useful to detect previous ZIKV infections in flavivirus endemic populations (171, 172). A limitation of this study is that its cross-sectional design makes it impossible to confirm whether the observed low ZIKV antibody titers in some participants are indeed due to waning, or if these participants never developed (high titer) antibodies against ZIKV. However, since it has been demonstrated that seroconversion after a ZIKV infection generally occurs, a lack of seroconversion is not likely to be the explanation for the low seroprevalence found in this cohort (171, 173).

DENV-2 seroprevalence and DENV-2 nAb titers were higher compared to ZIKV seroprevalence and nAb titers, possibly due to multiple previous DENV exposures. To try to understand why some participants had low or absent antibodies against ZIKV, we determined binding antibodies against different DENV antigens with a protein microarray. It is assumed that a heterotypic secondary DENV infection often leads to the reactivation of memory B- and T-cells that were induced during the original previous DENV infection(s) via a mechanism called original antigenic sin (OAS). Because of OAS, DENV pre-immunity may negatively impact the establishment of a ZIKV-specific immune response (164, 174). We indeed found that some participants with high DENV antibody titers had low ZIKV nAb titers. However, we also found high ZIKV nAb titers in sera from participants with high DENV antibody

titers. Therefore, we cannot conclude that OAS between DENV and ZIKV might play a role in establishing a long-lasting ZIKV immune response. However, there were several limitations with this analysis. Firstly, we looked at DENV antibodies three years after the participants had a ZIKV infection, which might not correlate to the situation at the time of ZIKV infection regarding (the amount of) DENV exposure. Secondly, flavivirus antibodies are notorious for cross-reactivity; this makes it very challenging to demonstrate an inverse correlation between the breadth and magnitude of ZIKV antibodies compared to DENV antibodies.

It is commonly assumed that a ZIKV infection results in long-term and possibly lifelong immunity against ZIKV; however, sufficient data to support this is currently lacking. Furthermore, for DENV serotypes 1 to 4, which are closely related to ZIKV, homotypic reinfections have been sporadically observed (172). We show that in sera from five participants (10.2%), no ZIKV neutralization was observed with the highest serum concentration tested (titer < 1:8). However, since the minimum nAb titer required for protection against (re-) infection with ZIKV remains unknown and we did not look at the presence of memory B- or T-cells in this study, we were not able to determine whether individuals with low nAbs are possibly susceptible to ZIKV reinfection. Although the questions of the duration of ZIKV immunity remain unanswered, it is plausible that the absence of circulating ZIKV nAbs results in the loss of sterilizing ZIKV immunity. The loss of sterilizing ZIKV immunity could result in transient viremia upon secondary ZIKV exposure, until the effector cells of the adaptive immune system are reactivated and clear the virus. This potential short period of viremia upon secondary ZIKV exposure in persons with low or absent ZIKV nAbs can be of special importance to pregnant women, in whom ZIKV, during viremia, can possibly cross the placenta and infect the fetus. Furthermore, if the protection against ZIKV reinfection is shorter than expected, this can have important implications for when a new ZIKV outbreak can be expected in the regions that were affected during the 2015–2016 outbreak (175).

Conclusions

In conclusion, we found that ZIKV seroprevalence was relatively low in this cohort of participants with a previous ZIKV infection. These results indicate that caution is warranted in the interpretation of ZIKV seroprevalence data several years after a ZIKV infection. In order to gain more knowledge on ZIKV antibody dynamics and duration of protection after a ZIKV infection, longitudinal studies need to be performed that, preferably, also use cellular assays to determine previous ZIKV exposure.

Supplementary figure

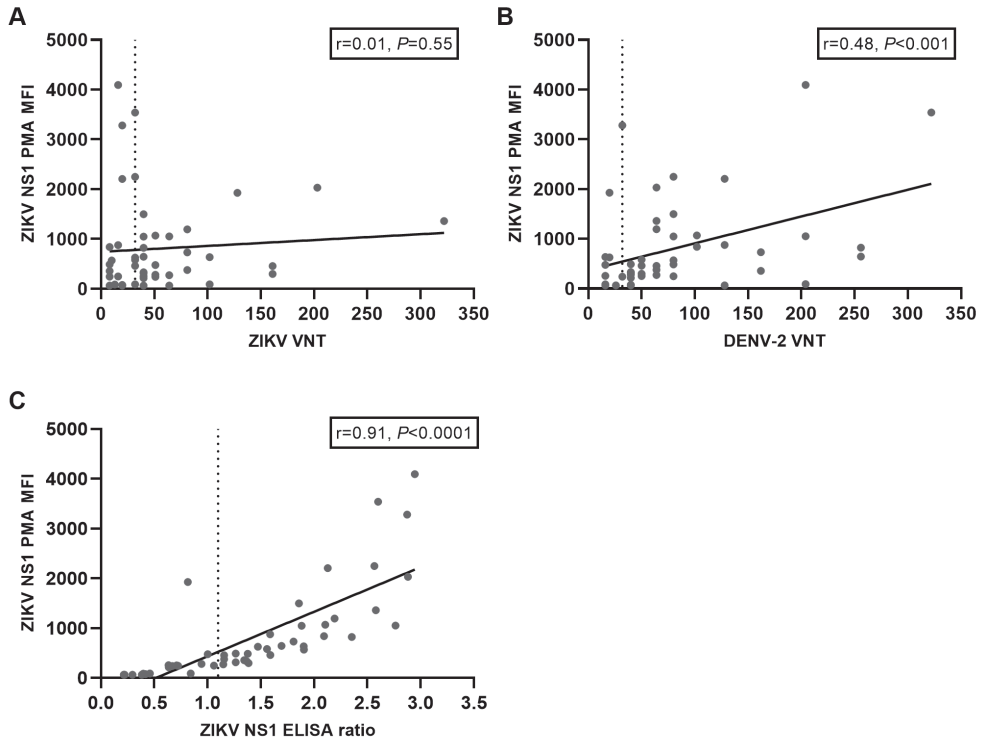
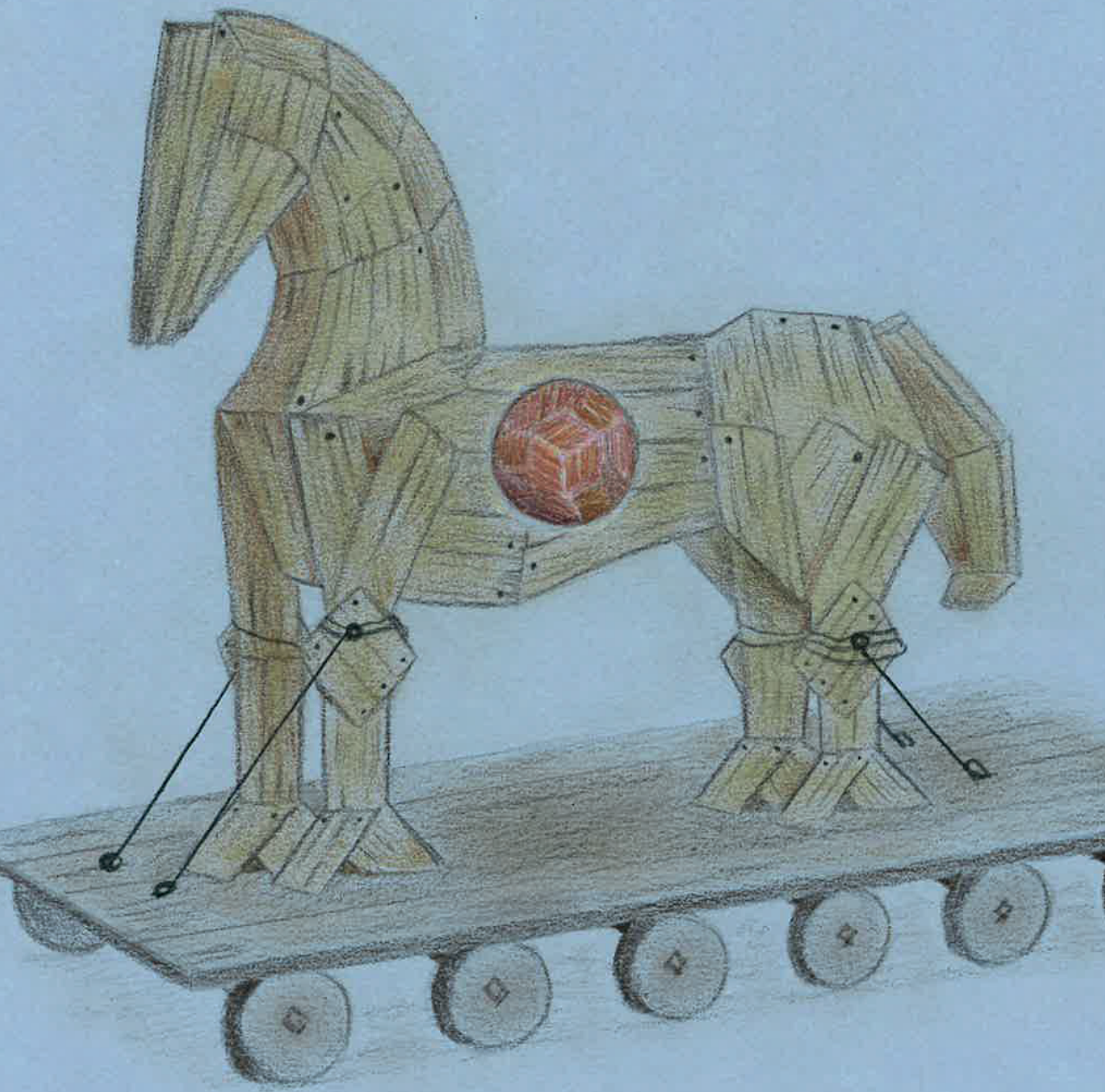


Figure S1. Correlations between ZIKV NS1 IgG titers determined with a protein microarray (PMA) compared to titers from other serological assays.
A: Correlation between ZIKV NS1 PMA IgG titers and titers from the ZIKV VNT. The dotted line indicates the cut-off value for a positive ZIKV VNT test result. **B**: Correlation between ZIKV NS1 PMA IgG titers and titers from the DENV-2 VNT. The dotted line indicates the cut-off value for a positive DENV-2 VNT test result. **C**: Correlation between ZIKV NS1 PMA IgG titers and ratios from the ZIKV NS1 IgG ELISA. The dotted line indicates the cut-off value for a positive ZIKV NS1 IgG ELISA test result.



PART III

Antibody-dependent enhancement of Zika viru

6

CHAPTER

The possible role of cross-reactive dengue virus antibodies in Zika virus pathogenesis

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Abstract

Zika virus (ZIKV) has been known for decades to circulate in Africa and Asia. However, major complications of a ZIKV infection have recently become apparent for reasons that are still not fully elucidated. One of the hypotheses for the seemingly increased pathogenicity of ZIKV is that cross-reactive dengue antibodies can enhance a ZIKV infection through the principle of antibody-dependent enhancement (ADE). Recently, ADE in ZIKV infection has been studied, but conclusive evidence for the clinical importance of this principle in a ZIKV infection is lacking. Conversely, the widespread circulation of ZIKV in dengue virus (DENV)-endemic regions raises new questions about the potential contribution of ZIKV antibodies to DENV ADE. In this review, we summarize the results of the evidence to date and elaborate on other possible detrimental effects of cross-reactive flavivirus antibodies, both for ZIKV infection and the risk of ZIKV-related congenital anomalies, DENV infection, and dengue hemorrhagic fever.

Introduction

Zika virus (ZIKV) is an arthropod-borne flavivirus in the family Flaviviridae, which includes several other arthropod-borne viruses of clinical importance, such as dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV) (176). ZIKV is a positive-sense single-stranded enveloped RNA virus. The genome encodes a polyprotein, which is processed into three structural proteins (the capsid [C], premembrane [prM], and the envelope [E] protein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (11). Until 2006, literature was limited, and no large outbreaks of ZIKV were reported (177). This changed in 2007 with the first report of a major outbreak of ZIKV on the island of Yap in Micronesia, followed by another large outbreak in French Polynesia in 2013 (26, 178). In May 2015, ZIKV infection was reported in Brazil, which was the first report of locally acquired ZIKV in South America and heralded an unprecedented outbreak across the Americas and the Caribbean. Phylogenetic studies estimate that between late 2013 and early 2014, ZIKV was introduced from the Pacific Islands into the northeast of Brazil, where it spread to other regions and countries (29, 151, 179). Several months after the start of the 2015–2016 ZIKV outbreak, unusually high numbers of Guillain-Barré syndrome (GBS) cases were observed in adults and of microcephaly cases in fetuses and newborn infants (180). For the congenital abnormalities, it became clear that microcephaly constituted the proverbial tip of the iceberg, and since then several other severe abnormalities have been associated with a congenital ZIKV infection, such as lissencephaly, ventriculomegaly, and ocular abnormalities (181-184).

Relatively early in the epidemic, the causal relationship between ZIKV and congenital abnormalities was established (9, 72). A burning question has been why these congenital abnormalities were only seen in the recent ZIKV outbreaks in the Americas. Could preexisting immunity to other flaviviruses explain this phenomenon? Recently, a considerable amount of research has been performed to investigate whether antibody-dependent enhancement (ADE) of ZIKV can explain the seemingly increased pathogenicity of ZIKV. The rationale behind this consideration is that, in the closely related DENV, ADE plays an important role in the increased risk of developing severe symptoms during secondary DENV infection (103). In this review, we discuss the evidence for ADE of ZIKV infection. Because of differences in tissue tropism of ZIKV and DENV, it should be taken into account that clinical presentations of infection enhancement by cross-reactive antibodies can differ between these viruses (17, 185). Therefore, we elaborate on a specific route by which cross-reactive dengue antibodies could have a detrimental effect in ZIKV infection, namely by facilitating vertical transmission of ZIKV from mother to fetus during pregnancy. Finally, we discuss the potential implications of cocirculation of ZIKV and DENV for the problem of DENV ADE.

The placental barrier

The most notorious complications of a ZIKV infection are the severe congenital abnormalities it can cause. In order to discuss whether and how cross-reactive dengue antibodies can play a role in these complications, it is important to understand how ZIKV can reach the fetus during pregnancy. One way for ZIKV to infect the fetus during pregnancy is through transplacental transmission. The placenta is an important protective barrier against pathogens for the fetus. The human placenta consists of many chorionic villi; the anchoring chorionic villi are attached to the mucosal lining of the uterus (decidua), whereas the floating chorionic villi float around in maternal blood in the intervillous space, where gas and nutrient exchanges take place. The chorionic villi are lined by two types of trophoblasts: an outer layer of terminally differentiated multinuclear syncytiotrophoblasts (STBs) and mononuclear cytotrophoblasts (CTBs), which are situated underneath the STB layer and can differentiate into STBs or extravillous trophoblasts (EVTs) that infiltrate the decidua in anchoring villi. The STB layer is important for protection against pathogens and has previously been demonstrated to be resistant to infection from many pathogens, including cytomegalovirus (CMV), *Toxoplasma gondii* (*T. gondii*), and *Listeria monocytogenes* (95, 186, 187). CTBs and EVTs, on the other hand, are susceptible to some pathogens, including *T. gondii* and CMV (187, 188). To enter the villus core and reach the fetal circulation, pathogens either have to cross the STB layer in floating villi or infect EVTs in anchoring villi. Recently, how and when ZIKV can cross the placenta have been investigated in experimental studies and clinical observations.

First-trimester placentas seem most permissive for ZIKV

Analysis of placentas of women with a suspected ZIKV infection showed that the relative level of ZIKV RNA was 25-fold higher in first-trimester placentas compared with second- and third-trimester placentas (86). In placentas of women infected with ZIKV, using in situ hybridization (ISH), ZIKV was consistently identified in only the Hofbauer cells (HBCs), which are the placental macrophages that are located in the chorionic villus core, and not in CTBs or STBs (86, 98). In addition, multiple in vitro studies that were performed with primary placental cells isolated from early- and late-pregnancy placental explants found that ZIKV replicates in CTBs isolated from first-trimester placenta explants (189-192), whereas in CTBs isolated from term placenta explants, only low replication of ZIKV was observed (99, 191). It was also demonstrated that STBs obtained from term placentas were resistant to ZIKV, possibly because of the production of type III interferons (96). These observations suggest that the placenta is more susceptible to ZIKV infection during the first trimester of pregnancy than during the second and third trimesters of pregnancy. In contrast to the differential sensitivity of CTBs and STBs from placentas in different stages of pregnancy, many of the above-mentioned studies found similar levels of replication of ZIKV in HBCs isolated from both early- and full-term placentas, suggesting that these cells can possibly serve as a replication reservoir for ZIKV once the virus has entered the chorionic villus core.

Results from cohort studies

In contrast to experimental studies that indicate (partial) resistance to ZIKV of the second- and third-trimester placentas, the results of clinical cohort studies show that ZIKV-associated congenital abnormalities also occur in infants from mothers who had a ZIKV infection in the second or third trimester of pregnancy, albeit less frequently (181, 193, 194). Preliminary data from the United States Zika Pregnancy Registry demonstrated that 8% of the infants from mothers who had laboratory confirmed ZIKV infection during the first trimester of pregnancy had birth abnormalities, with 5% and 4% in the second and third trimesters, respectively (194). A case-control study from Rio de Janeiro in 2016 reported that 55% of infants from mothers who were ZIKV PCR positive during the first trimester of pregnancy had birth abnormalities, compared with 52% and 29% during second and third trimesters (181). Finally, a cohort study performed in French territories in the Americas found that ZIKV-related congenital abnormalities were present in 12.7% of the infants of women who had a PCR-confirmed, symptomatic ZIKV infection during the first trimester of pregnancy, whereas this was 3.6% and 5.3%, respectively, for the second and third trimesters (193).

In conclusion, there seems to be a discrepancy between the results from experimental research, which indicates that ZIKV cannot efficiently replicate in the protective trophoblasts of the term placenta, and data from clinical cohort studies, which demonstrate that the risk of congenital abnormalities is still significant when a ZIKV infection occurs in the third trimester of pregnancy. One explanation for this discrepancy could be the presence of a cofactor that enhances the ability to infect placental cells—for instance, the presence of cross-reactive dengue antibodies. This is a factor that is not accounted for in experimental research but that is present in a large part of the population in the clinical cohort studies from the Americas. In the next paragraphs, we will discuss how dengue antibodies can potentially exert a detrimental effect on infections, either via "traditional" ADE or through different mechanisms that make these antibodies a potential risk factor for ZIKV congenital abnormalities.

ADE

Flavivirus antibodies pose a challenge for serological diagnostic tests, as they often bind not only to the virus a person was infected with but also to related flaviviruses. The presence of cross-reactive antibodies can also have a disease-enhancing effect via the principle of ADE. ADE of a flavivirus infection was first described in the 1960s, when it was observed that severe DENV infection occurred mainly during secondary infections and in infants that had subneutralizing levels of maternal antibodies, i.e., below the level needed to protect against a primary DENV infection (195). It was hypothesized that antibodies resulting from infection with one DENV serotype might enhance disease in a subsequent infection with a different DENV serotype by a process called ADE (196). According to the ADE hypothesis, antibodies produced during primary DENV infection can bind to a different DENV serotype but cannot neutralize it. These cross-reactive antibodies can facilitate the entry of the nonneutralized virus–antibody complexes (immune complexes), mainly via fragment crystallizable (Fc) gamma receptors (FcγRs), into the mononuclear phagocytic cells (MPCs). Antibody-mediated entry of virus in MPCs may result in either more infected cells (extrinsic ADE) or a more skewed T helper 2 (Th2) response (intrinsic ADE) (112, 197). Infected MPCs may then serve as a reservoir to facilitate the viruses to reach different tissues in the body, resulting in more widespread infection, increased number of viral progeny, and worsening of disease (196, 198). Different epidemiological studies have provided evidence that the incidence of severe DENV disease is higher among first-time-infected infants born to DENV-immune mothers and children who had developed a mild or asymptomatic dengue infection and became secondarily infected by a different DENV serotype (195, 199).

To test the hypothesis of ADE for DENV infections, a significant amount of experimental research has been performed (200-206). Most in vitro studies have repeatedly reported FcγR-mediated enhancement of DENV infection in the presence of subneutralizing concentrations of cross-reacting antibodies against different DENV serotypes (200, 201, 206). Mouse and nonhuman primate (NHP) models provided evidence for the potential clinical relevance of ADE for DENV. Mainly in mouse models, these studies showed increased viral loads and poor disease outcome during secondary DENV infection (201-205, 207).

Recently, results from a longitudinal cohort study with more than 6,000 children in Nicaragua confirmed that preexisting DENV antibodies were directly associated with disease severity in a dose-dependent manner (103). Furthermore, in a follow-up study from phase III clinical trials for the live-attenuated tetravalent dengue vaccine Dengvaxia (CYD-TDV), it was observed that three years after the administration of this vaccine, the risk of hospitalization for DENV was increased in children younger than nine years of age (104, 208). One of the hypotheses for this observation is that vaccine-related ADE of a subsequent DENV infection causes the increase in hospitalization for DENV in the vaccinated group of children younger than nine years old (209-211). These observations provide evidence that preexisting nonneutralizing, binding DENV antibodies are an important risk factor for the occurrence of severe DENV disease. As DENV and ZIKV are closely related, the observations to date raise concerns about the impact of preexisting flavivirus immunity in determining the disease outcome for closely related and cocirculating flaviviruses, such as ZIKV.

Evidence required for ADE of ZIKV

Soon after the 2015–2016 ZIKV outbreak in the Americas, research was initiated to investigate whether ADE of a ZIKV infection could occur in the presence of flavivirus-reactive antibodies (notably, antibodies to DENV) and whether this could explain the seemingly increased pathogenicity of ZIKV, as proposed in Fig. 1. Most of the results published so far are derived from experimental studies performed in myeloid cell lines or in animal models in which variables such as mortality, viremia, and proinflammatory cytokines are compared between flavivirus-preimmune and -naïve animals upon infection with ZIKV. As stated by Scott Halstead, one of the scientists who first described the ADE hypothesis, evidence that a microbial disease is worsened by ADE should not only come from experimental research, in which in situ replication of the causative organism in myeloid cells is demonstrated, but also come from epidemiological studies, as observations from animal experiments cannot always be extrapolated to the effects observed in infections in humans (212). In the next paragraphs, an overview of the results from epidemiological and experimental research that studied ADE in ZIKV will be given.

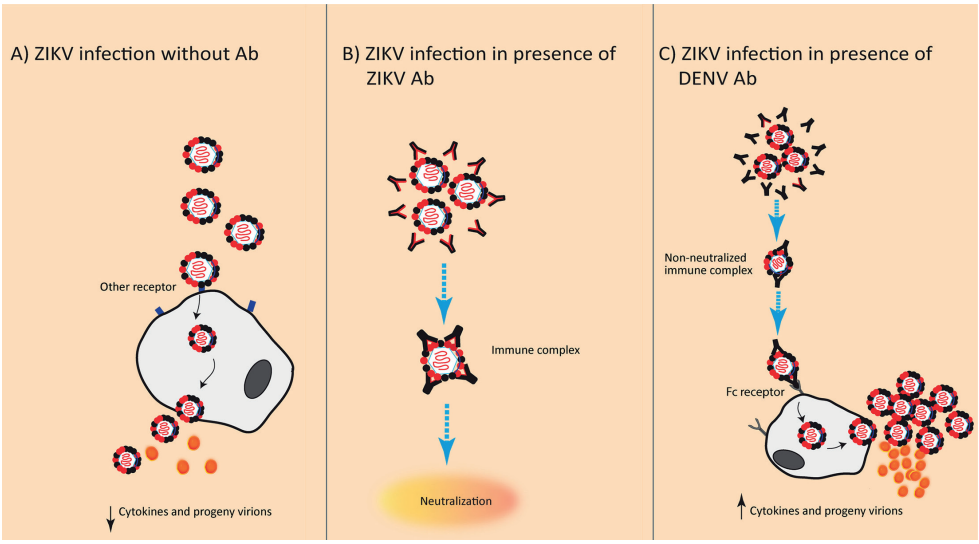

Figure 1. Proposed mechanism of ADE of ZIKV infection mediated by cross-reactive anti-DENV antibodies.
A: Primary ZIKV infection in naïve individuals. Entry occurs via other receptors and leads to virus and cytokine production. **B:** Secondary ZIKV infection in a ZIKV-preimmune individual. Neutralization occurs effectively. **C:** ZIKV ADE (black antibodies; preexisting antibodies against primary infecting DENV) Abs in immune sera can cross-react with ZIKV, allowing entry of the virus–antibody complexes into MPCs via the Fc receptor, leading to higher viral load along with higher levels of pro- and/or anti-inflammatory cytokines than cells infected in absence of antibodies.
Ab, antibody; ADE, antibody-dependent enhancement; DENV, dengue virus; Fc, fragment crystallizable; MPC, mononuclear phagocytic cell; ZIKV, Zika virus.

Need for epidemiological studies

Epidemiological studies investigating the occurrence of ADE of ZIKV infection are scarce, and epidemiological evidence for the traditional signs of ADE, such as an increased viral load or aberrant immune response leading to more severe disease, is currently lacking for ZIKV. Two epidemiological studies have determined the clinical outcomes of ZIKV infection in DENV-naïve and -preimmune patients (213, 214). One of the studies did not find significant differences in cytokine profiles and ZIKV viremia in DENV-naïve and -preimmune patients (214). Likewise, the other study also did not report any association between abnormal birth outcomes and preexisting DENV antibodies (213). However, both of these studies had a small sample size and, therefore, had a low power for detecting differences in viral loads, cytokines, disease severity, and birth outcomes between the groups. For comparison, the recent publication providing convincing evidence for DENV ADE at the population level was based on a cohort of more than 6,000 individuals (103).

Experimental studies: Contrary findings from in vitro and in vivo studies

In addition to epidemiological studies, several experimental studies using either preimmune sera/plasma or monoclonal antibodies (mAbs) have been conducted to investigate the enhancing role of flavivirus cross-reactive antibodies in ZIKV infection (Table 1). In different in vitro studies, human DENV-immune plasma and/or a panel of DENV-specific human mAbs were used to determine the cross-reactivity as well as neutralizing and infection-enhancing properties of these antibodies against ZIKV infection (114, 215-218). Similar to the results of in vitro studies with DENV, enhanced ZIKV titers were detected in the presence of both DENV-preimmune sera and DENV-specific human mAbs by using FcγR-bearing human monocytic cell lines. However, unlike in vitro studies, there are contrary findings in in vivo studies about the role of preexisting DENV antibodies in facilitating enhanced ZIKV pathogenesis (219-224). In most of the studies conducted so far, enhanced ZIKV pathology due to preexisting DENV antibodies via ADE has not been observed, with only one exception (219). Bardina and colleagues have reported an in vivo enhancement of ZIKV infection in mice, with increased morbidity and mortality in the presence of DENV and WNV human immune plasma (219). This study also suggested that preexisting ZIKV cross-reacting antibodies can either be protective or can enhance pathogenesis depending on the concentrations of these antibodies, in line with the observations from DENV research (219). A recent in vivo study using NHPs described that a ZIKV infection, 2.8 years post-DENV infection, did not produce any sign of ADE because of insignificant differences of viremia duration between ZIKV-infected naïve and DENV-preimmune NHPs (222), which is not in contrast with previous in vivo findings from DENV studies. The lack of clinical confirmation of the in vitro ADE results can be explained by multiple factors, such as the in vivo model used or the strain or serotype of primary infecting DENV and secondary infecting ZIKV. Another issue that could explain this is the different characteristics of antibodies binding to FcγR between humans and mice—i.e., distribution of immunoglobulin G (IgG) subclasses—and binding affinities of Fc to FcγR (225). Some studies suggest that binding affinities of human IgG–Fc to mouse FcγR are lower than to human FcγR, which would make the translation of ADE results obtained in the mouse model with human serum difficult and uncertain (110). However, more recent studies suggest that human IgG binds to mouse FcγR with similar affinities as to human FcγR (226). Additionally, the duration between primary versus secondary infections, dose and route of infection, and titers and biological properties of cross-reactive IgG antibodies (such as IgG subclasses and, presumably, Fc glycosylation of these antibodies) can influence the outcome of ADE studies. Unlike for DENV, the infection of MPCs by ZIKV-immune complexes has not been evaluated in vivo. Therefore, there is a need to determine whether the cell tropism of

ZIKV infections differs between DENV-naïve and -preimmune individuals and to assess the potential for disease enhancement through properly powered epidemiological studies.

Table 1. Overview of in vitro and in vivo studies investigating ADE in ZIKV infections.

Study	Pre-immune sera/plasma mAbs		*In vitro*		*In vivo*	
			Cell line	ADE	model	ADE
Dejnirattisai et. al (114)	DENV plasma	mAbs	U937	+		
Swanstrom et. al (223)		mAbs	U937	-	*IFNAR–/–*C57BL/6 mice	-
Paul et. al (227)	DENV sera	mAbs	K562	+		
Priyamvada et. al (217)	DENV sera	mAbs	U937	+		
Stettler et. al (224)	DENV	mAbs	K562	+	*AG129* mice	-
Charles et. al (216)		mAbs	THP1	+		
Bardina et. al (219)	DENV & WNV plasma		K562	+	*Stat2–/–* C57BL/6 mice	+
Casthana et. al (215)	DENV serum	mAbs	K562	+		
Slon Campos et. al (218)	DENV vaccinated sera		K562	+		
Kam et. al (220)		mAb	K562	-	*IFNAR–/–* mice	-
Pantoja et. al (222)	DENV sera		K562	+	Macaque	-
Duehr et. al (228)	TBEV sera		K562	+	*Stat2–/–* mice	-
McCracken et. al (221)	DENV, YFV sera		U937, K562	+	Macaque	-

ADE, antibody-dependent enhancement; AG129 mice, type I and II interferon receptor–lacking mice; DENV, dengue virus; IFNAR–/–, type I interferon receptor–lacking mice; mAb, monoclonal antibody; Stat2–/–, signal transducer and activator of transcription knockout mice; TBEV, tick-borne encephalitis virus; YFV, yellow fever virus.

Enhancement of DENV by ZIKV antibodies

Whereas most studies have focused on investigating the possibility of ZIKV ADE by DENV antibodies, ADE of DENV by preexisting ZIKV antibodies could be more clinically relevant. This is because of severe disease complications that are associated with DENV ADE, such as dengue hemorrhagic fever, dengue shock syndrome, and possibly also a worsened maternal and perinatal outcome when occurring during pregnancy (103, 229-233). Two in vivo studies demonstrated more severe disease symptoms and mortality in DENV-infected mice that were pretreated with a ZIKV mAb or that had maternally acquired ZIKV antibodies compared with mice without ZIKV antibodies (Fig. 2) (224, 234). In a study with rhesus macaques, it was observed that the macaques that were previously infected with ZIKV had a significantly higher DENV viral load and proinflammatory cytokine production upon DENV-2 infection compared with ZIKV-naïve macaques (235). However, no signs of dengue hemorrhagic fever were observed in these macaques; thus, only ADE of infection was observed, without changes in disease severity (235). Overall, these studies indicate that prior ZIKV exposure might be

a risk factor for DENV ADE. On the other hand, observations from arbovirus surveillance in Brazil suggest a decrease in DENV circulation after the ZIKV outbreak, possibly due to DENV crossneutralization by ZIKV antibodies (236). Additionally, there are indications that these crossneutralizing ZIKV antibodies can prevent DENV ADE (237, 238). However, for DENV, it is demonstrated that the risk of severe disease depends on the titer of preexisting DENV antibodies (103). Therefore, it is plausible that cross-neutralizing ZIKV antibodies can prevent DENV ADE, whereas cross-reactive, binding ZIKV antibodies can enhance DENV infection, stressing the importance of measuring the balance between neutralizing and nonneutralizing antibodies in studies on pathogenesis (237). The possibility of DENV ADE by ZIKV antibodies is especially of importance in DENV-naïve persons who live in DENV-endemic areas and who have had a previous ZIKV infection. Furthermore, the possibility of ZIKV vaccine–induced ADE of a DENV infection should be taken into account for the evaluation of a future ZIKV vaccine.

The role of cross-reactive antibodies in ZIKV-associated congenital abnormalities

The literature discussed thus far has focused on addressing the possibility of enhancement of ZIKV disease in an infected person with prior DENV exposure. However, an important question is whether—rather than the "DENV" mechanism of ADE, which focuses on cytokine production, viral load, or mortality—the clinical presentation of ZIKV infection enhancement by cross-reactive antibodies might be missed because ZIKV has a broader tissue tropism than DENV and can be detected in, among others, the placenta, the reproductive tract, the eyes, and brain tissue (17, 185). Even though there are no reports of worsened ZIKV disease in individuals with prior DENV exposure, is it possible that cross-reactive flavivirus antibodies can still be a risk factor for the ZIKV-associated congenital anomalies?

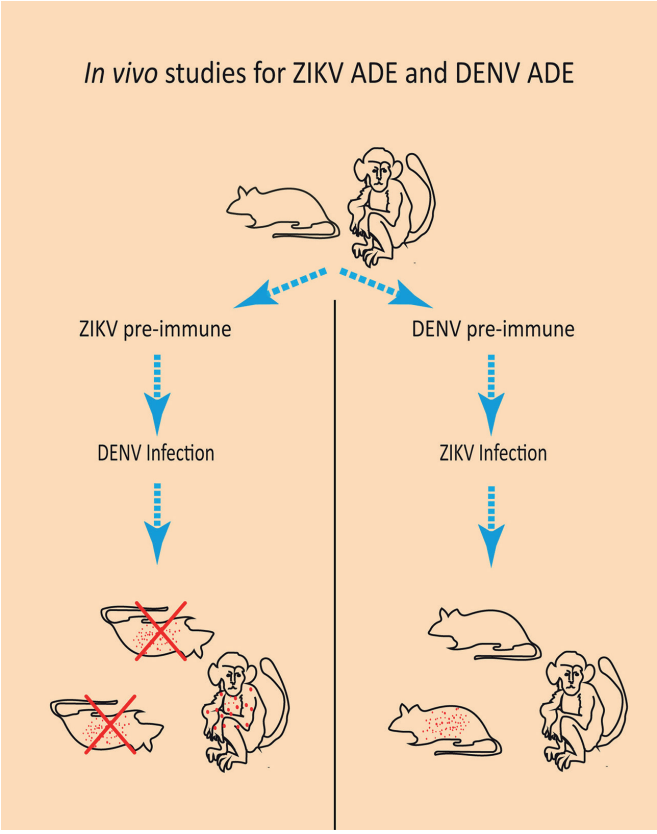

Figure 2. Results from in vivo studies investigating the role of ZIKV antibodies in DENV infection and on DENV antibodies in ZIKV infection**.**
Left panel: In mice that had ZIKV antibodies either maternally acquired or administered before DENV infection, increased DENV viral load, cytokine production, and mortality was observed. In macaques that were previously infected with ZIKV, an increased DENV viral load but no clinical symptoms or mortality was observed upon infection with DENV. Right panel: In some DENV-preimmune mice that were infected with ZIKV, an increased viral load and cytokine production but not mortality was observed. In DENV-preimmune macaques infected with ZIKV, no changes in viral load, cytokine production, or mortality were observed in DENV.
ADE, antibody-dependent enhancement; DENV, dengue virus; ZIKV, Zika virus.

Neonatal Fc receptor–mediated transcytosis across the placenta

From weeks 20–24 of pregnancy, when the placenta is fully developed, maternal IgG antibodies are actively transported across the placenta from mother to fetus through neonatal Fc receptor (FcRn)-mediated transcytosis in STBs (239-241). STBs internalize fluid containing maternal IgG at the apical surface; the Fc region of IgG can subsequently bind the FcRn in acidic endosomes, after which IgG is released at the basolateral surface at a neutral pH (242). The hypothesis that transcytosis of IgG–virion complexes across the placenta can

occur has been confirmed in in vitro studies that demonstrated that IgG–virion complexes of human immunodeficiency virus (HIV) and CMV can be transcytosed across FcRn-bearing epithelial cells and that this process can be inhibited or completely blocked when the FcRn is blocked or knocked down (188, 243). In an ex vivo study using placental explants, it was demonstrated that CMV could be transcytosed across the STB layer in the presence of both high and low neutralizing antibodies (188). However, in the presence of high neutralizing antibodies, CMV virions were captured by villus core macrophages and were unable to replicate, whereas in the presence of low neutralizing antibodies, viral replication was detected in CTB progenitors beneath an intact and uninfected STB layer (188). If this FcRn-mediated transcytosis is possible for ZIKV, Zika virions bound to maternal nonneutralizing, cross-reactive flavivirus antibodies could still be infective when released at the fetal side of the chorionic villus, similar to what has been found for CMV–IgG complexes (Fig. 3). Once in the chorionic villus, ZIKV will encounter, among others, CTBs and HBCs. Because ZIKV can readily replicate in the perivascular-located HBCs, ZIKV could disseminate from HBCs to the fetal capillaries and enter the fetal circulation (99, 191, 244, 245). A recent experimental study found indications that ZIKV can cross the trophoblast layer of the placenta through FcRn-mediated transcytosis. In this study, second-trimester placental explants were used to demonstrate that ZIKV infection of these explants was higher when ZIKV was preincubated with cross-reactive DENV mAbs, mainly IgG1 and IgG3 subclasses, compared with nonspecific influenza mAbs (246). Blocking of the FcRn with an FcRn-specific mAb inhibited ZIKV replication by 16.5-fold (246). The finding that ZIKV can infect placental explants more efficiently in the presence of DENV antibodies was confirmed by another recent study (247). In this study, there was no enhancement of infection observed in the placental explants when ZIKV was preincubated with sera containing YFV or chikungunya virus antibodies, but in the presence of DENV antibodies, there was faster ZIKV replication and more virus production compared with the absence of DENV antibodies (247). Furthermore, the clinical observation that, in several placentas of ZIKV infected women, ZIKV is only detected in HBCs and not in the trophoblasts lining the chorionic villi is another indication that transplacental FcRn-mediated transcytosis of ZIKV can occur in ZIKV-infected pregnant women (86, 98).

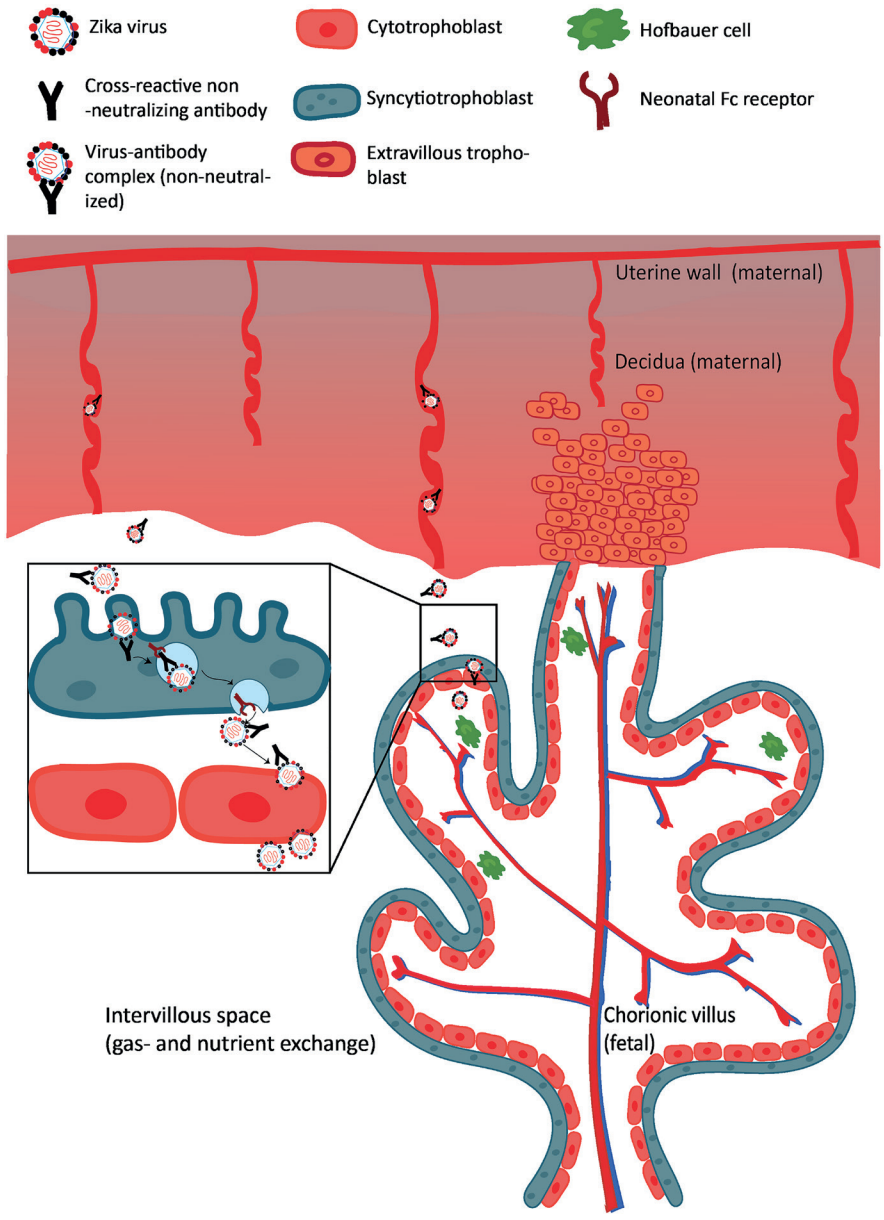

Figure 3. Proposed mechanism of FcRn-mediated transcytosis of a ZIKV–IgG complex in a chorionic villus. Illustrated is a chorionic villus that is anchored to the mucosal lining of the uterus (decidua). Through the circulation of the mother, ZIKV bound to maternal cross-reactive flavivirus IgG antibodies is present in the intervillous space. This IgG–virion complex can subsequently cross the syncytiotrophoblasts via FcRn-mediated transcytosis. When ZIKV is transcytosed across this trophoblast layer, it can infect the perivascular-located Hofbauer cells, after which viral progeny can cross the endothelial cell barrier, possibly with help from ZIKV NS1 protein, and reach the fetal circulation.
FcRn, neonatal fragment crystallizable receptor; IgG, immunoglobulin G; NS1, nonstructural protein 1; ZIKV, Zika virus.

Conclusion

The hypothesis that antibodies produced during a primary DENV infection may cause severe secondary DENV infection (through ADE) has been controversial for a long time. To date, the theory of ADE in DENV infection is more broadly accepted, mainly because a large epidemiological study provided clear evidence for enhanced risk of DENV complications in children with a specific range of preexisting antibodies. Studies performed to determine ZIKV ADE so far have found evidence for ADE in vitro, but compelling evidence in vivo is lacking, whereas ADE of a DENV infection in the presence of cross-reactive ZIKV antibodies is observed in several in vivo studies. Based on the current literature, there is not enough evidence to confirm or disprove definitively that the ADE observed in vitro plays an important role in ZIKV pathogenicity. It is unlikely that ADE of a ZIKV infection in humans would result in the same disease complications as seen in DENV, as current studies have not found any indications of this effect. However, there is a less-well-researched possibility that cross-reactive flavivirus antibodies can cause other detrimental effects in ZIKV infection, possibly by facilitating transplacental transmission through FcRn-mediated transcytosis. Currently, properly designed clinical studies that find strong associations of cross-reactive flavivirus antibodies and congenital syndrome are missing. Therefore, large longitudinal cohort studies with pregnant women in flavivirus-endemic areas are needed to assess the potential role of cross-reactive flavivirus antibodies in pathogenesis of fetal infection and disease when a ZIKV infection occurs during pregnancy. For these studies, serological discrimination of cross-reactive flavivirus antibodies will be crucial. Fundamental knowledge of the pathogenesis of this severe illness remains important, particularly in light of potential consequences for flavivirus vaccination.

7

CHAPTER

Comparative analysis of *in vitro* models to study antibody-dependent enhancement of Zika virus infection

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Abstract

During the 2015-2016 outbreak of Zika virus (ZIKV) in the Americas, a previously unknown severe complication of ZIKV infection during pregnancy resulting in birth defects was reported. Since the ZIKV outbreak occurred in regions that were highly endemic for the related dengue virus (DENV), it was speculated that antibody-dependent enhancement (ADE) of a ZIKV infection, caused by the presence of cross-reactive DENV antibodies, could contribute to ZIKV disease severity. Emerging evidence indicates that while *in vitro* models can show ADE of ZIKV infection, ADE does not seem to contribute to congenital ZIKV disease severity in humans. However, the role of ADE of ZIKV infection during pregnancy and in vertical ZIKV transmission is not well studied. In this study, we hypothesized that pregnancy may affect the ability of myeloid cells to become infected with ZIKV, potentially through ADE.

We first systematically assessed which cell lines and primary cells can be used to study ZIKV ADE *in vitro*, and we compared the difference in outcomes of (ADE) infection experiments between these cells. Subsequently, we tested the hypothesis that pregnancy may affect the ability of myeloid cells to become infected through ADE, by performing ZIKV ADE assays with primary cells isolated from blood of pregnant women from different trimesters and from age-matched non-pregnant women.

We found that ADE of ZIKV infection can be induced in myeloid cell lines U937, THP-1 and K562 as well as in monocyte derived macrophages from healthy donors. There was no difference in permissiveness for ZIKV infection or ADE potential of ZIKV infection in primary cells of pregnant women compared to non-pregnant women.

The results from these *in vitro* ZIKV ADE assays indicate that pregnancy does not alter the permissiveness of myeloid cells for ZIKV infection or ADE of ZIKV infection.

Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus that can infect humans, often resulting in an asymptomatic or self-limiting mild infection. However, a ZIKV infection during pregnancy can result in severe congenital birth defects such as microcephaly, arthrogryposis, and hypertonia (9, 72). Furthermore, ZIKV infections can sporadically trigger Guillain-Barré syndrome (66, 67, 165). ZIKV is closely related to dengue virus (DENV) which also is a mosquito-borne flavivirus that can cause dengue hemorrhagic syndrome and dengue shock syndrome (248). The severe presentation of a DENV infection occurs more often during a secondary DENV infection with a different serotype due to antibody dependent enhancement (ADE) (102, 103, 249). ADE is a paradoxical phenomenon in which antibodies, that normally act against pathogens and aid the immune response, can actually worsen an infection (101). In the case of DENV, antibodies against one serotype of DENV that can cross-react with, but not cross-neutralize, another DENV serotype can help the virus to enter phagocytic cells through Fcγ-receptor (FcγR) mediated uptake, after which the virus can replicate in these cells. This results in an increased number of infected cells (extrinsic ADE) and can also lead to increased virus production per infected cell due to a shift in immune response towards a more pro-viral state (intrinsic ADE) (111, 112, 250). Antigenic relatedness between DENV and ZIKV results in antibody cross-reactivity between these viruses (217, 251). Because of antibody cross-reactivity and the occurrence of the 2015-2016 outbreak of ZIKV in DENV (hyper)endemic regions, ADE of ZIKV infection was suggested as a possible mechanism behind the increased observations of ZIKV related congenital complications during this outbreak (252). While there are indications from animal studies that ADE of ZIKV infection can increase the risk of transplacental ZIKV transmission, this has not been studied in detail for human pregnancies (253, 254).

Multiple experimental studies investigated whether cross reactive DENV antibodies can cause ADE of ZIKV infection in FcγR bearing cells and in animal models (255). Although *in vitro* ADE of ZIKV infection has been observed in many of these studies, this has not been observed in several animal models and in clinical studies (115, 214, 255). Therefore, it seems that the result from *in vitro* ADE studies poorly predict the occurrence of *in vivo* ADE of disease. Studying ADE in humans is challenging as it requires sufficiently powered standardized prospective cohorts which is difficult for an infection that is mostly associated with asymptomatic- or mild disease. Furthermore, the methodological differences in the *in vitro* ADE studies make it difficult to compare the results between these studies and the conflicting results might be in part attributed to the lack of standardization of *in vitro* ZIKV ADE assays.

Therefore, to develop a standardized *in vitro* model to study ADE of ZIKV infection, we compare different cell lines and primary cell types and read-outs of *in vitro* ZIKV ADE assays to assess permissiveness for- and immunological response to ZIKV infection with and without presence of cross-reactive antibodies.

Because the most severe complications of a ZIKV infection can occur during pregnancy, and the surface FcγR expression on myeloid cells is progressively upregulated during pregnancy, we then tested if there is a difference permissiveness for ADE of ZIKV infection in myeloid cells from pregnant women compared to versus non-pregnant women (256).

The results of this study can ultimately provide a comparative understanding of various *in vitro* models employed to study ADE of ZIKV infection and gain insight into the risks of ZIKV infections during pregnancy in women who were previously exposed to other flaviviruses.

Methods

Human subjects

To assess the permissiveness of primary myeloid cells for ZIKV infection and ADE of ZIKV infection, buffy coats from three healthy blood donors were obtained from the blood bank of the Netherlands (Sanquin, the Netherlands). To investigate ADE in primary cells derived from pregnant women, whole blood was collected from pregnant women from different trimesters at the outpatient clinic of the department of Obstetrics and Gynaecology at Erasmus Medical Center, Rotterdam, the Netherlands after written informed consent was obtained.

Exclusion criteria for participation were current or recent (<1 month) use of immune suppressive medication, a current or recent (<1 week) infection or vaccination and a known immunodeficiency. For the control group, aged-matched, non-pregnant women who had not been pregnant in the 6 months prior to study participation, were recruited. Exclusion criteria for the non-pregnant women were the same as for the pregnant women. Approval for this study was granted by the Medical Ethical Committee of Erasmus Medical Center (MEC-2021-0134).

Cell lines

Cell lines tested to study ADE were selected based on previous studies regarding ADE of ZIKV and DENV infection (215, 257-260). K562 cells were obtained from ATCC (CCL-243) and were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, US), 100U/ml penicillin and 100μg/ml streptomycin (Lonza) at 37°C, 5% CO_2. U937 and THP-1 cells (kindly provided by the department of

Immunology of Erasmus Medical Center) were cultured in RPMI 1640 (Lonza) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2. Vero cells (African green monkey kidney epithelial cells, ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza) with 10% FBS and 100U/ml penicillin and 100μg/ml streptomycin and 2mM glutamine. All cell lines were tested mycoplasma free.

Primary human cells

For isolation of peripheral blood mononuclear cells (PBMCs), blood was layered on Ficoll-Paque PLUS density gradient media (GE Healthcare Life) and centrifuged for 20 minutes at 800 x g without brake. After centrifugation, the PBMC layer was isolated, washed three times with PBS and frozen at -135°C until further use. Monocytes were isolated from frozen PBMC's using magnetic based CD14 positive selection according to the manufacturer's instructions and the Pan Monocyte Isolation Kit (both Miltenyi Biotec). Purity of the monocytes, based on CD14 positivity was 87.6% as determined with flow-cytometry (Fig. S1). To differentiate monocytes into macrophages and dendritic cells, monocytes were plated at a density of 100.000 cells per well in 96 well plates in complete RPMI 1640 (Lonza) medium supplemented with 4200 IU/ml macrophage colony stimulating factor (M-CSF; R&D systems, US) for macrophages, and with 1000 IU/ml granulocyte macrophage colony stimulating factor (GM-CSF; R&D systems, US) and 500 IU/ml IL-4 for dendritic cells, for six days. For maturation of immature dendritic cells, lipopolysaccharide (Sigma-Aldrich) was added to the cells on day six for 48-hours in a concentration of 1μg/ml. Maturation of dendritic cells was confirmed by the observation that among the tested maturation markers the expression of maturation markers, including CD80 and CD83 was upregulated in mature dendritic cells compared to immature dendritic cells (Figure S2), while the differences in expression of CD86 and PD-L1 were limited.

Virus and virus quantification

An Asian lineage ZIKV strain was used for all experiments (Suriname ZIKVNL00013, EVAg no. 011V-01621). The virus was grown in Vero cells and passage number 3 was used for the current study. Viral titers in supernatants, expressed as 50% tissue culture infective dose (TCID50) was determined by 10-fold dilution endpoint titration on Vero cells and calculated with the method of Kärber (261).

ZIKV infection

For infection experiments, cells were seeded in 96-well plates at a density of 100.000 cells per well and infected with ZIKV at a multiplicity of infection (MOI) of 0.5, 1, 5 or 10 for 1 hour at

37°C, 5% CO2. After incubation, the supernatant was removed, and cells were washed three times. Cell supernatants were collected at day 0 and day 2 post infection. Supernatant was stored at −80°C until further use. Experiments were performed in triplicates.

Antibody-dependent enhancement assay

For ADE assays, ZIKV (MOI 0.5) was incubated with different concentrations of the humanized IgG1 pan-flavivirus antibody 4G2 (hu4G2, Native Antigen Company, UK) at 37°C to form immune complexes. After an hour of incubation, the virus-antibody mixture was added to the cells in 96-well plates and incubated for one hour at 37°C, 5% CO_2. Subsequently, cells were washed three times and supernatant was collected at day 2 post infection and frozen at -80°C until further use. For FcγR blocking experiments, cells were pre-incubated for one hour with 10μg/ml FcγRI, FcγRII and/or FcγRIII blocking antibodies (clones 10.1, 6C4 and 3G8 respectively, all eBioscience) at 37°C before infection with ZIKV or ZIKV+hu4G2.

Flow cytometry

In some ADE experiments, flow-cytometry was used as a read-out to determine the percentage of infected cells. For this, cells were collected at day two post infection and stained with live/dead stain prior to fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences, USA). Cells were blocked with 10% normal goat serum (NGS, Dako, Denmark) and total human Fc block (BD Biosciences). Intracellular staining for ZIKV E-protein was performed with a mouse 4G2 antibody (MAB10216, clone D1-4G2-4-15; Millipore, Germany) followed by an APC/Cy-7 conjugated goat anti-mouse IgG2a secondary antibody (Abcam, UK). Flow cytometry was performed with the FACS Lyric machine (BD Biosciences, USA). Data was analyzed using FlowJo 10.6.1, software (Ashland, OR, USA). All experiments were performed three times (biological replicates), and each experiment included duplicate (technical replicates) measurements from which the average was calculated and used for further analysis. To demonstrate that intracellular ZIKV-E staining represents productive infection and not just phagocytosis, some ZIKV infected cells were also stained with an anti-DENV NS3 antibody (E1D8, My Biosource, US) which has been shown to cross-react with ZIKV NS3 (262).

Cytokine detection in supernatant

Cytokines were detected in the supernatant of infected cells using a 13-plex bead-based fluorescence assay according to the manufacturer's instruction (LEGEND-plex Human Anti-Virus Response Panel, Biolegend). Briefly, beads conjugated with a specific antibody against cytokines were incubated with cell supernatant of either uninfected cells, ZIKV infected cells

or ZIKV+hu4G2 infected cells. Subsequently, a detection antibody cocktail was added, and read-out of the assay was performed with flow cytometry (FACS lyric, BD Biosciences, USA). The cytokines that could be detected with this assay were IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-α, IFN-β, IFN-λ1, IFN-λ2/3, IFN-γ, TNF-α, IP-10 and GM-CSF. Data was analyzed with LEGEND-plex data analysis software (Biolegend).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8. Significant differences between groups in the ADE assays were determined with either a student t-test or ANOVA with Dunnett's post-hoc test or for non-normally distributed data a Mann-Whitney U test or Kruskall-Wallis tests, followed by Dunn's post-hoc test. Correlations between viral titers and percentage of infected cells were determined with Spearman's rank correlation coefficient. A p-value ≤ 0.05 was considered a statistically significant difference.

Results

Permissiveness for ZIKV infection

We first studied the permissiveness for ZIKV infection of cells that are commonly used for *in vitro* ZIKV ADE assays; the cell lines U937 (217, 252), K562 (215, 224) and THP-1 (216, 263) as well as primary monocytes (264), monocyte derived macrophages, monocyte derived immature dendritic cells (immature DCs) and monocyte derived mature dendritic cells (mature DCs) (265, 266). Cells were infected with ZIKV at varying multiplicities of infection (MOI) for 48 hours after which viral titers were determined in supernatants. Infection of U937 cells with ZIKV with an MOI of 0.5, 1 or 5 did not result in viral replication while infection with ZIKV at an MOI of 10 resulted in low titers (147 $TCID_{50}$/ml, Fig 1A). THP-1 cells and especially K562 cells were more permissive for ZIKV infection (peak titer $4.1x10^3$ and $1.1x10^5$ $TCID_{50}$/ml, respectively, Figs 1B and 1C). For the primary cells, replication with low titers was observed in monocytes (peak titer 147 $TCID_{50}$/ml, Fig 1D), while monocyte derived macrophages and immature DCs were permissive to ZIKV infection resulting in high ZIKV titers in supernatants (peak titers $3.2x10^5$ and $6.8x10^5$ $TCID_{50}$/ml, respectively, Figs 1E and 1F). Mature DCs were less permissive than immature DCs and could only be infected with a high MOI of ZIKV (peak titer $6.8x10^3$ $TCID_{50}$/ml, Fig 1G). Vero cells were included as a reference since these cells are known to be highly permissive for ZIKV infection. As expected, peak viral titers in supernatants of infected Vero cells were high, regardless of the MOI of ZIKV (peak titer $1.5x10^7$ $TCID_{50}$/ml, Fig 1H).

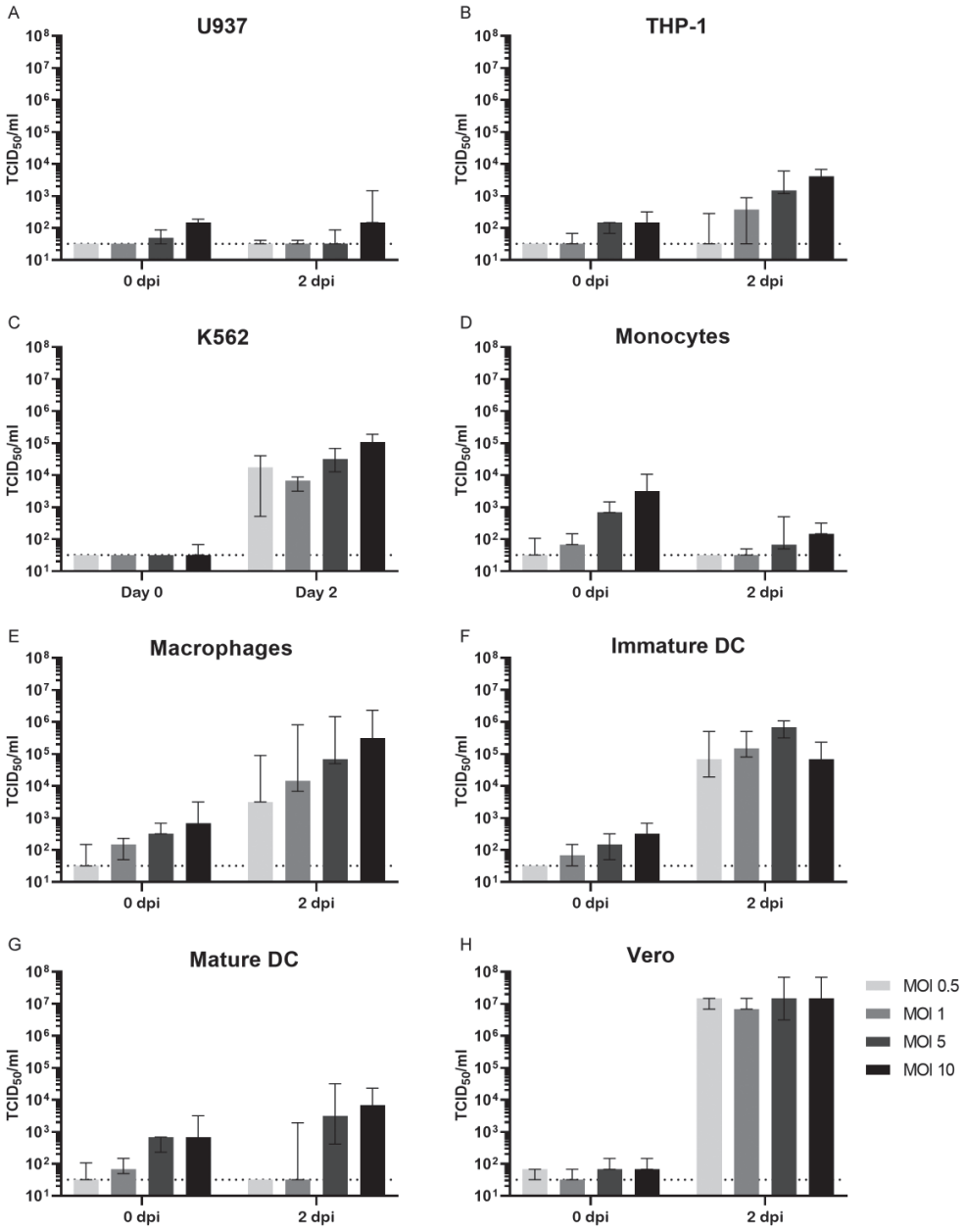

Figure 1. ZIKV infection of FcγR-bearing cell lines, primary myeloid cells and vero cells.
Infection of FcγR-bearing cell lines (**A,B,C**) and primary cells (**D,E,F,G**) with ZIKV at different MOI's for 48 hours.
I: Vero cells, which are known to be highly permissive to ZIKV infection, were infected with different MOI's of ZIKV for two days as a positive control. ZIKV titers were determined in supernatants. Bars represent median ZIKV titer ± interquartile range. DC; dendritic cells

ADE of ZIKV infection in myeloid cell lines and primary myeloid cells

To determine the potential for ADE of ZIKV infection of the above-mentioned cell lines and primary cells, we subsequently performed ADE assays with these cells. Cells were infected with ZIKV alone or ZIKV that was pre-incubated with increasing concentrations of a humanized pan-flavivirus monoclonal antibody (hu4G2) at the lowest MOI (0.5) that was used in the permissiveness experiments in Fig 1. After 48-hours, two commonly used read-outs for ADE assays were used; virus titration of supernatants and flow cytometry analysis to determine the percentage of infected cells.

Pre-incubation of ZIKV with hu4G2 resulted in a concentration dependent increase of infectious titer and percentage of infected cells in all three tested cell lines (Fig 2). Highest viral titers and percentage of infected cells were observed in K562 cells ($9.7x10^5$ $TCID_{50}$/ml and 29.6% infected cells) and lowest in U937 cells ($5.0x10^4$ $TCID_{50}$/ml and 2.1% infected cells) after infection with ZIKV+1μg/ml hu4G2. For all cell lines, there was a strong correlation between the viral titers in supernatants and the percentage of infected cells ($r=0.85$ for U937, $r=0.85$ for THP-1 and $r=0.82$ for K562, $P<0.0001$ for all**,** Figs S3A-C).

For the primary cells, ADE of ZIKV infection was not observed in monocytes nor in mature DCs, while for immature DCs, addition of hu4G2 even partially inhibited ZIKV infection (Figs 3C and 3G). We ruled out that CD14 positive selection of monocytes influences the permissiveness of these cells for ZIKV infection due to stimulation of CD14 by using a non-CD14 based negative selection kit to isolate monocytes from PBMCs. The permissiveness for ZIKV infection was comparable between the negatively and positively isolated monocytes when infected with ZIKV at a MOI of 0.5, 1 and 5 and slightly increased when infected with an MOI of 10 (2315 vs 147 $TCID_{50}$/ml $P=0.002$, Fig S6).

In monocyte derived macrophages, ADE of ZIKV infection was dependent on the concentration of hu4G2 as shown by increased in viral titers in supernatants and an increase in the percentage of infected cells ($1.5x10^6$ $TCID_{50}$/ml and 15.3% infected cells with 1μg/ml hu4G2, $P<0.01$, Figs 3B and 3F). Similar to the cell lines, a strong positive correlation was found in monocyte derived macrophages between the viral titers in supernatants and the percentage of infected cells ($r=0.82$, $P<0.0001$, Fig S3D).

Collectively these results demonstrate that ADE of ZIKV infection can be observed in the myeloid cell lines U937, K562 and THP-1. For primary cells, ADE of ZIKV infection is only observed in monocyte derived macrophages.

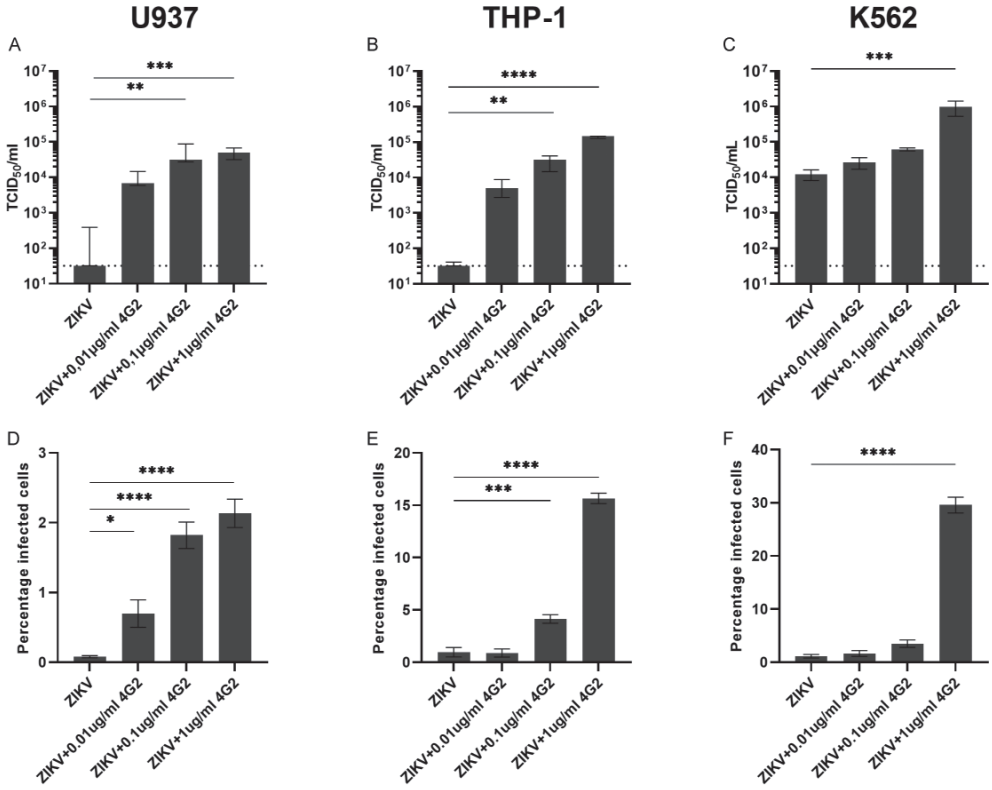

Figure 2. ADE of ZIKV infection in U937, THP-1 and K562 cells.
The FcγR-bearing human cell lines U937, THP-1 and K562 were infected for 48 hours with ZIKV at an MOI of 0.5 with and without increasing concentrations of the pan-flavivirus humanized monoclonal antibody 4G2 (hu4G2). ZIKV titers were determined in supernatants (**A-C**) and percentage of infected cells was determined with flow cytometry (**D-F**). Bars represent median titer ± IQR (**A-C**) and mean percentage of infected cells ± SEM (**D-F**). Statistical significance was determined with the Kruskall-Wallis test with Dunn's post hoc test or a one-way ANOVA with Dunnett's post hoc test, comparing the conditions with hu4G2 to infection with only ZIKV. * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

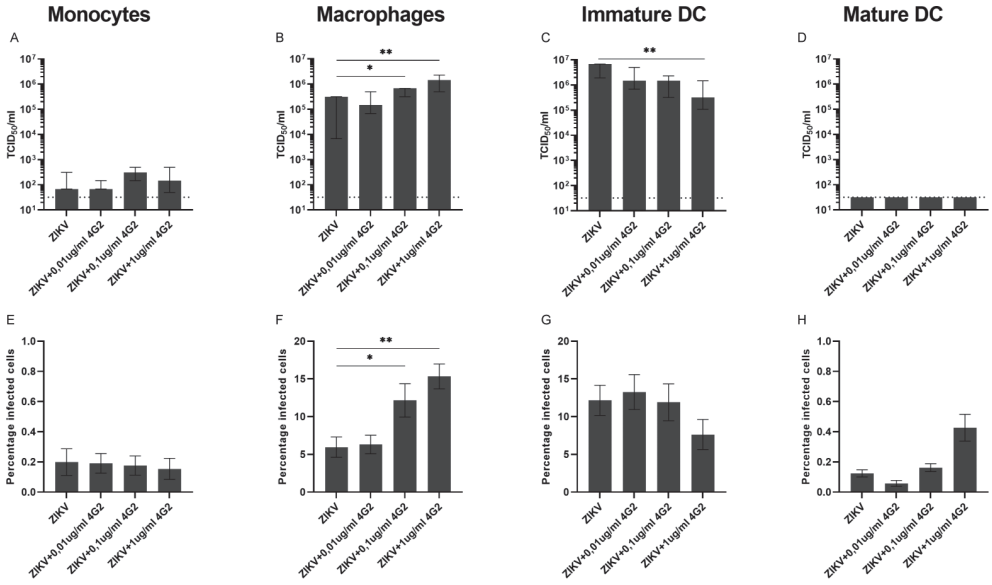

Figure 3. ADE of ZIKV infection in primary myeloid cells.
FcγR-bearing human myeloid cells were infected for 48 hours with ZIKV at an MOI of 0.5 with and without increasing concentrations of the pan-flavivirus humanized monoclonal antibody 4G2 (hu4G2). Viral titers in supernatants were determined with titration (**A-D**) and percentage of infected cells was determined with flow cytometry (**E-H**). Bars represent median titer ± IQR (**A-D**) and mean percentage of infected cells ± SEM (**E-H**). Statistical significance was determined with the Kruskall-Wallis test with Dunn's post hoc test or a one-way ANOVA with Dunnett's post hoc test, comparing the conditions with hu4G2 to infection with only ZIKV. * P<0.05, ** P<0.01. N=3 donors.

Fcγ receptors and ADE of ZIKV infection

One of the main relevant differences between the cell lines used for ADE assays in this study is the expression of Fcγ-receptors (FcγRs). U937 and THP-1 cells express FcγRI and –II (CD64 and CD32) while K562 cells only express FcγRII (Fig S4) (267). For primary myeloid cells, FcγRI and –II is expressed by monocytes, monocyte derived macrophages and can be induced in dendritic cells, while FcγRIII (CD16) is expressed by monocyte derived macrophages and dendritic cells and on intermediate- and non-classical monocytes (268-271). In the cells permissive for ADE of ZIKV infection, we tested which FcγRs were important for this ADE. Therefore, we performed ZIKV ADE assays with the highest tested concentration of hu4G2 (1 μg/ml) and pre-incubated the cells with monoclonal antibodies against FcγRI, FcγRII, FcγRIII or with a blocker of all Fc-receptors (total Fc Block) to block the interaction of hu4G2 with these FcγRs.

ADE of ZIKV infection in U937 and THP-1 cells was mainly inhibited by blocking FcγRI and by adding total Human Fc Block and to a lesser extent by blocking FcγRII while blocking

FcγRIII did not reduce viral titers or percentage of infected cells (Figs 4A, 4B, 4E and 4F). For K562, which only expresses FcγRII, ADE of ZIKV infection was significantly inhibited by blocking FcγRII but not by the other FcγR blockers (Fig 4 C&G). For macrophages, blocking FcγRI and FcγRII resulted in a slight, but not statistically significant, decrease in percentage of infected cells while adding total Fc Block did significantly reduce ADE of ZIKV infection in these cells (Fig 4 D&H). Collectively these data demonstrate that ADE of ZIKV infection, induced by a monoclonal antibody, can be inhibited by blocking FcγRI (except for K562) and to a lesser extent FcγRII.

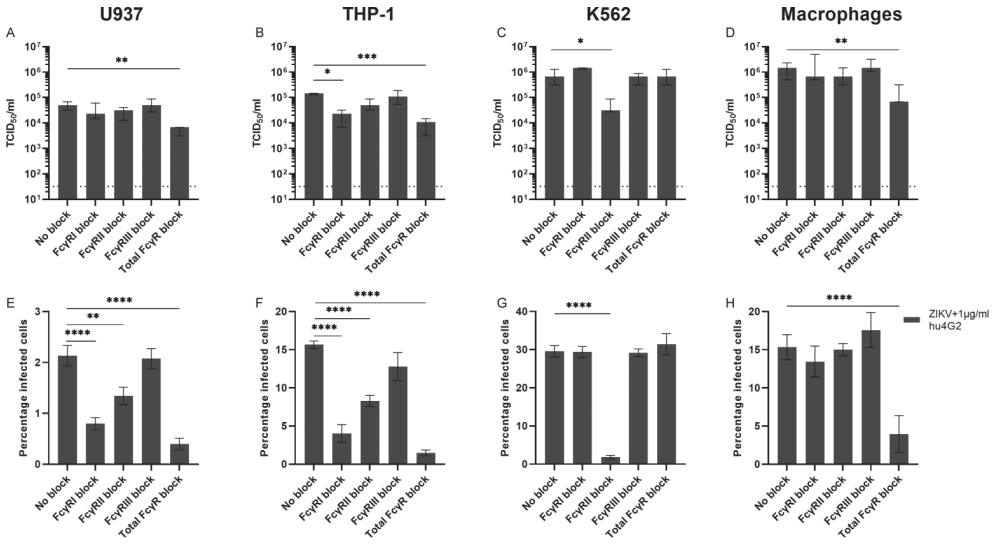

Figure 4. FcγRI and FcγRII contribute to ADE of ZIKV infection in FcγR cell lines and primary cells. FcγR-bearing human cell lines and myeloid cells that are permissive to ADE of ZIKV infection were pre-incubated with monoclonal antibodies against FcγRs or with Human BD Fc Block before adding ZIKV (MOI 0.5) that was pre-incubated with 1 µg/ml of the pan-flavivirus humanized monoclonal antibody 4G2 (hu4G2). Viral titers in supernatants were determined with titration (A-D) and percentage of infected cells was determined with flow cytometry (E-H). Bars represent median titer ± IQR (A-D) and mean percentage of infected cells ± SEM (E-H). Statistical significance was determined with the Kruskall-Wallis test with Dunn's post hoc test or a one-way ANOVA with Dunnett's post hoc test, comparing the conditions with u4G2 to infection with only ZIKV. * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001. N=3 donors for monocyte derived macrophages.

Cytokine quantification

ADE is believed to be one of the main mechanisms responsible for a cytokine storm that occurs during severe DENV infections, therefore we determined cytokine production of all cells during ADE of ZIKV infection (272).

Cytokines were determined in supernatants of cells that were permissive for ZIKV ADE, 48 hours after either mock infection, ZIKV infection or ADE of ZIKV infection. Only a limited number of cytokines could be detected in the supernatants of U937, K562 and THP-1 cells and differences between cytokine concentrations of uninfected cells compared to cells infected with ZIKV or ZIKV+hu4G2 were small (Figs 5 A-C). For THP-1 cells, IL-8 production of cells infected with ZIKV+4G2 was statistically significant higher compared to uninfected cells (average 43.7 vs. 35.7 pg/ml, P=0.007) while for K562 cells a decrease in IL-8 production was found during ADE of ZIKV infection compared to uninfected cells (426.4 vs 350.8 pg/ml, P=0.014). IL-6 production was lower in K562 infected with ZIKV+4G2 compared to uninfected K562 cells (24.2 vs. 28.8 pg/ml, P=0.005). Compared to the cell lines, more cytokines could be detected in supernatants of monocyte derived macrophages (Fig 5D).

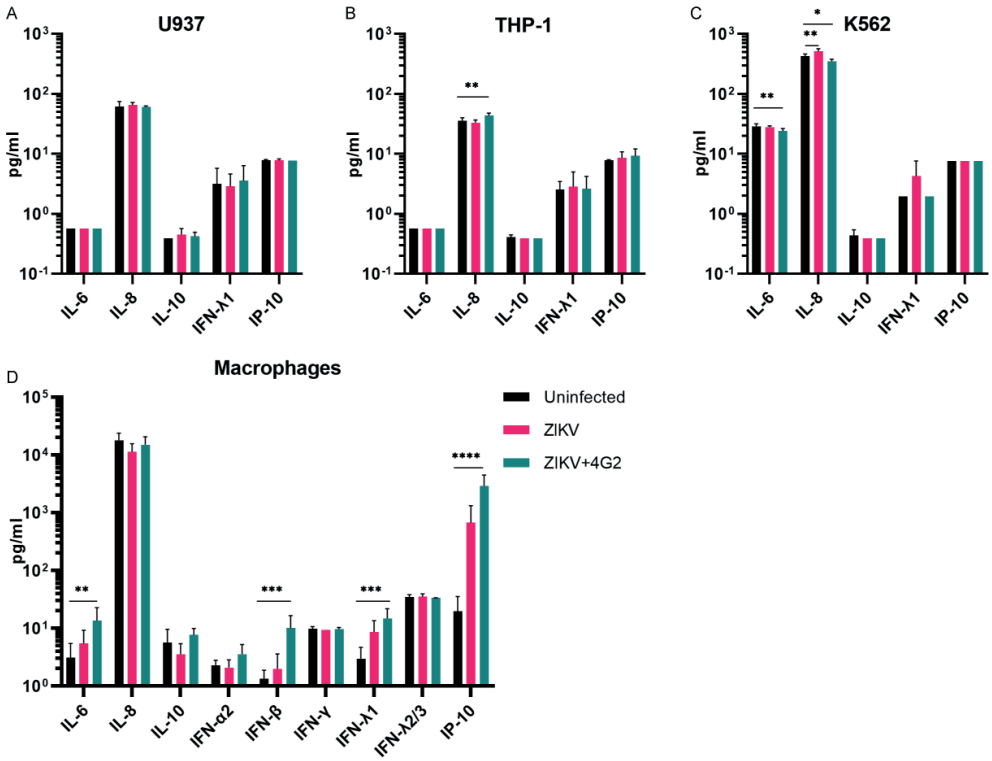

Figure 5. Modest increase of cytokine concentrations in supernatants of FcγR-bearing cells during ADE of ZIKV infection.
A panel of 13 cytokines were determined in FcγR-cells 48 hours after mock infection or infection with ZIKV or ZIKV that was pre-incubated with 1 μg/ml of the pan-flavivirus humanized monoclonal antibody 4G2 (hu4G2). Only the cytokines that could be detected in the supernatants of the cells are illustrated in the graphs. Bars represent mean ± SEM, statistical significance was determined with a one-way ANOVA with Dunnett's post hoc test, comparing uninfected cells with ZIKV infected and ZIKV+hu4G2 infected. *P<0.05, **P<0.01, ***P<0.001, ***P<0.0001.

In line with a previous observation, there was an increased production of IL-6 in monocyte derived macrophages during ADE of ZIKV infection compared to mock infection (13.6 vs. 3.1 pg/ml, P=0.003, Fig 5D) (266). Furthermore, production of IFN-β, IFN-λ1 and IP-10 was significantly increased during ADE of ZIKV infection compared to uninfected cells (10.1 vs. 1.3 pg/ml for IFN-β, 14.7 vs. 3.0 pg/ml for IFN-λ1 and 2889 vs. 19.8 pg/ml for IP-10, P<0.001, P<0.001 and P<0.0001, respectively, Fig 5D). An increase in IL-10 production during ADE of infection, which is thought to play an important role in intrinsic ADE of DENV infections, was not observed in any of the cells.

ZIKV permissiveness in myeloid cells from pregnant women

Due to the potential detrimental effects of a ZIKV infection during pregnancy, we wanted to test whether FcγR bearing primary myeloid cells, collected during different trimesters of pregnancy, are more permissive for ZIKV infection and ADE of ZIKV infection compared to non-pregnant women. Therefore, we isolated PBMCs from 30 pregnant women during each of the three trimesters, as well as from 10 non-pregnant women. Based on the above presented results, we decided to use monocyte derived macrophages to test potential differences in permissiveness of (ADE of) ZIKV infection. Even though in monocytes of three healthy donors we only found low grade ZIKV infection and no ADE (Fig 1D and Figs 3A and 3E), we also infected monocytes of the pregnant and non-pregnant women with ZIKV because a higher susceptibility of monocytes for ZIKV infection has been observed during the first trimester of pregnancy (273). We decided to only use the viral titers in supernatants as a readout because with this readout, an increase in infected cells (extrinsic ADE as well as an increase of viral production per infected cell (intrinsic ADE) can be detected which is not the case when determining the percentage of infected cells with flow cytometry.

No difference was found in permissiveness for ZIKV infection in monocytes or macrophages over the different trimester of pregnancy nor between pregnant women compared to non-pregnant women (Figs 6A and 6B). ADE of ZIKV infection was observed in monocytes and monocyte derived macrophages from pregnant women and non-pregnant women. There was no difference in peak ADE titers in supernatants of primary cells from pregnant women, independent of gestational age, compared to non-pregnant women (Figs 6A and 6B). Furthermore, no difference was found in ZIKV permissiveness and ADE potential between monocytes and macrophages from women at different gestational ages at the time of sample collection (first-, second- or third trimester) (Figs 6C and 6D).

Collectively, these data demonstrate that no enhanced susceptibility of ZIKV infection and ADE of infection is present in monocytes and monocyte derived macrophages collected from pregnant women compared to non-pregnant women.

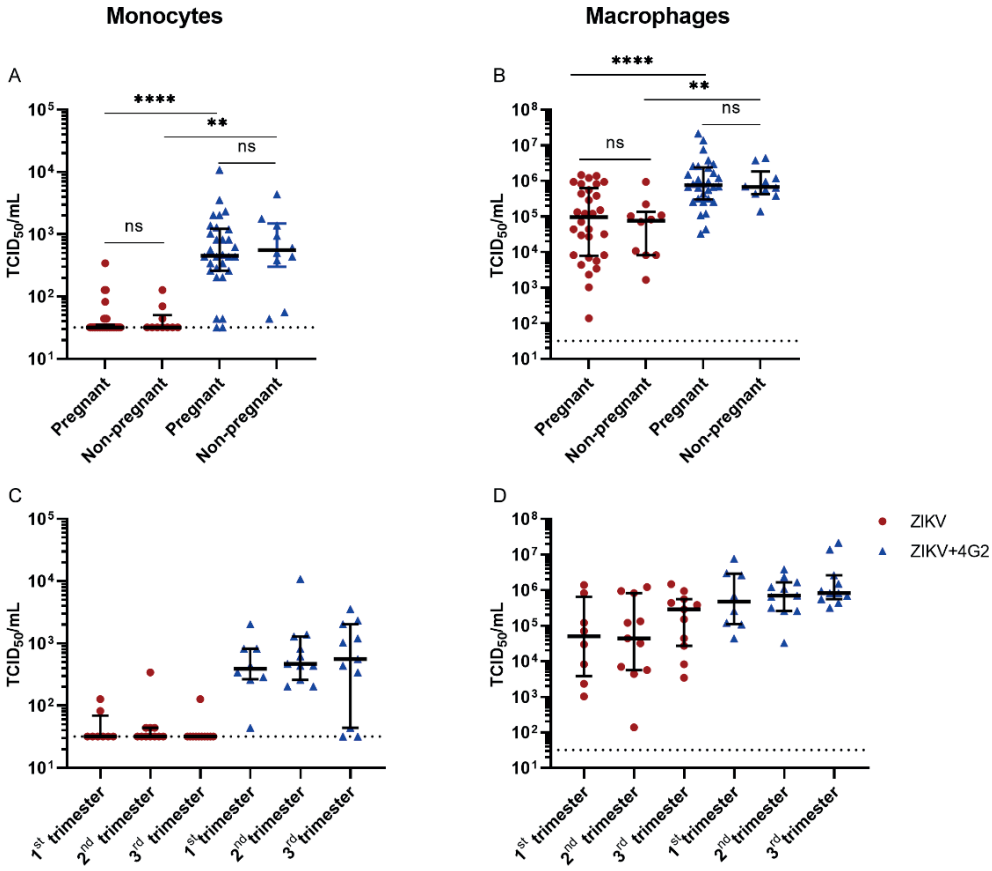

Figure 6. No differences in permissiveness for ZIKV infection and ADE of ZIKV infection in myeloid cells from pregnant women compared to non-pregnant women.
PBMC's were isolated from whole blood from 30 pregnant women and 10 non-pregnant women and monocytes were isolated and part of those were differentiated into macrophages. Cells were subsequently infected for 48 hours with ZIKV at an MOI of 0.5 with and without 1 µg/ml pan-flavivirus humanized monoclonal antibody 4G2 (hu4G2) and viral titers in supernatants were determined by titration. Bars represent median titer ± IQR. Statistical significance was determined with the Kruskall-Wallis test with Dunn's post hoc test ** P<0.01, ****P<0.0001.

Discussion

Here we found that the myeloid cell lines U937, K562 and THP-1, were all permissive for ADE of ZIKV infection as has been shown by others (114, 216, 227). The highest ZIKV titers were obtained in K562 cells, followed by THP-1 and U937 cells, the latter of which was non-permissive to ZIKV infection in the absence of cross-reactive antibodies. Blocking of FcγRI significantly reduced ADE of ZIKV infection in both U937 and THP-1 cells which, for U937 cells, is in line with findings for DENV ADE (206, 274). However, contrary to our observations, FcγRIIa has been reported to be the main FcγR responsible for ADE of DENV and ZIKV infections (264, 271, 275). In these previous studies, ADE of DENV or ZIKV infection was mainly induced by convalescent polyclonal sera while in this study, we only use a humanized IgG1 monoclonal antibody (hu4G2). A higher affinity of hu4G2 for FcγRI compared to FcγRII could possibly explain why ADE of ZIKV in these experiments mainly depend on FcγRI.

To relate the findings from in vitro cell line models to potential primary cell targets, we evaluated the permissiveness for (ADE) of ZIKV infection in primary human myeloid cells which are generally considered to be more representative of the in vivo human situation compared to cell lines. Of the primary myeloid cells isolated from healthy blood donors, only monocyte derived macrophages and immature dendritic cells were highly permissive to ZIKV infection while ADE of ZIKV infection was only observed in monocyte derived macrophages. High permissiveness of monocyte derived macrophages for ZIKV infection and ADE of ZIKV infection has been previously observed (260). The high permissiveness of immature dendritic cells for ZIKV infection but not for ADE of ZIKV infection has been previously observed for both DENV and ZIKV and might be caused by high expression of the flavivirus entry cofactor DC-SIGN by these cells, which has shown to be inversely correlated to the rate of ADE of infection (11, 264, 276). In the monocyte derived macrophages, ADE of ZIKV infection seemed to be dependent on both FcγRI and FcγRII since blocking of these receptors individually resulted in a non-statistically significant reduction of ADE of infection while blocking all FcγRs with total Fc block did result in a significant reduction of ADE. The low permissiveness of monocytes for ZIKV infection that we found in this study is in contrast with results from other studies that showed that monocytes are the main targets of ZIKV infection in blood (262, 264, 273, 277). One possible explanation for this can be that there is a variation in the tropism for monocytes between the different Asian lineage ZIKV isolates used in the previous studies and this study (278).

The main difference in cytokine production of the cell lines upon (ADE of) ZIKV infection was observed for IL-8, which was induced in THP-1 cells while reduced in K562 cells. IL-8

is a chemoattractant for neutrophils which are important effector cells of the innate immune system. A reduction of IL-8 production during ADE of ZIKV infection could reflect a dampened innate antiviral immune response as is observed during ADE of ZIKV infection in fetal macrophages (246, 279). The induction of IFN-β, IFNλ1 and IP-10 in monocyte derived macrophages during ZIKV ADE is in line with a pro-inflammatory immune response while an induction of IL-6 can indicate a T-helper cell 2 biased immune response as is seen during intrinsic ADE (113, 279, 280). However, as with the cell lines, we did not detect an increase in IL-10 production in monocyte derived macrophages during ADE of ZIKV infection, which has been suggested to be one of the hallmarks of intrinsic ADE for DENV and seems to contribute to disease severity in severe DENV infections (38, 111-113, 281). It is possible that a non-increased IL-10 production during ADE of infection is specific for ZIKV as we and others have not observed increased IL-10 production during ADE of ZIKV infection in fetal macrophages and in primary human (246, 282, 283).

When combining the results regarding peak viral titer and percentage of infected cells during ADE of ZIKV infection, THP-1 and K562 cells resemble the monocyte derived macrophages better than U937 cells, while U937 cells resemble monocytes. Regarding FcγRs expression, monocyte derived macrophages are resembled by U937 and THP-1 cells but not by K562 cells since these only express FcγRII. In general, these data suggest that the THP-1 cell line resembles monocyte derived macrophages the best for ADE of ZIKV infection concerning titers and FcγRs responsible for ADE of ZIKV infection while primary monocytes are best resembled by U937 cells. Since ADE of ZIKV infection can be induced in all three the cells lines, all three of these cells can be used for in vitro screening of antibodies for ADE potential.

The similar ZIKV titers that we found in ZIKV infected monocytes from pregnant women and non-pregnant women is in line with a previous study by Foo et. al (273). One difference is, however, that in this previous study, a small increase in permissiveness for ZIKV infection was found in monocytes from pregnant women in the first trimester compared to non-pregnant women based on ZIKV NS1 viral load, while this is not observed in the current study (273). This could possibly be explained by differences in the experimental set-up e.g., infection of whole blood vs. infection of PBMCs derived monocytes and the specific ZIKV isolate that was used. As with the monocytes, no differences were found in monocyte derived macrophages for ZIKV infection and ADE of ZIKV infection. These results indicate that monocytes and monocyte derived macrophages from pregnant women are not more permissive for ZIKV infection and ADE of ZIKV infection compared to non-pregnant women, independent of trimester.

Though our findings are consistent and validated at various levels, there are limitations to this study. First, to avoid the variability among the tested in vitro models because of IgG subclasses, we only used humanized 4G2 as enhancing antibody and not e.g., polyclonal sera containing DENV antibodies. However, it is likely that there are differences in affinity for FcγRs between monoclonal hu4G2 and polyclonal serum consisting of multiple IgG subclasses that may also have an impact on the final outcome of ADE assay (284). Furthermore, differences in glycosylation status of this monoclonal antibody compared to polyclonal sera are also likely to influences the affinity for different FcγRs (285, 286). The exact role of interactions of immune complexes with different FcγRs on the outcome of ADE of infection and the role of glycosylation of these antibodies on this is an interesting topic that should be studied in more detail. Furthermore, we only used one MOI (0.5) and one concentration of hu4G2 for all infection experiments to limit the number of variables. It could be that with a higher MOI or with a (even) higher concentration of hu4G2, ADE of ZIKV infection would be observed in e.g., monocytes and mature dendritic cells. However, it is not likely that these conditions will occur in vivo. Lastly, for the pregnant cohort due to limited sample availability, we have tested only PBMCs derived myeloid cells to validate ZIKV-ADE in in vitro culture system. This does not allow us to study the possible effects of changes in hormonal levels that occur during pregnancy on permissiveness for (ADE of) ZIKV infection which needs further validation in a well-defined cohort

In conclusion we found that U937, THP-1 and K562 cell lines can be used for in vitro ADE assays for ZIKV as well as monocyte derived macrophages. Regarding permissiveness for ZIKV infection and ADE of ZIKV infection, THP-1 cells mimic the results that are obtained with monocyte derived macrophages while U937 cells mimic the results of monocytes. No increased permissiveness for ZIKV infection and ADE of ZIKV infection was found in primary myeloid cells from pregnant women compared to age-matched non-pregnant women. These data are in line with data from a study with pregnant non-human primates and indicate that during pregnancy, pre-existing DENV immunity is not likely to be a risk for a more severe ZIKV infection in the mother (287). A possible facilitating role of DENV immunity on the risk of transplacental ZIKV transmission is not ruled out based on the results of this study and is suggested in multiple experimental studies and should be studied in more detail in human studies (116, 246, 253, 254, 283).

Supplemental information

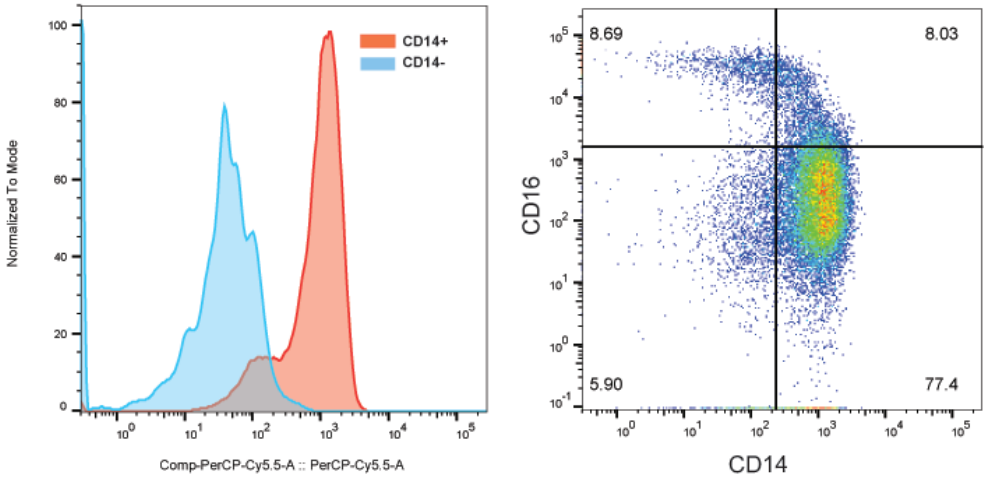

Figure S1. CD14+ expression of monocytes that were magnetically isolated from frozen PBMCs using anti- human CD14 microbeads. Cells in negative flow through (blue) and positive selection (red) were labelled with CD14-PerCP.Cy.5 and analyzed by flow cytometry.

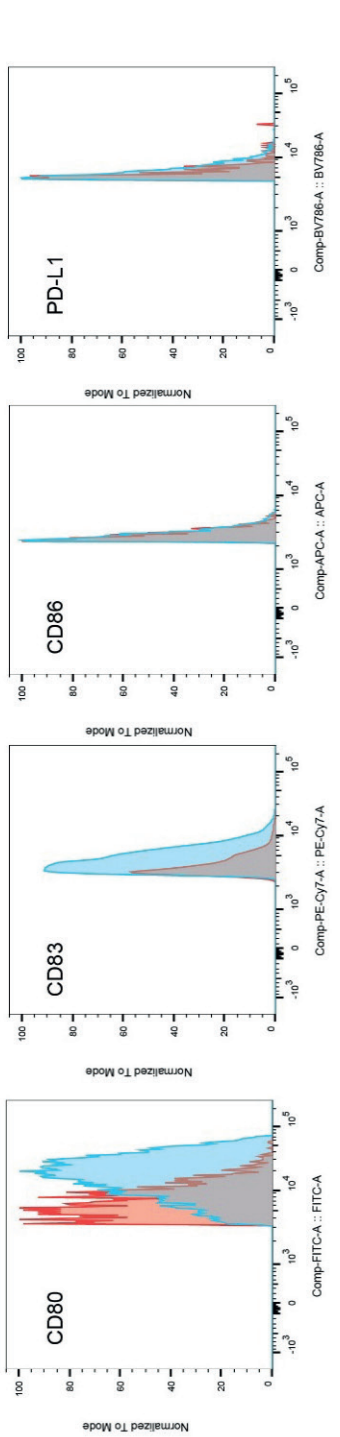

Figure S2. Characterization of dendritic cell surface expression markers using flow cytometry.
A-D: Flow cytometry histograms represent levels of CD80, CD83, CD86 and PD-L1 in immature (red) and mature (blue) dendritic cells. For comparison, the degree of maturation is represented by the expression of CD80 and CD83: immature DCs are largely negative (red) compared to mature DCs (blue).

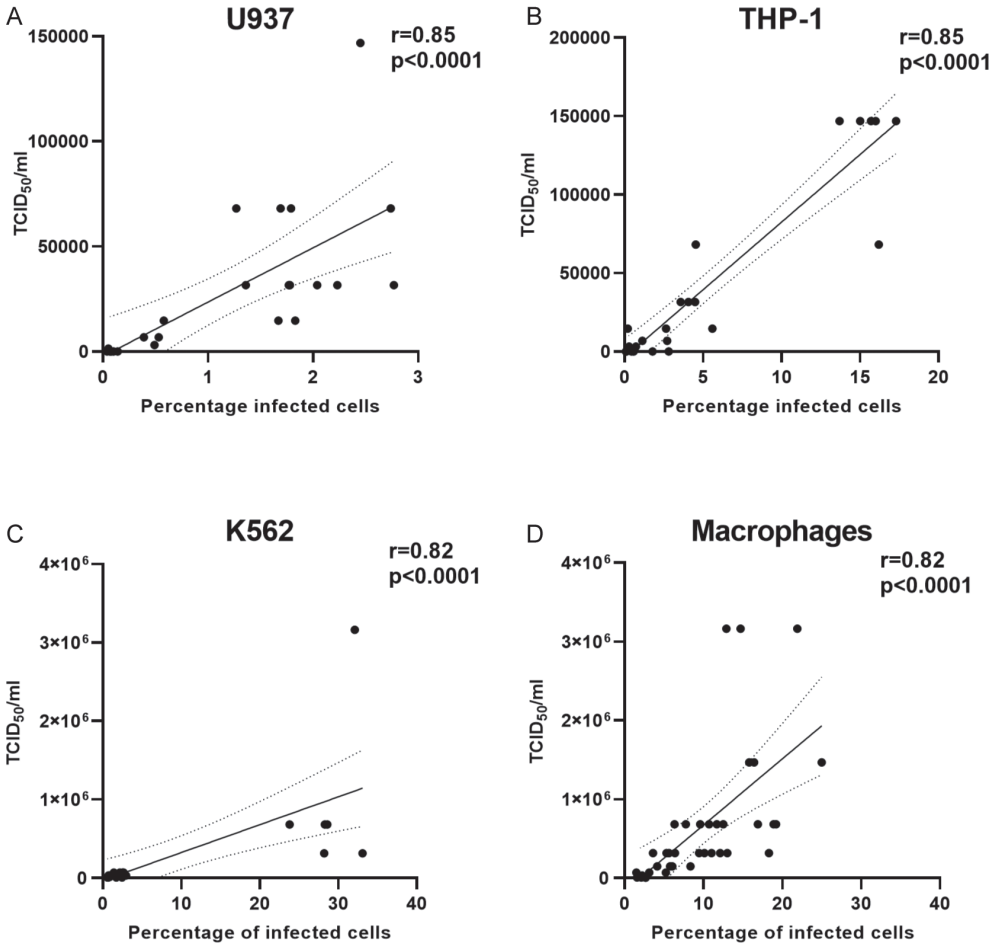

Figure S3. Strong correlation between viral titers in supernatants and percentage of infected cells U937 cells (**A**), THP-1 cells (**B**), K562 cells (**C**) and monocyte-derived macrophages (**D**). Correlations were calculated using Spearman's rank correlation coefficient.

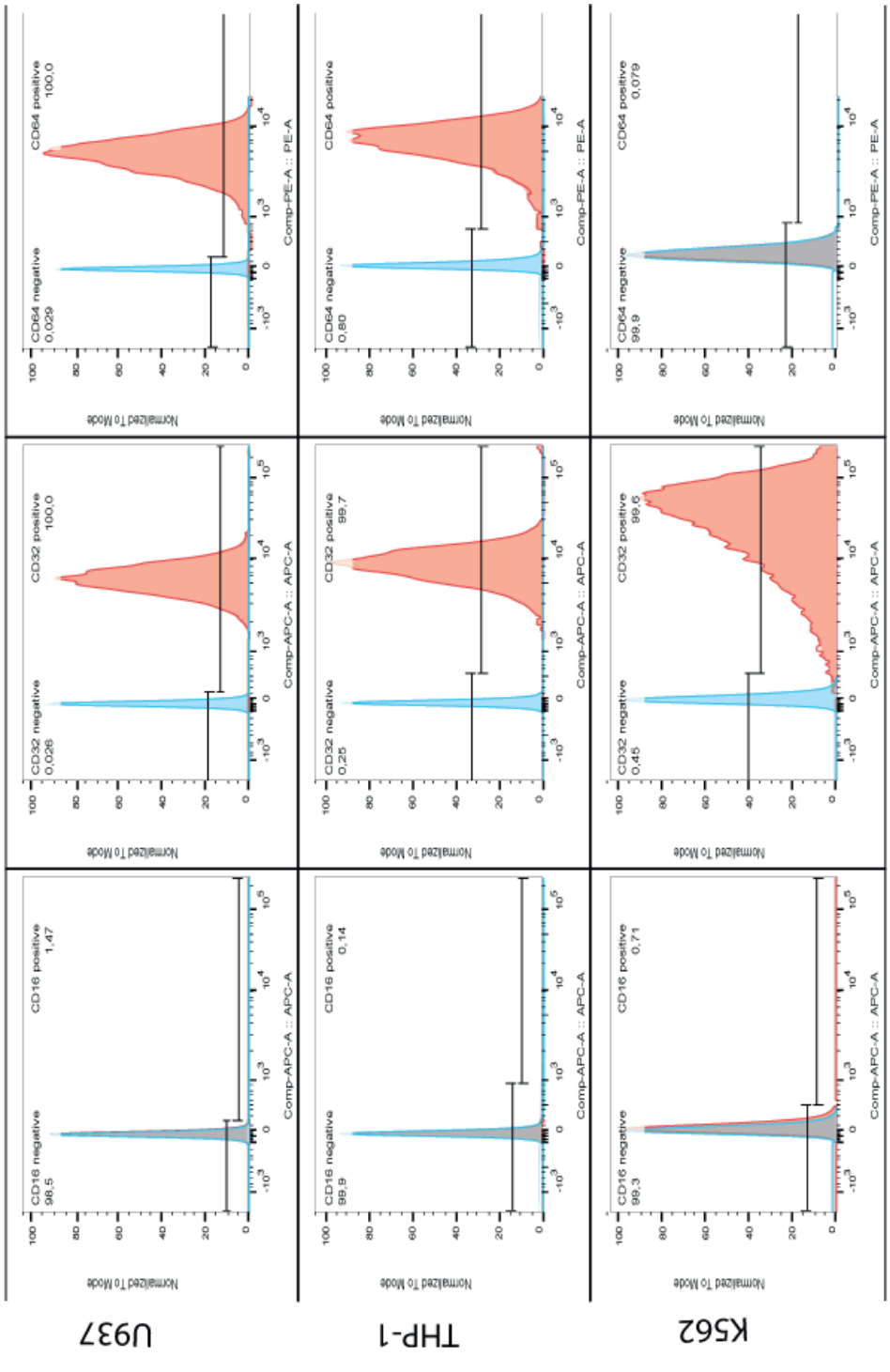

Figure S4. FcγR expression in U937, THP-1 and K562 cells. Cells are illustrated in the red graphs while isotype controls are illustrated in blue. CD16; FcγRIII, CD32; FcγRII, CD64; FcγRI

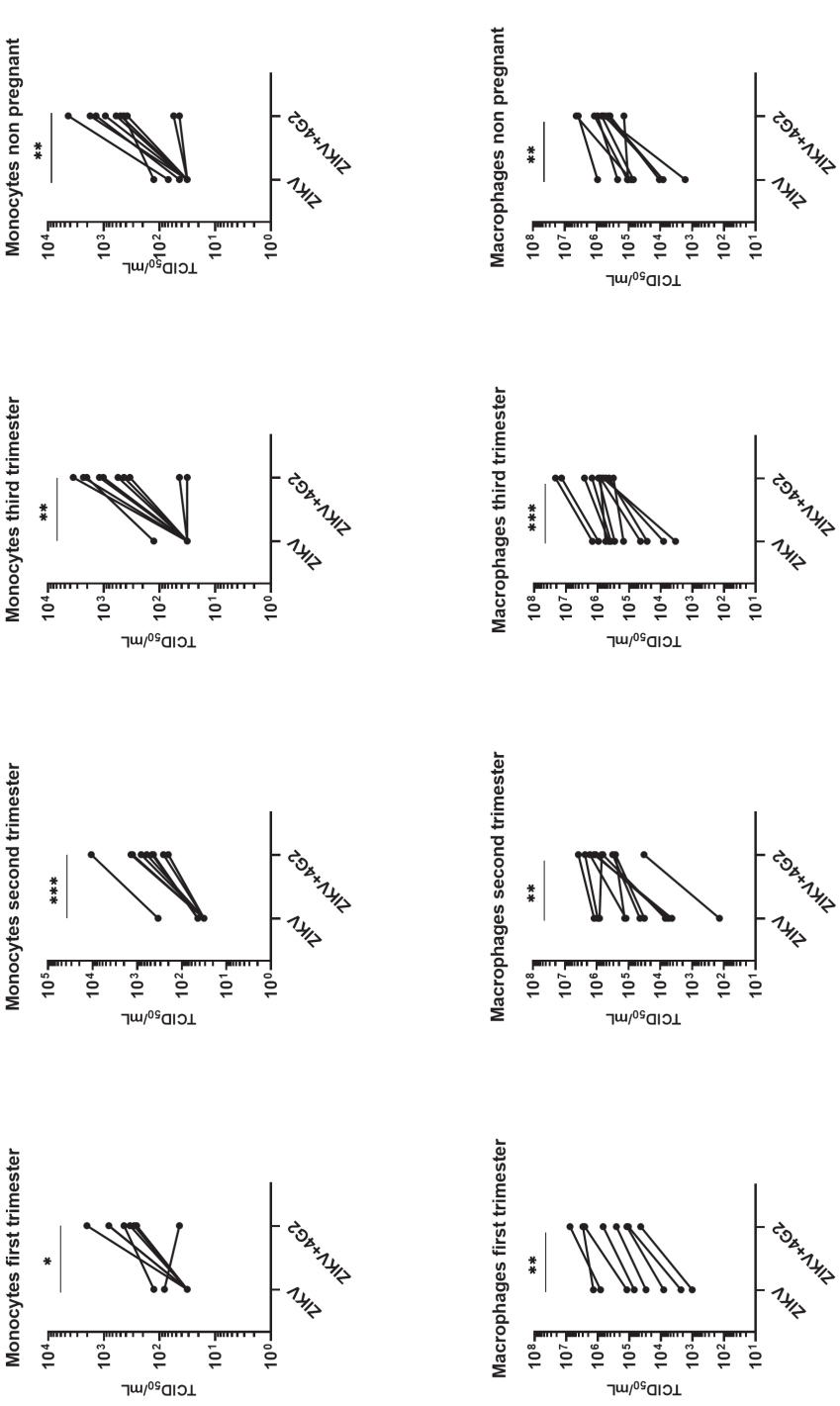

Figure S5. Paired ZIKV titers determined in supernatants of monocytes and macrophages from pregnant women (per trimester) and non-pregnant women infected with ZIKV and with ZIKV+hu4G2 to induce ADE of infection.

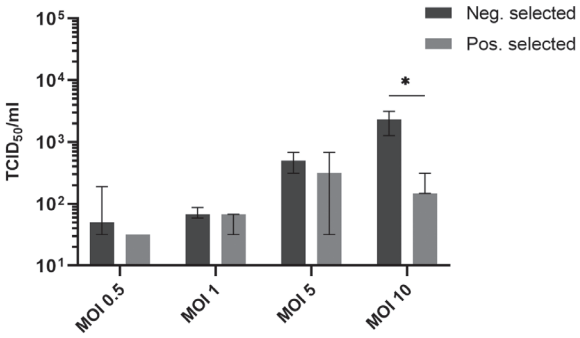

Figure S6. Monocytes were isolated from PBMC's with a CD14+ isolation kit (pos. selected) or with a pan monocyte isolation kit in which monocytes are negatively selected (untouched, neg. selected). Isolated monocytes were subsequently infected with ZIKV at various MOI for 48 hours after which viral titers were determined in the supernatants. Bars represent median +- IQR. The Mann-Whitney U test was used to assess statistical significance. *P<0.05.

8

CHAPTER

Transplacental Zika virus transmission in ex vivo perfused human placentas

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Abstract

A Zika virus (ZIKV) infection during pregnancy can result in severe birth defects such as microcephaly. To date, it is incompletely understood how ZIKV can cross the human placenta. Furthermore, results from studies in pregnant mice and non-human primates are conflicting regarding the role of cross-reactive dengue virus (DENV) antibodies on transplacental ZIKV transmission. Elucidating how ZIKV can cross the placenta and which risk factors contribute to this is important for risk assessment and for potential intervention strategies for transplacental ZIKV transmission.

In this study we use an *ex vivo* human placental perfusion model to study transplacental ZIKV transmission and the effect that cross-reactive DENV antibodies have on this transmission. By using this model, we demonstrate that DENV antibodies significantly increase ZIKV uptake in perfused human placentas and that this increased uptake is neonatal Fc-receptor-dependent. Furthermore, we show that cross-reactive DENV antibodies enhance ZIKV infection in term human placental explants and in primary fetal macrophages but not in primary trophoblasts.

Our data supports the hypothesis that presence of cross-reactive DENV antibodies could be an important risk factor for transplacental ZIKV transmission. Furthermore, we demonstrate that the *ex vivo* placental perfusion model is a relevant and animal friendly model to study transplacental pathogen transmission.

Author Summary

Zika virus is a mosquito-transmitted virus that can cause severe birth defects such as microcephaly when the infection occurs during pregnancy. Understanding how Zika virus crosses the placenta during pregnancy is important for future prevention strategies for vertical Zika virus transmission. Despite significant efforts to study this, to date it remains incompletely understood how Zika virus can cross the placenta and which risk factors contribute to this form of transmission. In this study we use an *ex vivo* placental perfusion model to study transplacental Zika virus transmission. The *ex vivo* placental perfusion model is a highly physiological and animal friendly model that mimics the *in vivo* conditions during pregnancy. We found that antibodies against the closely related dengue virus can significantly enhance placental uptake of Zika virus and Zika virus infection of human placental explants and fetal macrophages. These findings indicate that presence of cross-reactive dengue virus antibodies could contribute to transplacental Zika virus transmission.

Introduction

Zika virus (ZIKV) is a mosquito-transmitted virus from the *Flaviviridae* family, genus flavivirus. A ZIKV infection during pregnancy can result in severe birth defects such as microcephaly and in fetal loss (9, 245). It is estimated that this occurs in 2-20% of ZIKV infections during pregnancy (81, 83). To date it remains incompletely understood how ZIKV can cross the placenta which normally serves as an important physical and immunological barrier to prevent maternal-fetal pathogen transmission (116). More insight on how ZIKV crosses the placenta and risk factor identification is important for risk inventory and to create prevention strategies for transplacental ZIKV transmission.

The human placenta consists of many chorionic villi, which are tree-like structures that are the functional units of the placenta. These villi are either floating in maternal blood in the intervillous space or are anchored to the maternal part of the placenta (decidua) through extravillous trophoblasts. Multinucleated syncytiotrophoblasts line the chorionic villi, forming a cellular barrier against pathogens. Cytotrophoblasts are located below the syncytiotrophoblasts and can differentiate in extravillous trophoblasts and syncytiotrophoblasts. The stroma of chorionic villi consists of fibroblasts, fetal macrophages (Hofbauer cells) and endothelial cells. From the second pregnancy trimester, maternal blood flows in the intervillous space where it comes into contact with the chorionic villi (288). From this pregnancy stage, maternal immunoglobulin G (IgG) is actively transported across the placenta through neonatal Fc-receptor (FcRn) mediated transcytosis in syncytiotrophoblasts (289, 290).

Presence of antibodies against dengue virus (DENV), a flavivirus that is closely related to ZIKV, has been suggested to be a risk factor for transplacental ZIKV transmission through FcRn-mediated transcytosis of ZIKV – DENV antibody immune complexes across syncytiotrophoblasts in the placenta. This mechanism of transplacental transport has previously been observed for cytomegalovirus (CMV) while for ZIKV, FcRn-mediated transplacental transcytosis has been observed in pregnant mice but not in pregnant non-human primates (NHP) (94, 253, 254, 287, 291). A possible explanation for these conflicting results is that there are important structural differences between the rodent and NHP placenta such as the presence of trophoblasts giant cells in the rodent placenta while these cells are not present in the NHP and human placenta. Furthermore, deep trophoblast invasion, which in humans is essential for optimal nutrition of the fetus, is limited in especially the rodent- but also the NHP placenta (292, 293). Therefore, there is a need to study the possible harmful effects that cross-reactive DENV antibodies may have on transplacental ZIKV transmission in a model that better represents the human *in vivo* conditions during pregnancy.

In this study, we use the human *ex vivo* dual placental perfusion model to study the effects of cross-reactive DENV antibodies on transplacental ZIKV transmission. This physiologically representative and dynamic model is well suited to study placental uptake and transplacental transport with conserved structural integrity (294, 295). By using this model and human placental explants, we demonstrate that cross-reactive DENV antibodies enhance placental ZIKV uptake and infection.

Methods

Ethics statement

Placentas of women with uncomplicated singleton pregnancies who underwent an elective cesarean section were collected immediately after delivery at the Erasmus Medical Center, Rotterdam, the Netherlands. Patients with retained placenta, viral infections (HIV, hepatitis B, ZIKV), the presence of fetal congenital abnormalities and any form of diabetes were excluded. The study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418). Written informed consent was obtained from all patients prior to donating their placenta for this study.

Virus strain

The Asian lineage of Zika virus (Suriname 2016, GenBank KU937936, third passage) was used for all infection experiments. For the placental perfusion experiments, this virus was

inactivated by incubation with 0.02% β-propiolactone (BPL, Ferak Berlin GmbH) for three days at 4°C followed by three hours incubation at 37°C to hydrolyze BPL. Inactivation of the virus was confirmed by inoculation of Vero cells with high concentrations of the inactivated virus for five days at 37°C which resulted in no infection based on absence of cytopathogenic effect (CPE) and staining of ZIKV E-protein assessed with fluorescent microscopy.

Placental perfusion experiments

The *ex vivo* dual placental perfusion model used in this study was performed as described in detail by Hitzerd et al. and was located in a biosafety level 1 laboratory (296). In short, placentas were collected directly after cesarean section and were attached to the perfusion machine. The double set-up of this system allowed for perfusion of two cotyledons at the same time if placentas had two cotyledons that were in sufficient condition to be attached to the perfusion machine. The maternal and fetal compartments were perfused with 200 mL Krebs-Henseleit buffer supplemented with 5000 IU heparin and 95% O_2, 5% CO_2 at 37°C. After an equilibration and washout period of approximately 60 minutes, $ZIKV^{BPL}$ that was pre-incubated for 60 minutes with 333 µl pooled flavivirus naïve serum or pooled serum containing DENV-2 nAbs (both 1:250 dilution) was added to the maternal circulation in a concentration of $1x10^5$ $TCID_{50}$ equivalent/mL. To block the interaction between immune complexes and FcRn, protein G was added to $ZIKV^{BPL}$ – immune complexes at a concentration of 3 µg/ml and 9 µg/ml, 60 minutes prior to adding the complexes to the maternal circulation. Placentas were perfused for either 40 or 120 minutes and samples from the maternal and fetal circulation were collected every 10 or 15 minutes, respectively, for ZIKV RNA detection with RT-PCR. After 40 or 120 minutes of perfusion, placentas were flushed with fresh perfusion medium in an open circulation for 10 or 30 minutes, respectively, to wash away residual, unbound ZIKV. Next, 4mm tissue biopsies were taken from the perfused cotyledon of the placenta after which the tissue was placed in a 4% formaldehyde solution for fixation for subsequent immunohistochemistry.

As a quality control for capillary leakage, which results in leakage of perfusion fluid from the fetal compartment to the maternal compartment, FITC-dextran (40kDa, 36mg/L) was added to the fetal circulation before start of perfusion. Samples were taken every 30 minutes from the fetal and maternal circulation and FITC-dextran levels in these samples were determined using a Multiwell Plate Reader (Victor X4 Perkin Elmer). In all perfusion experiments that are used for analysis, the fetal-to-maternal FITC-dextran ratio was below the cut-off for capillary leakage (0.03). Experiments in which capillary leakage did occur during perfusion were excluded from analysis. Antipyrine (100 mg/L) was added to the maternal circulation and

detected in the fetal circulation using ultraviolet-visible spectroscopy (Shimadzu UV-1800) to confirm adequate overlap between the maternal and fetal circulation. ZIKV RT-PCR in the fetal circulation was only performed if the fetal-to-maternal antipyrine ratio was >0.65 after 120 minutes of perfusion, which was the case in 65% of the perfusions.

ZIKV quantification

The amount of ZIKV in supernatants, expressed as 50% tissue culture infective dose per milliliter supernatant ($TCID_{50}$/mL), was determined by 10-fold dilution endpoint titration on Vero cells and the method of Kärber was used to calculate $TCID_{50}$ titers from three replicates (261).

For determination of ZIKV RNA in placenta tissue, the tissues were homogenized with a ceramic bead in a FastPrep-24 5G sample disruptor instrument (MP Biomedicals). ZIKV RT-PCR was performed with the 1086/1162c/1107-FAM primers/probes set described by Lanciotti et. al (120). $TCID_{50}$ equivalent values were extrapolated from a standard curve that was generated by making a 10-fold dilution of a ZIKV virus stock with a known virus titer ranging from 10^6 $TCID_{50}$ to 10^1 $TCID_{50}$.

Human sera

Sera that were obtained from a previously performed ZIKV seroprevalence study in Suriname that did not contain ZIKV nAbs were tested for ADE potential with an *in vitro* ZIKV ADE assay using U937 cells (Department of Immunology, Erasmus MC, the Netherlands) as described previously (114, 136). Infection experiments with placental explants were performed with one serum containing DENV-2 nAbs which reached a peak ADE titer at 1:200 dilution. ADE was defined as a statistically significant increase in viral titer in supernatants in presence of serum containing cross reactive antibodies compared to conditions without serum or with control serum. Because of the relatively large volume of the perfusion medium (200 mL), placental perfusion experiments were performed with pooled serum from six donors. This pooled serum reached a peak ADE titer at 1:200 dilution. DENV-2 nAb titers were determined in these sera as described before and were >1:100 for all sera, see S2 Table (251). Two sera in which no antibodies against all clinically relevant flaviviruses (among others ZIKV, DENV, yellow-fever virus, West Nile virus, tick-borne encephalitis virus and Japanese encephalitis virus) were detected with a protein microarray, were used as a negative control (162).

Infection of human placental explants

Placentas were collected within 30 minutes after an elective caesarian section. Tissues were extensively washed with PBS to remove blood and antibodies. After the removal of the decidua

and chorionic plate, tissues containing chorionic villi were cut into blocks of approximately 3x3 mm which were placed in a 24-wells plate in 1 mL of DMEM/F12 medium (Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma), 5.7mL 7.5% Sodium Bicarbonate (Lonza) and 50 mg/mL Primocin (InvivoGen). Subsequently, the villi were infected with either $1.0x10^5$ $TCID_{50}$/mL ZIKV, ZIKV+DENV nAb containing serum, ZIKV+1 µg/mL humanized 4G2 (hu4G2, IgG1, The Native Antigen Company) or ZIKV+flavivirus naïve serum in 750µl medium. At six days post infection (dpi), half of the tissues were homogenized for RNA isolation and ZIKV RT-PCR, and the other tissues were fixed in a 4% formaldehyde solution for immunohistochemistry and *in situ* hybridization (ISH).

For Fc-gamma receptor (FcγR) blocking experiments, explants were pre-incubated for two hours with monoclonal antibodies against FcγRs (clones 3G8, 6C4 and 10.1), 3 µg/well. After two hours, $ZIKV^{BPL}$–DENV nAbs immune complexes were added to these tissues and incubated for another two hours after which the tissues were washed three times, and the explants were incubated in 1 mL culture medium for 48 hours. For blocking the interaction of IgG with FcRn, recombinant protein G (ThermoFisher, 1.5 µg/mL) was added to $ZIKV^{BPL}$ – DENV nAbs immune complexes and incubated for one hour at 37°C before adding this to placental explants for two hours. After two hours, explants were washed three times and incubated in 1 mL culture medium for 48 hours.

ZIKV RNA detection with *in situ hybridization and immunohistochemistry co-staining*

ISH for ZIKV RNA was performed with RNAscope (Advanced Cell Diagnostics) on 5 µm thick slides of formalin fixed, paraffin embedded tissues, according to the manufacturer s instructions and as described before (80). Controls included a positive and negative control probe (ubiquitin C and DapB respectively) and uninfected placenta tissue on which the ZIKV probe was used. For immunohistochemistry, tissues were deparaffinized and rehydrated and boiled for 15 minutes in an EDTA buffer. Tissues were subsequently incubated with anti-cytokeratin-7 antibody (Abcam, ab52870, 1:350 dilution) followed by a HRP labeled goat anti mouse antibody (DAKO 1:100 dilution) to visualize trophoblasts. To visualize Hofbauer cells (HBCs), tissues were incubated with an anti-CD163 antibody (ThermoScientific, 1:100 dilution) followed with a rabbit anti mouse IgG antibody labeled with biotin (DAKO, E03354 1:100 dilution) followed by adding streptavidin-HRP (DAKO, D0397, 1:300 dilution). AEC (3-Amino-9-ethylcarbazole, Abcam) was used as HRP substrate, and counterstaining with Mayer's hematoxylin (Merck) was performed for all tissues.

Isolation of Hofbauer cells and trophoblasts

HBCs and trophoblasts were isolated as previously described before with few alterations (297). After the decidua basalis was thoroughly removed and tissues were extensively washed with PBS, tissues were minced and incubated in digestion medium (RMPI-1640 medium (Lonza) supplemented with 10% FBS and 1 mg/mL collagenase IV (Worthington Biochem. Corp), 300 µg/mL DNAse 1 (Worthington Biochem. Corp)) at 37°C. Four digestion cycles of 30 minutes were performed and after each cycle, tissue was mechanically dissociated using gentleMACS Tissue Dissociator (Miltenyi Biotec). The cell suspension after the first digestion cycle, containing mainly dead cells and erythrocytes, was discarded. Mononuclear cells were isolated by using 30-70% Percoll density gradient centrifugation (GE Healthcare Life Sciences). HBCs were isolated from the interphase fraction using CD14-positive magnetic isolation as per manufacturer's instructions (Miltenyi Biotec). The CD14-negative fraction containing cytotrophoblasts was collected and plated in a 96-wells plate that was treated with fibronectin and incubated overnight, after which non-adherent cells were washed away with fresh medium. Purity of the isolated cells, determined with confocal laser scanning microscopy, was >95% for HBCs and >80% for trophoblasts.

Infection and visualization of Hofbauer cells and cytotrophoblasts

HBCs and trophoblasts were seeded at a density of $1.0x10^5$ cells per well and infected with ZIKV or ZIKV+DENV nAbs at a multiplicity of infection (MOI) of 0.5 for 48 hours. FcγR-blocking antibodies were added to the cells one hour prior to infection at a concentration of 10 µg/mL. In some conditions, protein G (1.5 µg/mL) was added to ZIKV - DENV nAbs immune complexes and incubated for one hour before adding this to the cells. For visualization with confocal laser scanning microscopy (Zeiss), cells were fixed and permeabilized (BD Cytofix/Cytoperm) and stained with primary antibodies against CD68 (DAKO, 1:75 dilution), cytokeratin-7 (Abcam, ab52870, 1:350 dilution) and 4G2 (Merck Millipore, 1:200) and secondary antibodies goat-anti mouse IgG1 A647, goat-anti mouse IgG2A AF488 and donkey anti-rabbit AF555 (all Invitrogen, 1:400 dilution). Nuclei were stained with Hoechst 33342 (Invitrogen). Percentage of infected cells and purity of cells were calculated using ImageJ. Processing of the confocal laser scanning microscopy images was done with the ImageJ plugin QuickFigures (298).

Cytokine detection in supernatants

Cytokines were quantified in supernatants of primary placental cells with a 13-plex bead-based assay (Biolegend, LEGENDplex) according to the manufacturer's instructions. Readout was

performed with flow cytometry (BD FACSlyric), and data was analyzed with LEGENDplex data analysis software (Biolegend). Cytokine levels in supernatants of uninfected placental cells were compared to cytokine concentrations in supernatants of cells infected with ZIKV+control and ZIKV+DENV nAbs.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (Graphpad Software Inc). For non-normally distributed variables, the Kruskall-Wallis test with Dunn's post hoc test was used while for normally distributed variables a one-way ANOVA with Dunnett's post hoc test was used. For the placental perfusion experiments, ZIKV $TCID_{50}$ equivalents/mL in the maternal circulation were compared at all time points using multiple t-tests with the Holm-Šdák correction for multiple testing. A P-value <0.05 was considered to be statistically significant.

Results

Ex vivo perfused placentas efficiently take up ZIKV immune complexes

To study the role of DENV antibodies on placental uptake of ZIKV in a physiologically relevant model, we used the *ex vivo* dual perfused human placenta model. In this model, a cotyledon of the human placenta is attached to a perfusion machine directly after birth to recreate separate maternal and fetal circulations (Fig 1A). In comparison to placental explants, a commonly used model to study placental infection, the placental perfusion model has intact structural integrity and resembles the dynamic *in vivo* route of viral infection at the maternal-fetal interface better (294, 299-301).

Placental perfusion experiments were performed with β-propriolactone (BPL) inactivated Asian lineage ZIKV ($ZIKV^{BPL}$) because of biosafety regulations. We confirmed that $ZIKV^{BPL}$ was replication incompetent but that the viral RNA could still be detected with RT-PCR and with ISH (S1 Fig). Experiments were performed with pooled serum containing DENV-2, but not ZIKV, neutralizing antibodies ($ZIKV^{BPL}$+DENV nAbs) that was shown to optimally enhance ZIKV infection in U937 cells at a 1:200 dilation (S2 Fig). As a negative control, pooled serum without flavivirus antibodies was used ($ZIKV^{BPL}$+control). Clinical data of participants from whom the placenta was used for perfusion are summarized in S1 Table.

A rapid reduction of ZIKV RNA levels was observed in the maternal circulation of placentas perfused with $ZIKV^{BPL}$+DENV nAbs, indicating efficient placental uptake of $ZIKV^{BPL}$ immune complexes (43.0-fold reduction in $TCID_{50}$ equivalent/mL after 120 minutes, Fig

1B). ZIKV RNA levels remained stable in the maternal circulation of placentas perfused with ZIKV^{BPL}+control (1.2-fold reduction in $TCID_{50}$ equivalent/mL after 120 minutes, Fig 1B) indicating limited placental uptake of ZIKV^{BPL} in absence of DENV nAbs. In both conditions, ZIKV RNA could not be detected in the fetal circulation after 120 minutes of perfusion. To assess whether FcRn-mediated transcytosis of ZIKV immune complexes was the main mechanism of the efficient placental uptake of ZIKV immune complexes, protein G was added to the ZIKV immune complexes prior to placental perfusion. Protein G binds IgG at the FcRn binding domain (the hinge proximal region of the CH2 domain) and therefore blocks the interaction between FcRn and IgG but not between IgG and FcγRs (302, 303). Protein G reduced the placental uptake of ZIKV^{BPL}+DENV nAb immune complexes in a dose dependent manner with a 7.2-fold decrease in ZIKV RNA levels in the maternal circulation with the highest protein G concentration compared to a 43.0-fold decrease without protein G after 120 minutes of perfusion (P<.001, Figs 1C and S3A).

After 120 minutes of perfusion and a 30 minute washout period with fresh perfusion medium to remove any residual unbound ZIKV, tissue biopsies were taken from the perfused cotyledon of the placenta. ZIKV RT-PCR was performed on the homogenized tissue biopsies and surprisingly, no difference was found in ZIKV RNA levels in placentas perfused for 120 minutes with ZIKV^{BPL}+control, ZIKV^{BPL}+DENV nAbs or ZIKV^{BPL}+DENV nAbs+protein G (mean 1193 vs. 740 vs. 441 $TCID_{50}$ equivalent/100 mg tissue, respectively, P>0.05, S3B Fig). We ruled-out that ZIKV^{BPL} – DENV nAbs immune complexes adhered to the tubes of the placental perfusion machine by demonstrating that ZIKV RNA levels remained stable during circulation of ZIKV^{BPL}+control and ZIKV^{BPL}+DENV nAbs through the perfusion machine to which no placenta was attached (S3C Fig).

It has previously been demonstrated that BPL can successfully inactivate ZIKV while containing the antigenicity of the particle (304). However, reduced viral membrane fusion has been observed with BPL inactivated influenza virus (305, 306). We hypothesized that if reduced viral membrane fusion would also occur after BPL inactivation of ZIKV, this can result in rapid breakdown of ZIKV^{BPL} – DENV nAbs immune complexes in placental cells which could explain the lack of difference in ZIKV RNA levels in placentas perfused with ZIKV^{BPL}+control compared to ZIKV^{BPL}+DENV nAbs. Therefore, we performed shorter perfusions of 40 minutes followed by a washout period of ten minutes. Again, we observed rapid placental uptake of ZIKV^{BPL}+DENV nAbs from the maternal circulation compared to ZIKV^{BPL}+control (18.3 vs. 0.87-fold decrease in $TCID_{50}$ equivalent/mL, respectively, P<.0001, Fig 1D). In these settings, we did detect significantly more ZIKV RNA in placentas perfused with ZIKV^{BPL}+DENV nAbs compared to ZIKV^{BPL}+control (average 773 vs. 117 $TCID_{50}$

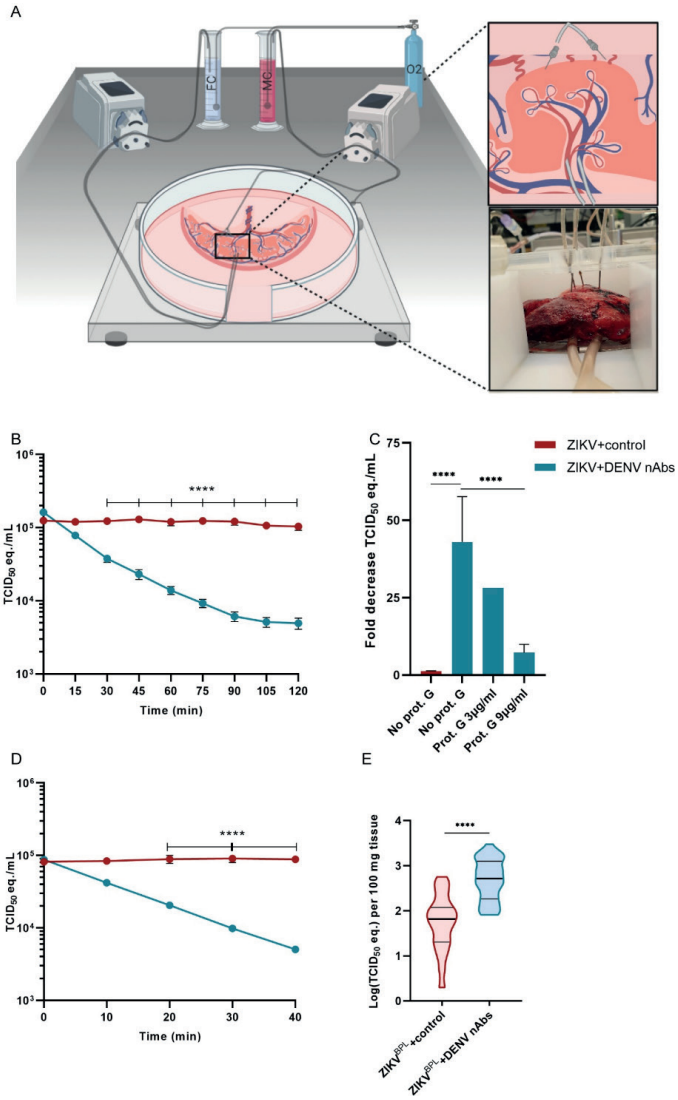

Figure 1. Efficient uptake of ZIKV immune complexes by *ex-vivo* perfused placentas.
$1*10^5$ $TCID_{50}$ equivalent/mL inactivated ZIKV ($ZIKV^{BPL}$) was incubated with either flavivirus naïve serum or serum containing DENV-2 nAbs and added to the maternal circulation (MC) of the placental perfusion model and perfused for 40 or 120 minutes. **A:** Schematic overview of the *ex vivo* dual placental perfusion model. FC; fetal circulation. Created with Biorender.com. **B:** ZIKV RNA levels in the MC were determined every 15 minutes with RT-PCR up to 120 minutes to detect placental uptake of $ZIKV^{BPL}$. Dots represent mean ±SEM, N=3-4 donors per condition. **C:** Mean fold reduction (+SEM) in ZIKV $TCID_{50}$ equivalent in the MC after 120 minutes of perfusion with and without different concentrations of protein G (Prot. G) to block the interaction of IgG with FcRn. **D:** ZIKV RNA levels in the MC were determined every 10 minutes with RT-PCR up to 40 minutes. Dots represent mean ±SEM, N=2 donors per condition. **E:** ZIKV RNA was detected in tissue biopsies of the perfused placentas after 40 minutes of perfusion.
Horizontal lines represent median and the 10^{th} and 90^{th} percentile cut-off. N=2 donors per condition. Data from the MC were analyzed with multiple t-tests with the Holm-Šidák correction and data from tissue lysates were analyzed with the Mann-Whitney U test. ****P<0.0001.

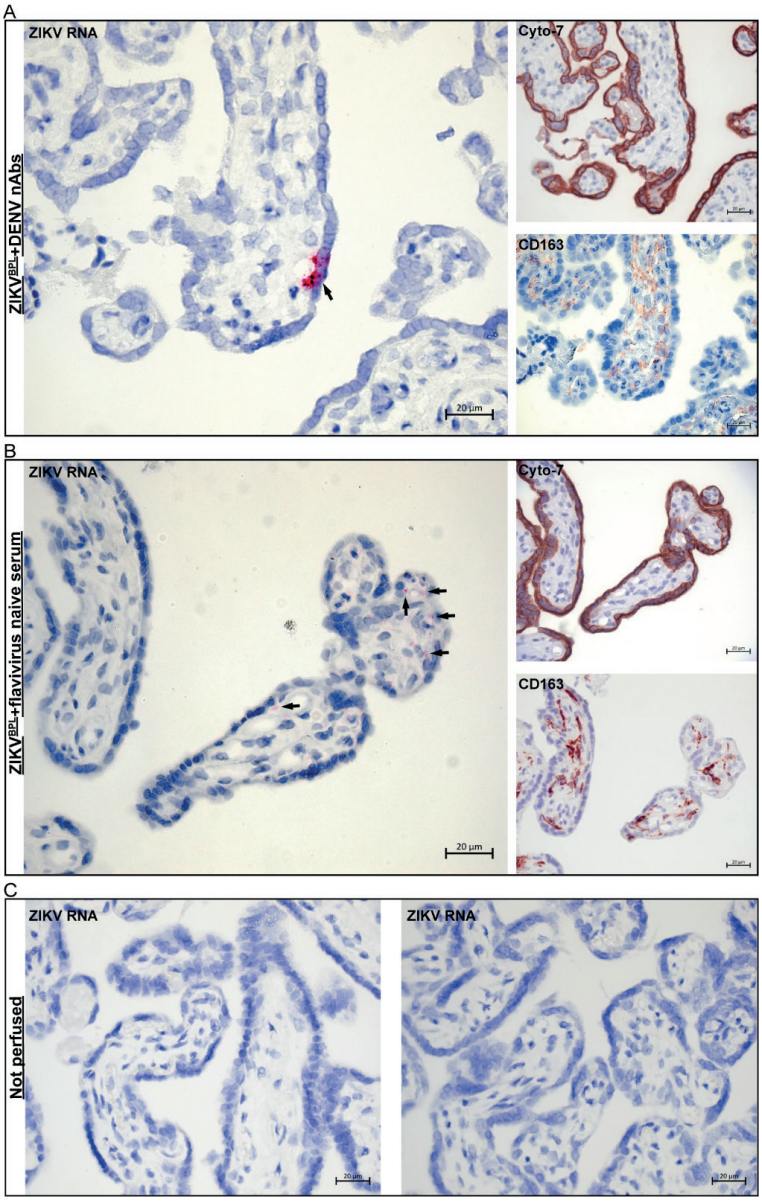

Figure 2. Detection of ZIKV RNA in chorionic villi of placentas perfused with ZIKV.
ZIKV RNA was detected with ISH in formalin fixed, paraffin embedded tissue of perfused placentas. Chromogenic staining for cytokeratin-7 (cyto-7) and CD163 was performed in sequential slides to detect trophoblasts and Hofbauer cells, respectively. **A:** Staining for ZIKV RNA, trophoblasts and Hofbauer cells in a placenta that was perfused for 40 minutes with $ZIKV^{BPL}$+DENV nAbs. **B:** Staining for ZIKV RNA, trophoblasts and Hofbauer cells in a placenta that was perfused for 120 minutes with $ZIKV^{BPL}$+flavivirus naïve serum. **C:** Staining for ZIKV RNA in a placenta that was obtained directly after birth and was not perfused.
Arrows indicate positive signal for ZIKV RNA.

equivalent/100 mg tissue, respectively, P<.0001, Fig 1E). Collectively, these data suggest that placental uptake of ZIKV immune complexes is highly efficient and FcRn dependent. One possible explanation for the observation that after 40 minutes but not after 120 minutes of perfusion, more ZIKV RNA was found in placentas perfused with $ZIKV^{BPL}$+DENV nAbs compared to $ZIKV^{BPL}$+control is that BPL inactivation of ZIKV reduces the ability of endosomal escape resulting in lysosomal breakdown. We did, however, not investigate this hypothesis in detail in the current study.

Lastly, we performed ISH for ZIKV RNA on the perfused cotyledons to try to detect ZIKV RNA. As expected from replication incompetent virus, the signal intensity for ZIKV RNA was low. We did, however, sporadically detect ZIKV RNA in trophoblasts and chorionic villi in placentas that were perfused for 40 minutes with $ZIKV^{BPL}$ +DENV nAbs and placentas perfused for 120 minutes with $ZIKV^{BPL}$+control (Figs 2A and 2B) but not in non-perfused placentas (Fig 2C). These results suggest that ZIKV can cross the placental barrier of term placentas during *ex vivo* perfusion.

FcRn-mediated ADE of ZIKV infection in placental explants

To confirm the results from the placental perfusion model with infectious virus, placental villus explants were isolated from term placentas and infected with ZIKV alone, ZIKV+DENV nAbs or ZIKV+control. As a positive control for ADE, ZIKV was pre-incubated with a humanized, monoclonal pan-flavivirus antibody (ZIKV+hu4G2) that is known to induce ADE of flavivirus infection *in vitro* (276).

Significantly higher ZIKV titers were found in supernatants of explants infected with ZIKV+DENV nAbs and ZIKV+hu4G2 compared to explants infected with only ZIKV or ZIKV+control at all the time points but mainly at two dpi (49.4-fold and 45.9-fold increase in median titer compared to only ZIKV, respectively, P<.0001, Fig. 3A). Part of the explants were homogenized at six dpi for ZIKV RNA detection with RT-PCR and ZIKV RNA levels were significantly higher in explants infected with ZIKV+hu4G2 but not with ZIKV+DENV nAbs compared to only ZIKV at six dpi (12.2-fold and 2.9-fold increase in $TCID_{50}$ equivalent, respectively, P=.03 and P=.99, Fig 3B).

We next studied the contribution of different FcγRs and FcRn on ADE of ZIKV in placental explants by pre-incubating the explants with monoclonal antibodies that block the interaction with IgG and FcγRs (307-311). Furthermore, protein G was added to the ZIKV immune complexes prior to adding them to the explants to block the interaction between IgG and FcRn. We confirmed that adding protein G to ZIKV – DENV nAbs immune complexes did not inhibit ADE of ZIKV infection in the monocytic cell line U937 that is commonly used for

in vitro ADE assays (S4 Fig). Since we mainly observed ADE of ZIKV infection in placental explants at two dpi, Fc-receptor blocking experiments were performed until two dpi.

Protein G significantly reduced ADE of ZIKV infection in placental explants (41-fold reduced median ZIKV titer and a 78-fold reduced median RNA levels, P<.0001, Fig 3C). Blocking FcγRII resulted in a 16-fold reduction in ZIKV titer in supernatants but this difference was not statistically significant (P=.07).

Collectively, these data confirm that serum containing DENV antibodies can enhance ZIKV infection in placental explants and that this enhancement is FcRn dependent.

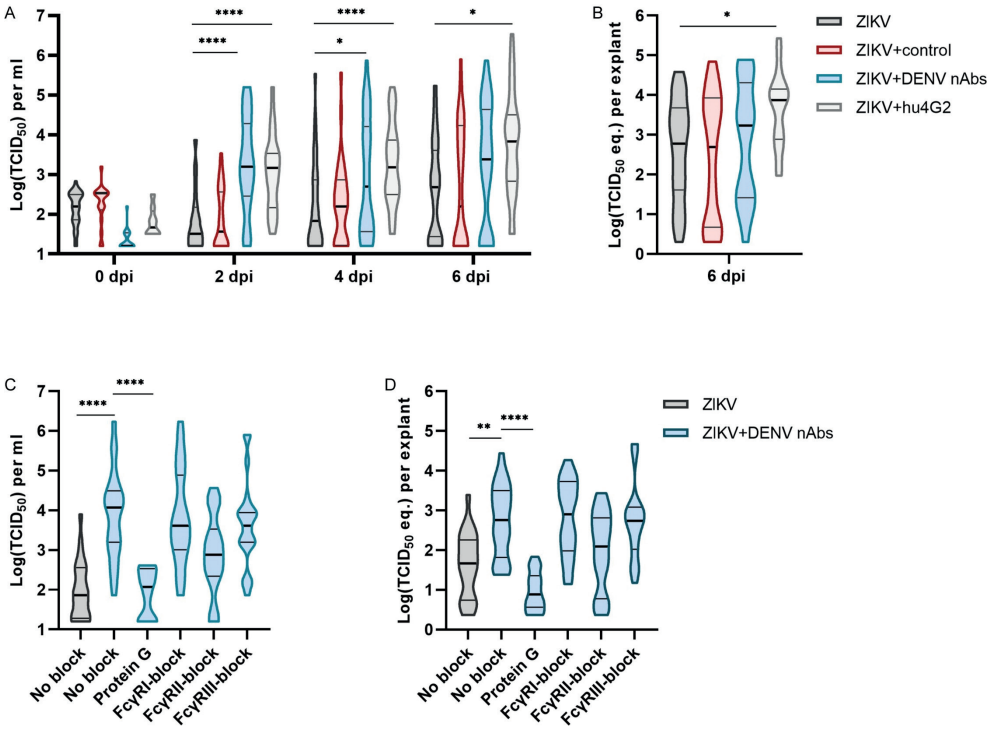

Figure 3. Cross-reactive flavivirus antibodies enhance ZIKV infection in placental explants.
A&B: Term human placental explants were infected with $1.0x10^5$ $TCID_{50}$/mL ZIKV alone or ZIKV that was preincubated with flavivirus naïve serum (ZIKV+control) or serum containing DENV-2 neutralizing antibodies (ZIKV+DENV nAbs), both in a dilution of 1:250, or with a humanized panflavirus monoclonal antibody (hu4G2, 1μg/mL). ZIKV titers were determined in supernatants **(A)** and ZIKV RNA levels were determined with RT-PCR in tissue lysates **(B)**. **C&D**: Term human placental explants were pre-treated with FcγR blocking antibodies or protein G was added to ZIKV – DENV nAbs immune complexes. Subsequently, the explants were infected with either $1.0x10^5$ $TCID_{50}$/mL ZIKV or ZIKV+DENV nAbs. ZIKV titers were determined in supernatants at two dpi **(C)** and ZIKV RNA levels were determined in tissue lysates with RT-PCR at two dpi **(D)**. N=3-4 donors per condition. Horizontal lines in the violin plots represent median and the 10th and 90th percentile cut-off. Statistical significance was determined using the Kruskal-Wallis test followed by Dunn's post hoc test. * P<0.05, **P<0.01, ****P<0.0001.

ZIKV infects Hofbauer cells and trophoblasts in placental explants

Next, we investigated which placental cells got infected with ZIKV in the placental explants. We performed ISH for ZIKV RNA in the placental explants that were infected with ZIKV, ZIKV+control, ZIKV+DENV nAbs and ZIKV+hu4G2 for six days. ZIKV RNA was more often detected and of higher intensity in placental explants infected with ZIKV+DENV nAbs or ZIKV+hu4G2 compared to explants infected with ZIKV or ZIKV+control in which ZIKV RNA signal was only sporadically detected (Figs 4A-F). To determine which type of placental cells got infected with ZIKV, sequential slides were stained for CD163 and cytokeratin-7 as markers for HBCs and trophoblasts, respectively. In the explants infected with ZIKV+hu4G2 and ZIKV+DENV nAbs, ZIKV RNA was detected in HBCs (Figs 4A-C) and in villous trophoblasts (Fig 4A). These findings demonstrate that during ADE of ZIKV infection in term placental explants, both villous trophoblasts and HBCs can get infected.

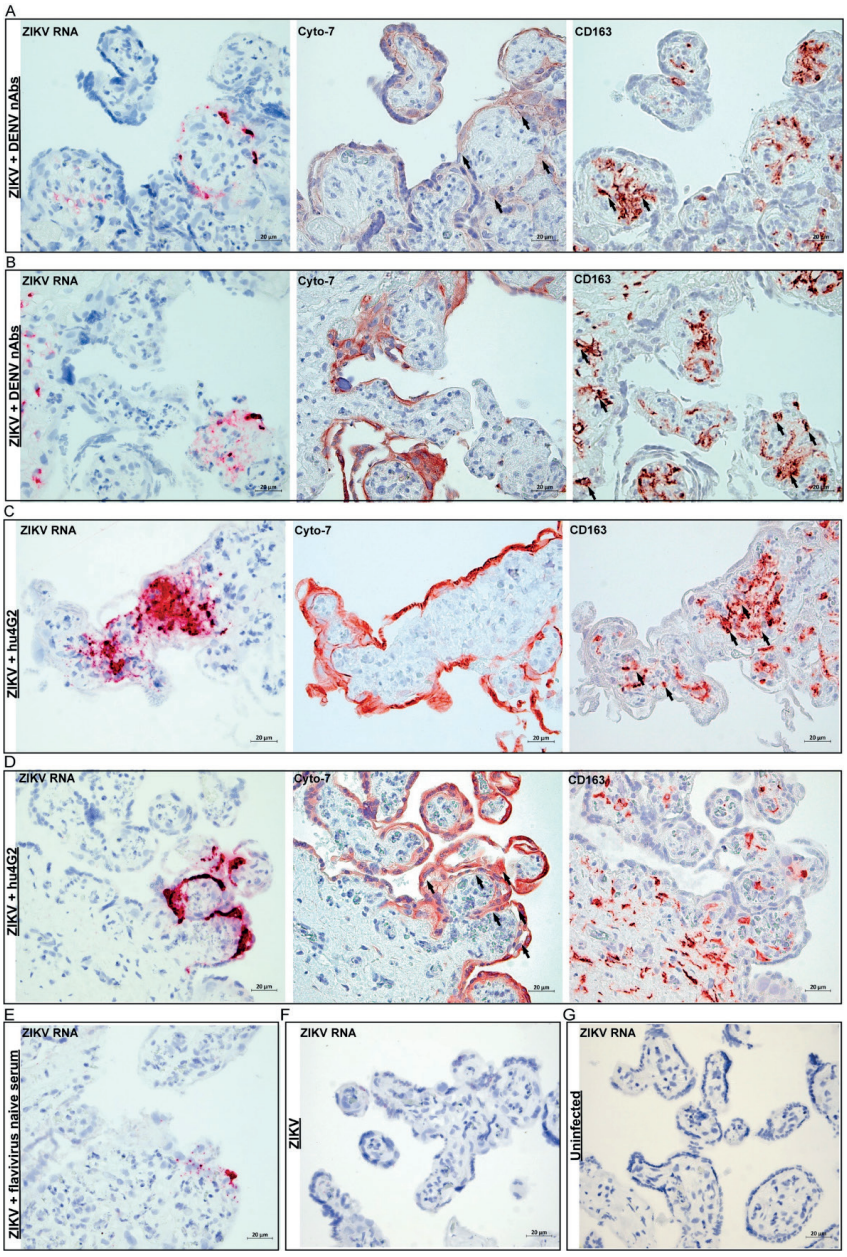

Figure 4. ZIKV infects Hofbauer cells and trophoblasts in placental explants in presence DENV nAbs. **A-D:** Representative pictures of ZIKV infection in Hofbauer cells (CD163) and trophoblasts (Cyto-7), in term human placental explants infected with ZIKV that was pre-incubated with human serum containing DENV-2 nAbs (**A&B)** or a humanized pan-flavivirus monoclonal antibody (hu4G2, **C&D**). Arrows indicate cells stained with either Cyto-7 or CD163 that correspond with cells in which ZIKV RNA was detected. **E-G:** Staining for ZIKV RNA in placental explants infected with ZIKV+flavivirus naïve serum **(E)**, only ZIKV **(F)** or uninfected **(G)**. All tissues were stained six days after (mock) infection.

Primary HBCs are permissive for ZIKV infection and ADE of ZIKV infection

To further confirm the role of HBCs and trophoblasts in ZIKV infection and ADE of ZIKV infection, we isolated and infected HBCs and trophoblasts from term human placentas.

Infection of HBCs with ZIKV+control resulted 2.2% infected cells while this increased to 8.8% when HBCs were infected with ZIKV+DENV nAbs (P=.0001, Figs 5A and 5C). Infection of HBCs with ZIKV+DENV nAbs also resulted in significantly enhanced ZIKV titers in supernatants compared to infection with ZIKV+control (21.5-fold increased median ZIKV titer at two dpi, P<.0001, Fig. 5D). ADE of ZIKV infection in HBCs was partially inhibited by pre-treating the cells with FcγRII blocking antibodies (13.7-fold reduced median ZIKV titer in supernatant compared to no FcγRII-block, P=.003, Fig 5D). For trophoblasts, percentage of infected cells and viral titers in supernatants were negligibly low for cells infected with ZIKV+control (0.6% and median 32 $TCID_{50}$/mL) and ZIKV+DENV nAbs (0.9% and median 498 $TCID_{50}$/mL) (Figs 5B, 5C and 5E).

To determine the effect of ZIKV infection and ADE of ZIKV infection on cytokine production, we used a 13-plex bead-based assay to determine concentrations of cytokines and chemokines in the supernatants of HBCs and trophoblasts at two days post (mock) infection. ADE of ZIKV infection in HBCs significantly induced production of TNF-α and IP-10 compared to uninfected HBCs (1.7-fold increase and 4.1-fold-increase respectively, P=.014 and P=.0001, Figs 5F and 5G). No differences were seen for other cytokines, notably not for cytokines that are associated with intrinsic ADE such as IL-6 and IL-10 (Figs 5H and 5I and S5A Fig) (112, 113). Cytokine concentrations in supernatants of trophoblasts that were infected with ZIKV+control or ZIKV+DENV nAbs did not differ from uninfected trophoblasts (S5B Fig).

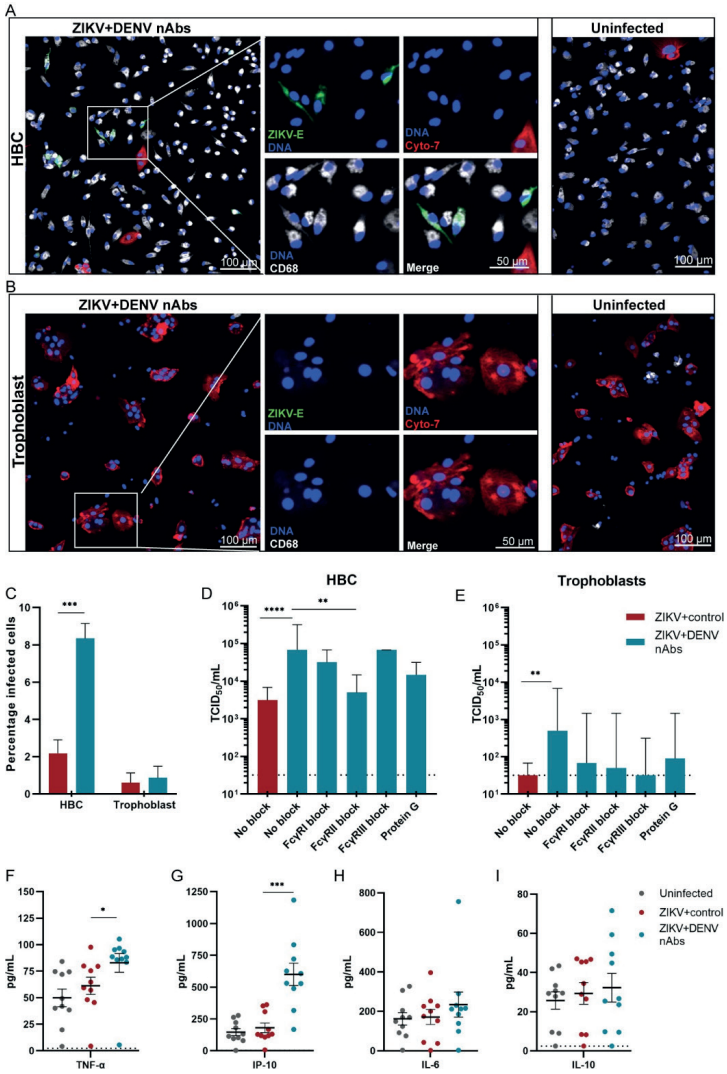

Figure 5. Primary Hofbauer cells are permissive for ZIKV infection and ADE of ZIKV infection.
Hofbauer cells (HBCs) and trophoblasts were isolated from term human placentas and infected with ZIKV+flavivirus naive serum (ZIKV+control) or ZIKV+DENV nAbs at an MOI of 0.5 in presence or absence of FcγR blocking antibodies and protein G, for 48 hours. **A:** Confocal laser scanning microscopy image of ZIKV+DENV nAbs infected HBCs and uninfected HBCs. **B:** Confocal laser scanning microscopy image of ZIKV+DENV nAbs infected trophoblasts and uninfected trophoblasts. HBCs were visualized by fluorescent staining for CD68, trophoblasts for cytokeratin-7 (Cyto-7), ZIKV by staining for ZIKV envelope protein (ZIKV-E) and nuclei with Hoechst 33342 staining (DNA). **C:** Percentage of infection of HBCs and trophoblasts was determined with confocal laser scanning microscopy. Bars represent mean+SEM. Significance was determined with a Student's T-test. **D&E:** ZIKV titers were determined in supernatants of HBCs and trophoblasts. Bars represent median+95%CI. Significance was determined using the Kruskal-Wallis test followed by Dunn's post hoc test, comparing ZIKV+DENV nAbs without block to the other conditions. **F-I:** Cytokines were determined in the supernatants of HBCs with a multiplex bead-based assay. Each dot represents one value of experiments performed in triplicate/quadruplicate, lines represent mean ±SEM. Significance was determined using one-way ANOVA with Dunnett's post hoc test. N=2-3 donors per condition for all experiments. *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

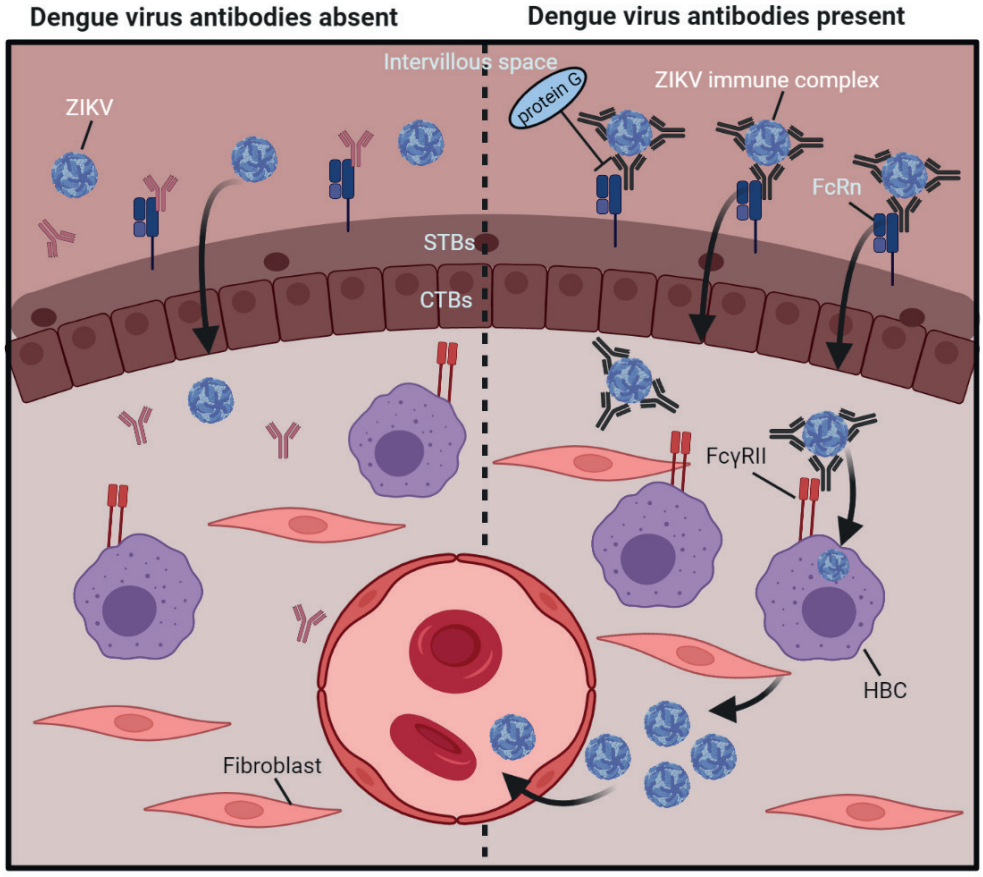

Figure 6. Proposed mechanism of ADE of ZIKV infection in the term human placenta.
Left panel: In absence of cross-reactive DENV antibodies, ZIKV crosses the placental barrier less efficiently than in presence of cross-reactive flavivirus antibodies through a mechanism that is not fully elucidated yet.
Right panel: In presence cross-reactive DENV antibodies, ZIKV immune complexes can be transported across the syncytiotrophoblasts layer through FcRn-mediated transcytosis. In the villus core, the non-neutralized complexes are taken up by the perivascular located HBCs through FcγRII, after which ZIKV can replicate in these cells. To reach the fetal circulation, ZIKV needs to subsequently cross the fetal endothelial barrier, possibly by infecting these cells. IVP; intervillous space, FcRn; neonatal Fc-receptor, STBs; syncytiotrophoblasts, CTBs; cytotrophoblasts, HBC; Hofbauer cell, FcγRII; Fcγ-receptor II. Created with Biorender.com.

Discussion

Here, we demonstrate that ex vivo perfused human placentas efficiently take up ZIKV– DENV nAbs immune complexes and that this is likely FcRn-dependent. Furthermore, we show that ZIKV infection in placental explants and in HBCs is enhanced by anti-flavivirus antibodies. Collectively, these data support the hypothesis of enhanced ZIKV transplacental transmission

through FcRn-mediated transcytosis of ZIKV – DENV nAbs immune complexes and through peripheral ADE of ZIKV infection in HBCs in the villus core as illustrated in Fig 6.

Because of the aforementioned limitations of placental explants and in vivo studies with pregnant mice, the pregnant NHP model is considered the most relevant to study transplacental pathogen transmission (116, 312). Compared to the pregnant NHP model, the ex vivo human placental perfusion model is a species specific, animal friendly and less expensive model that can be used to study transplacental pathogen transmission. This model has been previously used to study transplacental transmission of CMV, Coxsackie B-3 and ECHO-11 virus, however, transplacental transmission was not observed in these studies (313, 314). A limitation of this model is that the placenta can only be perfused for several hours. This limited perfusion time often does not allow for viral replication. Furthermore, perfusion experiments had to be performed with inactivated ZIKV because the placental perfusion model is situated in a biosafety level I laboratory. As expected with inactivated ZIKV and after a relative short perfusion time, we did not detect ZIKV RNA in the fetal circulation of perfused placentas. It is likely that viral replication in the villus core or endothelial cells is required for ZIKV to cross the endothelial barrier and reach the fetal circulation (39).

Besides ZIKV, FcRn-mediated transplacental transcytosis of virus-antibody immune complexes been observed for CMV (94). Furthermore, studies have demonstrated that immune complexes in general can cross the placenta (315, 316). This indicates that FcRn-mediated placental uptake or transplacental transcytosis of pathogen-antibody immune complexes likely is a more generalized phenomenon that is not restricted to ZIKV. However, in most cases, the pathogen will be neutralized by the antibodies bound to it and will not be able to escape the syncytiotrophoblasts or will subsequently be phagocytosed and cleared by HBCs (94, 317). Because of antibody cross-reactivity, non-neutralized virus-antibody complexes can easily arise in vivo for flaviviruses such as ZIKV. The reason why ZIKV is the only flavivirus associated with birth defects might be that ZIKV is the only flavivirus that can infect fetal endothelial cells (40).

FcRn-mediated transplacental transcytosis can only occur from the second pregnancy trimester onwards, since IgG is not transported across the placenta before this time. The risk of ZIKV induced congenital malformations in humans, however, is highest when a ZIKV infection occurs during the first trimester of pregnancy when the placenta is still developing (97). Therefore, FcRn-mediated transplacental transcytosis of ZIKV immune complexes might be an additional mechanism contributing to transplacental transmission of ZIKV in the second and third trimester of pregnancy while in the first trimester, ZIKV can infect

multiple proliferating placental cells (318). The reason why increased fetal pathology has not been observed in DENV immune pregnant NHP that got infected with ZIKV could be that these NHP were infected in the first trimester of pregnancy when maternal IgG is not yet transported efficiently across the placenta (287, 291, 319).

Based on previous studies and clinical observations, HBCs seem to be the main target for ZIKV infection in the human placenta while cytotrophoblasts and especially syncytiotrophoblasts seem less permissive (86, 98, 99, 245, 246, 320). However, in first trimester mouse placentas and first- and second trimester human placental explants, ZIKV was mainly detected in trophoblasts (41, 253, 254). Here, we found that during ADE of infection both trophoblasts and HBCs could be infected with ZIKV in term placental explants. After isolation of these cells from placental tissue, we found that HBCs are permissive for ZIKV infection and ADE of ZIKV infection while this was negligible for trophoblasts. Combining results from this study and previous studies, HBCs seem the main target for ZIKV infection and ADE of ZIKV. Trophoblasts mainly seem permissive for ZIKV infection early in pregnancy while later in pregnancy, they become more resistant to ZIKV infection (190-192, 253).

It has been reported that type I and type III interferons are important in restricting ZIKV infection in HBCs and trophoblasts, respectively (96, 246). However, we did not observe evident induction of these interferons during ZIKV infection or ADE of ZIKV infection in either HBCs or trophoblasts. In general, we found that ZIKV infection and ADE of ZIKV infection results in modest induction or inhibition of the cytokines that we tested, possibly because of the relatively low percentage of HBCs and trophoblasts that got infected.

There are several studies that indicate that DENV pre-immunity might not have a negative impact on disease severity of a ZIKV infection and might even offer protection against ZIKV infection (115, 321, 322). However, the data of this study indicates that caution is warranted for the potential harmful effects of cross-reactive DENV antibodies on transplacental transmission of ZIKV. This is relevant because of the co-circulation of ZIKV and DENV in many geographical regions and in the context of multivalent DENV vaccines that can induce an antibody repertoire that is partially cross-reactive with ZIKV (116, 249). Establishing the range of pre-existing DENV antibody titers that can increase the risk of transplacental ZIKV transmission in vivo, can be an important next step for risk assessment of vertical ZIKV transmission during pregnancy. Furthermore, we suggest that the ex vivo placental perfusion model is a highly relevant and animal friendly alternative for the pregnant NHP model to study transplacental pathogen transmission.

Supplementary data

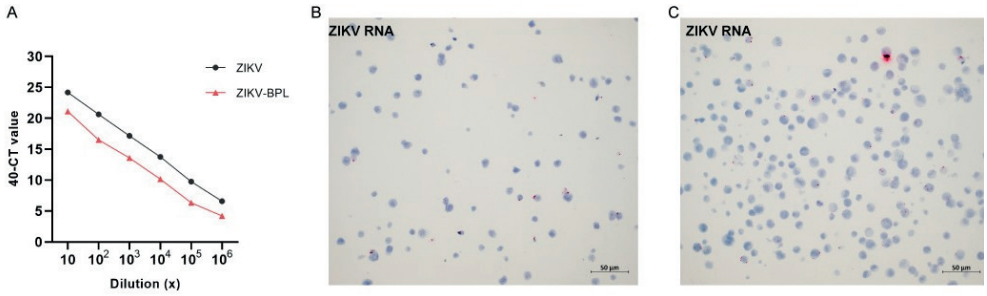

Figure S1. β-propriolactone inactivated ZIKV can be detected with RT-PCR and *in situ* hybridization. **A**: β-propriolactone inactivated ZIKV (ZIKV-BPL) can still detected with RT-PCR for ZIKV RNA, albeit with a lower sensitivity (~3 CT-value's lower). **B&C**; ZIKV **(B)** and ZIKV-BPL **(C)** can both be detected with *in situ* hybridization for ZIKV RNA after being incubated (MOI 2) with the monocytic cell line K562 for two hours on ice.

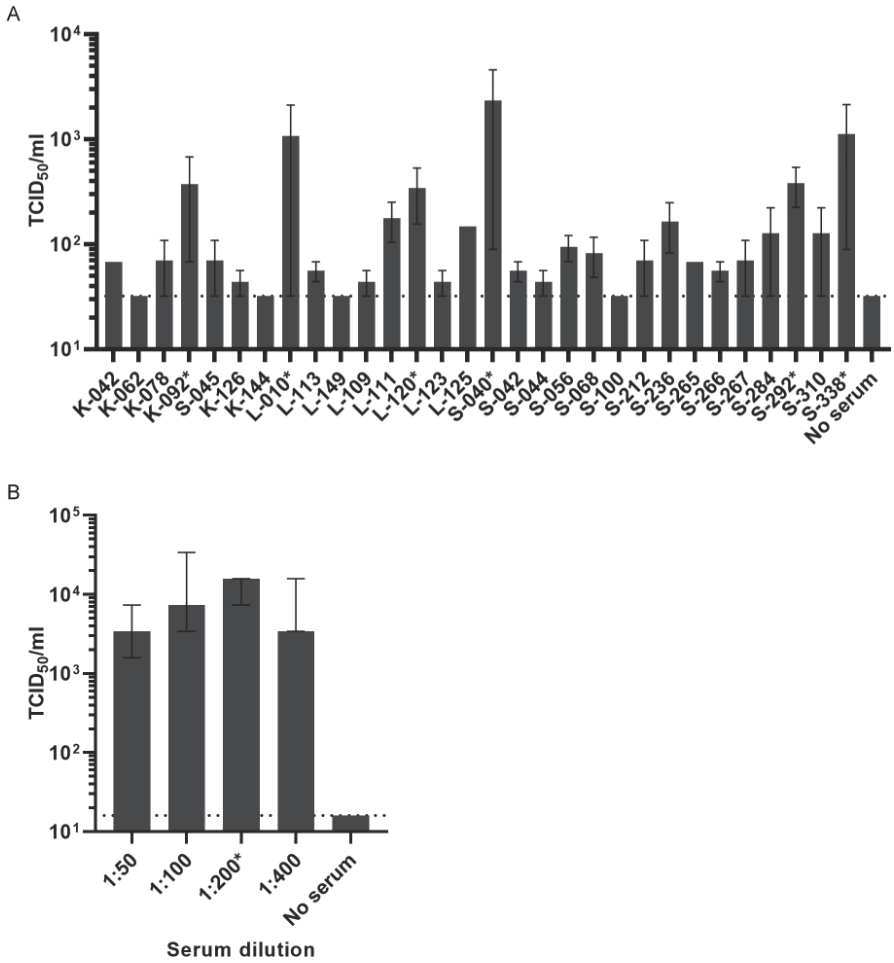

Figure S2. Selection of pooled sera for placental perfusion experiments.
A: Thirty sera that did not contain ZIKV nAbs, from a ZIKV seroprevalence cohort, were tested for ADE potential by pre-incubation of the sera at a 1:100 dilution with ZIKV (MOI 0.5) prior to adding this to U937 cells for 48 hours. **B:** Sera marked with an asterisk in panel A were pooled and pre-incubated with ZIKV (MOI 0.5) at four different dilutions prior to adding them to U937 cells for 48 hours. Bars represent median ZIKV titers ±IQR in supernatants.

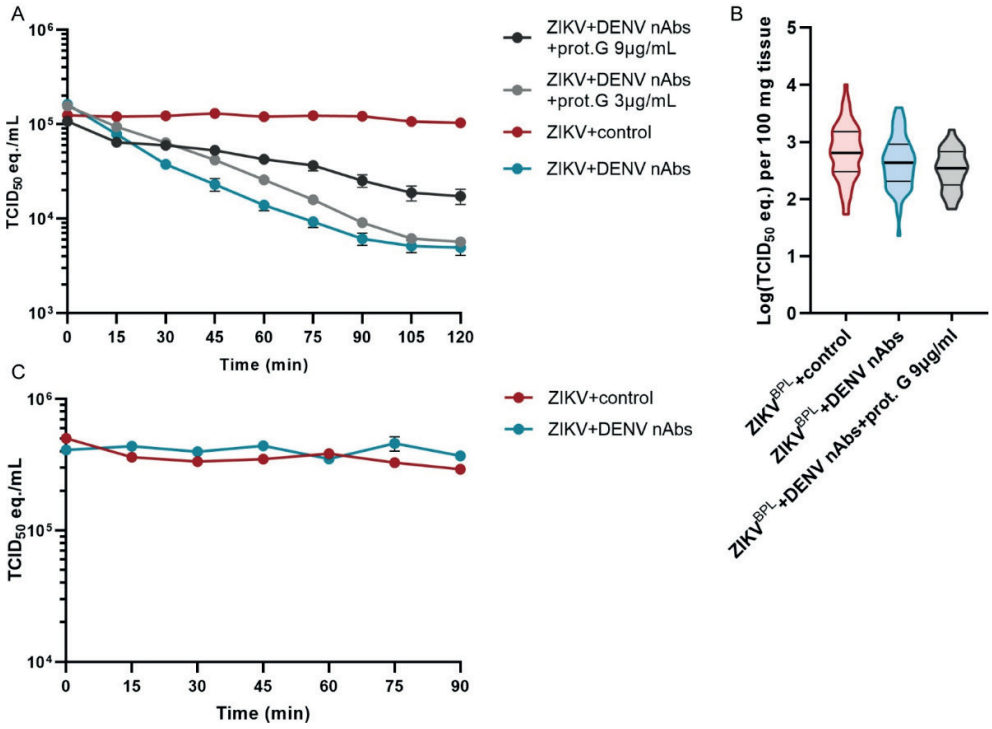

Figure S3. Uptake of ZIKV immune complexes by *ex vivo* perfused placentas is partially blocked by protein G.
A: Protein G was added to ZIKV^{BPL}+DENV nAbs in a concentration of 3 µg/mL and 9 µg/mL (N=1 and N=2 donors, respectively) and incubated for 60 minutes before adding this to the maternal circulation (MC) of the placental perfusion model. ZIKV RNA levels in the MC were determined every 15 minutes with RT-PCR up to 120 minutes. **B:** ZIKV RNA was detected in tissue biopsies taken from placentas that were perfused for 120 minutes. Horizontal lines represent median and the 10th and 90th percentile cut-off. Statistical significance was determined using the Mann-Whitney U test. **C:** ZIKV^{BPL}+flavivirus negative serum (ZIKV^{BPL}+control) and ZIKV^{BPL}+DENV nAbs were circulated through the perfusion machine to which no placenta was attached to test for tube adherence of the immune complexes. ZIKV RNA levels in the MC were determined every 15 minutes with RT-PCR up to 90 minutes.

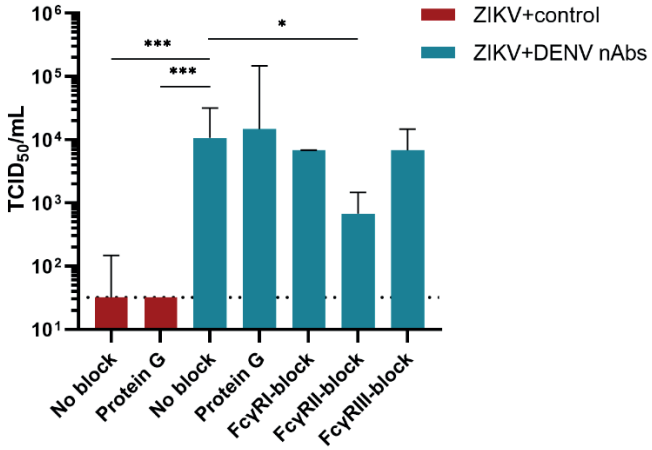

Figure S4. Adding protein G to ZIKV+DENV nAbs does not inhibit ADE of infection in U937 cells. U937 cells, expressing FcγR-I& -II, were infected with ZIKV (MOI 0.5) that was pre-incubated with flavivirus naïve serum (ZIKV+control) or serum containing DENV nAbs (both 1:250 dilution) with or without protein G. Cells were also pre-treated with FcγR blocking antibodies. ZIKV titers were determined in supernatants at two dpi. Bars represent median+95%CI. Significance was determined using the Kruskal-Wallis test followed by Dunn's post hoc test, comparing ZIKV+DENV nAbs without block to the other conditions. * P<.05, ***P<.001.

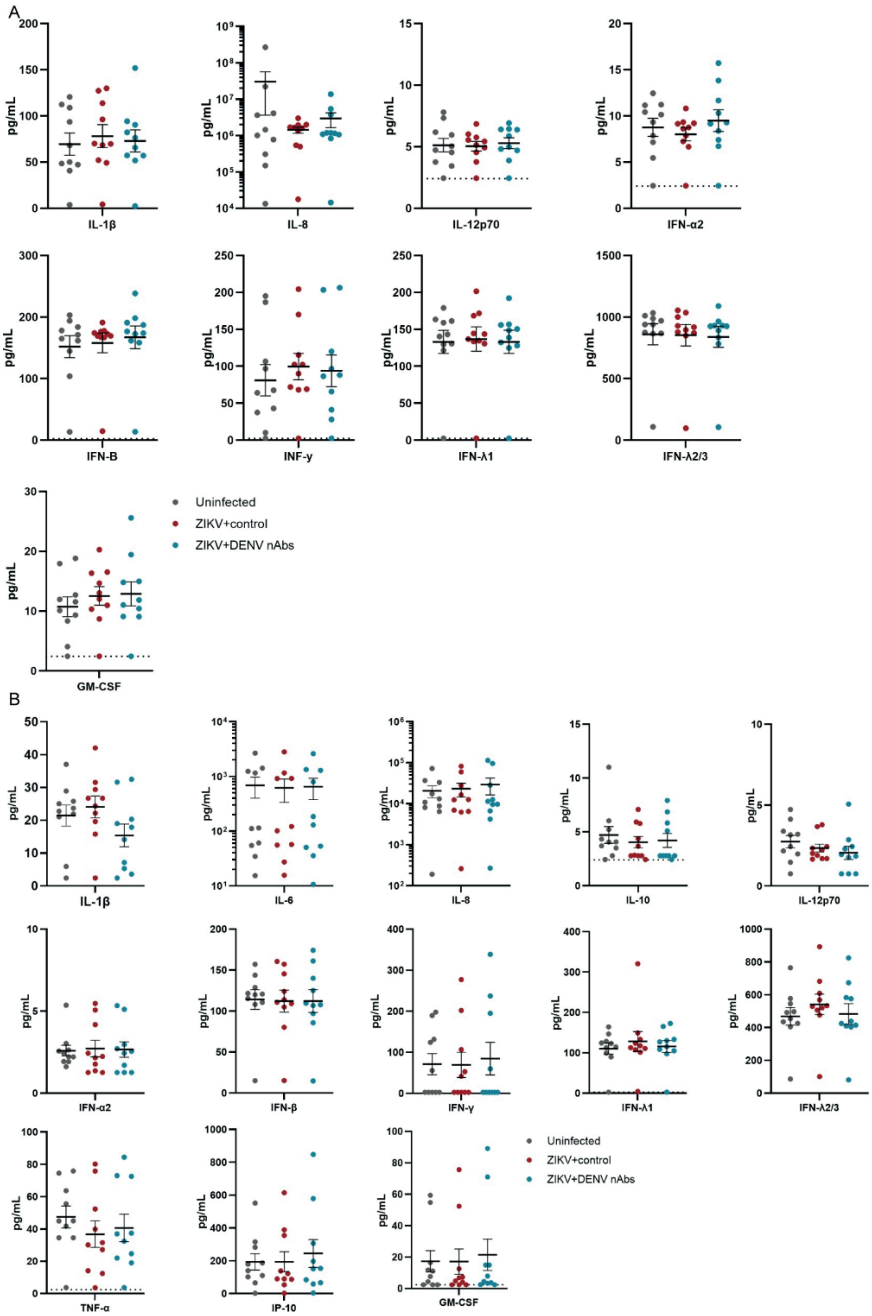

Figure S5. No significant changes in cytokines produced by Hofbauer cells and trophoblasts during ZIKV infection. Cytokines were determined in the supernatants of Hofbauer cells (**A**) and trophoblasts (**B**), 48 hours after infection with ZIKV+control or ZIKV+DENV nAbs at an MOI of 0.5. Each dot represents one value of experiments performed in triplicate/quadruplicate, lines represent mean±SEM. Significance was determined using one-way ANOVA with Dunnett's post hoc test. N=3 donors per condition.

S1 Table. Clinical characteristics of donors whom placentas were used for perfusion experiments.

Donor	Condition	Maternal age, years	Gravidity	Parity	Gestational age, weeks+days	Fetal sex	Birth weight, g	Placental weight, g
1	ZIKV+control 120 min.	34	2	1	39+1	Male	3090	650
2*	1. ZIKV+control, 120 min. 2. ZIKV+DENV nAbs, 120 min.	32	4	1	38+4	Female	3540	737
3	ZIKV+DENV nAbs, 120 min.	38	6	3	39+0	Female	3685	617
4	ZIKV+DENV nAbs, 120 min	30	2	1	39+2	Male	3320	551
5*	1. ZIKV+DENV nAbs, 120 min. 2. ZIKV+DENV nAbs+Prot. G 3µg/mL, 120 min.	38	5	3	38+4	Male	3625	726
6*	1. ZIKV+control, , 120 min. 2. ZIKV+DENV nAbs+Prot. G 9µg/mL, 120 min.	34	2	1	39+3	Male	4005	657
7	ZIKV+DENV nAbs+Prot. G 9µg/mL, 120 min.	27	5	1	38+4	Female	3340	691
8	ZIKV+DENV nAbs, 40 min.	28	2	1	39+0	Male	3545	736
9*	1. ZIKV+control, 40 min. 2. ZIKV+DENV nAbs, 40 min.	33	9	5	38+6	Male	4040	762
10	ZIKV+control, 40 min.	35	3	2	39+3	Female	3385	667

In placentas from donors marked with *, two cotyledons of the same placenta could be perfused at the same time and therefore, two conditions could be tested with the same placenta.

S2 Table. Results from ZIKV and DENV-2 VNT assays and ZIKV and DENV NS1 IgG ELISA's performed with sera used for enhancement experiments.

Serum	ZIKV nAb titer	ZIKV NS1 IgG ELISA ratio	DENV-2 nAb titer
L-010	<8	4.14	161
L-055	<8	3.99	256
L-120	<8	2.31	128
S-040	<8	1.12	161
S-292	<8	4.05	128
S-338	<8	4.63	>1024

Cut-off for positive ZIKV and DENV-2 VNT result: titer >1:32 Cut-off for positive ZIKV and DENV NS1 IgG ELISA result: ratio >1.1

CHAPTER 9

Summarising discussion

Zika virus (ZIKV) was first isolated in a sentinel monkey in Uganda in 1947. However, since then, with 160 publications in 60 years, it has not been extensively studied (5, 323). This is because it was generally considered a virus of limited clinical significance, due to its mild, self-limiting disease presentation in humans. The magnitude of the 2015-2016 ZIKV outbreak in the Americas, particularly the severe complications of a ZIKV infection that manifested itself during this outbreak, came as a surprise to many people working in the fields of virology, public health, and healthcare. After 2016, to improve our understanding of the pathogenicity, epidemiology, and interactions of ZIKV with other flaviviruses, scientific research on ZIKV intensified. More than six years of intensive global research efforts on ZIKV has resulted in a substantial increase in our understanding of this virus. However, many unanswered questions remain, such as the population immunity level that will be needed to prevent new ZIKV outbreaks, the potential pathogenic role that flavivirus antibodies can have on ZIKV-associated birth defects, and the exact mechanism behind vertical ZIKV transmission and ZIKV-associated birth defects. In this thesis, we have studied the neurological complications of a ZIKV infection, the epidemiology of ZIKV and serological response after a ZIKV infection and the possible negative effects that cross-reactive flavivirus antibodies can have on a ZIKV infection during pregnancy.

Zika virus and Guillain-Barré syndrome

The increased incidence of Guillain-Barré syndrome (GBS) during the 2015-2016 ZIKV outbreak indicated that a ZIKV infection may be associated with GBS (49, 324). Reports of increased GBS incidence from French Polynesia, where there was a ZIKV outbreak in 2013-2014, further strengthened the possibility of an association between ZIKV and GBS (67, 119). In Suriname, an increase in the number of GBS patients was also noted at the start of the ZIKV outbreak in this country at the end of 2015. To investigate whether ZIKV infections were the cause of this increase in GBS incidence, and to study the clinical characteristics, preceding infections and outcomes of GBS in Suriname, we carried out an observational study on GBS in Paramaribo, Suriname. Patients who were admitted to one of the three hospitals in Paramaribo with suspected GBS were recruited for this study. The first three GBS patients in the study with a suspected ZIKV-associated GBS, early in 2016, are described in **Chapter 2**. Based on serological assays, no evidence of recent infections with pathogens that commonly trigger GBS were found in these patients. A recent ZIKV infection was confirmed in one patient, based on a positive ZIKV-PCR test in urine. Infection was also suspected in the two other patients, based on a positive ZIKV virus neutralisation test (VNT). Since ZIKV had only just been identified in Suriname at the time, a positive ZIKV VNT result could indicate a recent ZIKV infection. However, a limitation in this study is that DENV VNTs were not

performed. After a recent DENV infection, cross-neutralisation of ZIKV can occur and this can result in a false-positive ZIKV VNT, which can complicate the interpretation of the ZIKV VNT (45, 46). The clinical characteristics and aetiology of the complete cohort of GBS patients recruited for this study in Suriname, both during and after the ZIKV outbreak, are described in **Chapter 3**. In this study, in an attempt to identify the preceding infection that triggered GBS, we performed more extensive serological testing than in **Chapter 2**. VNTs for DENV (DENV-2) were performed and we identified a confirmed recent DENV infection in one GBS patient, based on a >4-fold rise in DENV nAb titre while ZIKV nAbs were undetectable. Although GBS is generally not considered to be a complication of DENV infections, emerging observations like ours indicate that DENV infections can sometimes also be complicated by GBS (325-327).

One of the challenges of diagnosing a preceding infection that triggers GBS, is that there is usually a delay between the initial infection and the onset of GBS symptoms. On average, this delay is 1-2 weeks, which means that molecular detection methods of finding a pathogen are often unhelpful and serological assays have to be used (51). Despite the extensive diagnostic testing algorithm with the serological assays that we applied in **Chapter 3**, we were still unable to identify a possible, probable, or confirmed preceding infection in 50% of the GBS patients. It could be that GBS in these patients, who were not recently vaccinated, was triggered by pathogens that were not included in our diagnostic algorithm. This, in turn, might indicate that the variety of pathogens that can trigger GBS in tropical regions, is wider than currently assumed. It needs to be mentioned here that due to the limited therapeutic resources that are available in Suriname, the treatment of GBS with intravenous immunoglobulins (IVIg) and plasma exchange (PE), according to international guidelines, is also limited. It has been shown that IVIg and PE accelerates GBS recovery. Therefore, it is important that this treatment option becomes more generally available in Suriname (50, 51, 328).

ZIKV epidemiology and immunity

In 2017, ZIKV circulation in the Americas decreased almost as quickly as it increased during the 2015-2016 outbreak (329). One of the hypothesised explanations for this rapid decrease was herd immunity. Given that the majority of ZIKV infections are asymptomatic and ZIKV diagnostics were largely unavailable in the Americas at the beginning of the ZIKV outbreak, the number of confirmed ZIKV infections, and therefore the estimation of population immunity, was greatly underestimated (330). In an attempt to determine the real magnitude of the 2015-2016 ZIKV outbreak and get an indication of the risk of future ZIKV outbreaks, as well as the potential benefit that a ZIKV vaccine could have in these regions, in **Chapter**

4 we performed a seroprevalence study in Suriname. We found that, on average, 35.1% of participants had ZIKV-neutralising antibodies. Furthermore, we found that the ZIKV seroprevalence was 36.7% and 24.5% in two remote villages in the Surinamese rainforest, indicating that the ZIKV outbreak in Suriname was widespread across the county. The exact population immunity threshold needed to stop an arbovirus outbreak is not known; but it has been suggested to be above 60% for ZIKV and chikungunya virus (153, 331). Therefore, it is plausible to assume that ZIKV population immunity was not the only factor contributing to the rapid decrease of ZIKV circulation in Suriname. Other factors, such as mosquito density varying over time and human behavioural changes in protecting against mosquito bites, might also have played an important role. In this seroprevalence study, we also found that the ZIKV VNT is a useful assay for the specific detection of ZIKV antibodies in DENV-endemic countries. Flavivirus serology is notoriously challenging, because of antibody cross-reactivity between the different viruses, such as ZIKV and DENV (167). DENV is (hyper) endemic in many of the regions where ZIKV emerged, and because of this cross-reactivity many serological assays cannot distinguish between DENV antibodies and ZIKV antibodies. We observed this in the above-mentioned study by testing serum samples that were collected in Suriname before ZIKV was circulating there (2012-2014), using a commercial ELISA assay to detect IgG against ZIKV non-structural protein 1 (NS1). The specificity of this assay was only 47.7%, assuming that everyone in this cohort was indeed ZIKV-naïve, given that ZIKV had not yet been identified in the Americas yet. In contrast, and as reported in other studies, the specificity of the VNT when performed on sera from non-acutely ill patients, was 100% (45). Therefore, the ZIKV VNT seems to be the preferred diagnostic test to distinguish ZIKV from DENV antibodies.

The samples that were tested with the ZIKV VNT in **Chapter 4**, were collected about a year after the ZIKV outbreak peaked in Suriname. To test whether the ZIKV VNT can be used as a diagnostic assay to determine previous ZIKV exposure that occurred longer ago, in **Chapter 5** we describe how we collected sera from inhabitants of Suriname who had had a PCR-confirmed ZIKV infection more than three years ago. The ZIKV VNT was positive in only 59.2% of this group of 49. With the anti-ZIKV NS1 IgG ELISA, this seroprevalence was 63.3%. These results suggest that due to low sensitivity, the ZIKV VNT, which we found to be highly specific in **Chapter 4**, is less likely to detect a ZIKV infection several years after infection. Therefore, using this test to predict the ZIKV seroprevalence in regions that did not experience a recent (<1-2 years) ZIKV outbreak, might underestimate previous ZIKV exposure. One way to overcome this low sensitivity is to decrease the cut-off for a positive signal. This will, however, increase the false-positivity rate because of cross-neutralisation by antibodies against other flaviviruses, and therefore also decrease the test specificity.

Consequently, assays that are not based on serology, such as cellular assays, might be a better alternative in determining previous ZIKV exposure. Using these assays, the presence of long-lived, ZIKV-specific memory B-cells and T-cells can be determined. For T-cells this can be done by exposing memory T-cells to an optimised, overlapping peptide pool covering the ZIKV proteome and measuring T-cell activation markers such as interferon-γ, as was done for SARS-CoV-2 (332). For B-cells this can be done by the *ex-vivo* activation of isolated memory B-cells and the detection of anti-ZIKV antibodies in the supernatants of these cells, or by the detection of ZIKV-specific memory B-cells with flow cytometry (333). The performance regarding sensitivity and specificity of the above-mentioned cellular assays are unknown, since these tests are still experimental and not commonly used for flavivirus diagnostics. However, due to the afore-mentioned limitations of serological assays, further development and testing of cellular assays for previous ZIKV (or flavivirus) exposure might be worthwhile.

The low ZIKV VNT titres in the cohort described in **Chapter 5** might indicate a waning of ZIKV immunity, although it was not possible to confirm this in the current study because of its cross-sectional design. However, relatively fast antibody waning after a ZIKV infection has been observed in longitudinal studies (160, 173). Therefore, it is plausible to assume that antibody waning below the limit of detection is the cause of the low ZIKV seroprevalence in this cohort. This raises the question of whether persons with these low antibody titres are susceptible to ZIKV re-infection. Because we did not assess the presence and frequency of circulating memory T-cells and B-cells in this study, we cannot ascertain whether participants with low ZIKV antibody titres are susceptible to reinfection with ZIKV. However, it is likely that the absence of circulating ZIKV-neutralising antibodies will result in a loss of sterilising immunity. It could result in a short-lasting viremia upon exposure to ZIKV. This is relevant for pregnant women because it may result in virus transmission to the foetus, which will have no passive maternal immunity so it could, potentially, result in birth defects. Given that the duration of protection against ZIKV is essential for ascertaining when a new ZIKV outbreak can be expected in the regions affected by the 2015-2016 ZIKV outbreak, the duration of immunological protection after a ZIKV infection should be studied in more detail in longitudinal studies (175).

Antibody-dependent enhancement of ZIKV

Because of the potency of cross-reactivity between ZIKV and DENV antibodies and given that ZIKV emerged in DENV-endemic regions, an important question for the ZIKV outbreak is whether DENV (or flavivirus) pre-immunity poses a risk for more severe ZIKV infections resulting from antibody-dependent enhancement (ADE). In **Chapter 6**, we review the results

of studies carried out into ZIKV ADE up until the beginning of 2019. In these (mainly experimental) studies, ADE of a ZIKV infection is readily induced *in-vitro* but less often observed *in-vivo* and not observed in clinical cohorts. In this chapter, we also review whether ADE of a DENV infection can be induced by ZIKV antibodies, as both viruses co-circulated in the Americas since 2015. We conclude that there was insufficient evidence to support or reject the hypothesis of DENV ADE by ZIKV antibodies, and vice versa. Emerging evidence from a paediatric cohort in Nicaragua indicates that a previous ZIKV infection can increase the risk of a subsequent severe DENV infection (115). For ZIKV, in contrast with DENV, severe disease was not reported in clinical observations, regardless of flavivirus pre-immunity status (115, 214, 334). Although previous flavivirus exposure does not seem to exacerbate ZIKV symptoms, the possible consequences of flavivirus pre-immunity on ZIKV infections and complications during pregnancy are less clear. For example, ADE of ZIKV during pregnancy could result in a higher viral load in blood. And while this might not be accompanied by increased disease severity (ADE of ZIKV disease), it can still have detrimental effects for the foetus if the virus crosses the placenta.

To better understand the potential risk posed by ZIKV ADE during pregnancy, in **Chapter 7** we study the role of cross-reactive flavivirus antibodies on *in-vitro* ZIKV infection in myeloid cells obtained from pregnant women. To prevent the rejection of the semi-allogenic foetus, pregnancy induces complex immunological changes, one of which is an increase in the intermediate monocyte ($CD14^{+}CD16^{+}$) population, cells that have been shown to be susceptible to ZIKV infection. Because there is quite some variation in methodologies used in *in-vitro* ZIKV ADE assays, mainly regarding the cells and the readout used for the assay, in **Chapter 7** we have also tried to assess the influence these variables have on the results of an *in vitro* ADE assay. For this, we tested whether, and which, myeloid cell lines and primary cells can be infected with ZIKV and are thus susceptible to ADE of ZIKV infection. We found that all three of the tested myeloid cell lines, U937, THP-1 and K562, could be used for *in-vitro* ZIKV ADE assays, while in primary myeloid cells, ADE of ZIKV infection could mainly be induced in monocyte-derived macrophages. The strong correlation between the percentage of infected cells and the viral titre in the supernatant indicates that both readouts can be used for this assay. We subsequently studied whether monocytes and monocyte-derived macrophages of pregnant women are more susceptible to ZIKV infection and ADE of ZIKV infection, compared with those of non-pregnant women. We collected whole blood from 30 pregnant women and 10 non-pregnant women, all of childbearing age, and isolated the peripheral blood mononuclear cells (PBMCs). ZIKV ADE assays were carried out with monocyte-derived macrophages and monocytes and viral titre in supernatants was used as a readout, because this makes it possible to detect the combined effects of extrinsic ADE (more infected cells)

and intrinsic ADE (more virus production per cell). We found no differences in susceptibility to ZIKV infection or ADE of ZIKV infection between cells from pregnant women compared with non-pregnant women. This study demonstrates that with *in-vitro* assays, there are no indications that pregnancy changes myeloid cells' susceptibility to ZIKV infection and ADE of ZIKV infection.

While the results in **Chapter 7** suggest that pregnancy does not affect ADE in maternal myeloid cells, we hypothesise in **Chapter 6** that complexes of cross-reactive flavivirus IgG bound to ZIKV can possibly be actively transported across the placenta through neonatal Fc-receptor (FcRn) mediated transcytosis. This mechanism of transplacental transcytosis has been observed with cytomegalovirus, while for ZIKV this phenomenon has been observed in pregnant mice and in human placental explants, but not in pregnant non-human primates (94, 246, 253, 254, 335, 336). For this reason, we tested the hypothesis of FcRn mediated transplacental transcytosis of ZIKV immune complexes in **Chapter 8**. Firstly, we isolated and infected placental explants from full-term human placentas and infected them with ZIKV. In line with results from other studies, we found that ZIKV infection of placental explants is enhanced in the presence of serum containing DENV antibodies and that this enhancement could be reduced by blocking the interaction between IgG and FcRn with protein G (246, 253, 336). While placental explants are a convenient model to study virus infections of the placenta, they do have some disadvantages regarding studying the transplacental transmission of a virus. The structural integrity of these explants is harmed during isolation and culturing. Moreover, the mechanism of infecting these explants is static compared with the dynamic *in-vivo* situation in which maternal blood flows in the intervillous space of the placenta, where it comes into contact with the chorionic villi. To study transplacental ZIKV transmission and the role played by DENV antibodies on this transmission in a more representative way, we developed an *ex-vivo* dual placental perfusion model to study transplacental ZIKV transmission. This is a representative model with intact structural integrity and a dynamic infection mechanism. Furthermore, experiments were performed with human placentas, which is important, because there are several structural differences in placenta anatomy between species (292). Lastly, this model is animal friendly and less expensive compared with animal models.

In using this model with a flavivirus for the first time, we found that DENV antibodies markedly increased the placental uptake of ZIKV. This uptake was FcRn-mediated. We demonstrated this by showing that adding protein G to ZIKV-immune complexes, prior to adding these to the maternal circulation of the placental perfusion model, significantly reduces placental ZIKV uptake. The results of this study support the hypothesis that DENV antibodies can be

a risk factor for transplacental ZIKV transmission. However, for several reasons these results cannot be directly translated to the *in-vivo* human situation during pregnancy. Firstly, the very rapid placental uptake of approximately 90% of ZIKV-DENV antibody immune complexes during perfusion is likely, in part, because the perfusion medium contains low levels of maternal IgG, compared with whole blood. Therefore, there is significantly less competition for the ZIKV-DENV nAb immune complexes to bind FcRn, compared with when maternal IgG is present in high concentrations. Secondly, these experiments had to be performed with inactivated virus, due to biosafety restrictions since the placental perfusion model is situated in a biosafety level I laboratory. This limitation could be overcome by setting-up this model in a biosafety level II or III facility. This would make it possible to study infectious ZIKV, as well as other viruses that are associated with foetal or placental damage, such as CMV, rubella and, most recently, SARS-CoV-2 (337, 338). If placentas could be perfused with infectious virus, it would increase the validity of the experiment because the virus is not altered. Furthermore, it would make viral detection in perfused placental tissue less challenging, especially if perfused placental tissue were to be incubated overnight in a culture medium to allow for viral replication. Lastly, in this study we only used one dilution of pooled sera containing DENV nAbs, selected for its high ZIKV ADE potential. It is likely that only a range of DENV nAbs titres can induce an increased risk of transplacental ZIKV transmission, as has been observed with an ADE of DENV infection in a paediatric cohort (103).

Importantly, the mechanism of the FcRn-mediated transplacental transcytosis of ZIKV would be an additional route as to how ZIKV can cross the placenta from the second pregnancy trimester onwards (maternal IgG is not transported across the placenta before this stage). Given that the risk of transplacental ZIKV transmission is at its greatest when the infection occurs during the first pregnancy trimester and that ZIKV-associated birth defects have been observed in flavivirus-naïve women, it is evident that this mechanism is not an absolute requirement for ZIKV to cross the placenta.

Future perspectives

Looking ahead, an important question concerning ZIKV is: when it comes to early detection, diagnostics, prevention and treatment, just how prepared are we for a new ZIKV outbreak? As we have seen with other virus outbreaks, such as the chikungunya virus outbreak in 2013-2014, the 2013-2016 Ebola outbreak and the current SARS-CoV-2 pandemic, the answer to this question is that we are not prepared (339, 340). Despite the fact that six years of intensive international research into ZIKV has generated significantly more knowledge about this virus, this research has not yet resulted in sufficient new tools to prevent or mitigate future ZIKV

outbreaks (341). It is difficult to predict if, when and where ZIKV will re-emerge. Currently, ZIKV circulation worldwide is low and this has resulted in a decreased ZIKV population immunity, because of the introduction of many ZIKV-naïve individuals (new-borns). Add to this a possible waning of ZIKV immunity and it becomes plausible to assume that future (smaller) ZIKV outbreaks will occur and that these will be accompanied by an increased incidence of severe birth defects. Several aspects regarding how prepared we are for future ZIKV outbreaks are discussed below.

Fundamental and clinical observational studies

The many high-quality fundamental studies into ZIKV that have been performed since 2015 have provided substantially more knowledge about ZIKV pathogenicity. However, the implications of several findings of these studies, e.g., that ZIKV can infect testes and that cross-reactive DENV antibodies might facilitate transplacental transmission, have not yet been extensively studied in an observational context in humans (254, 342). The main reason for this is the worldwide low circulation of ZIKV these past few years. To try to answer the remaining questions regarding ZIKV pathogenicity in humans, it is important that protocols for observational studies are in place that will allow these studies to be immediately started up as soon as ZIKV re-emerges. One of the main unanswered questions that should be studied in clinical observational studies is whether DENV antibodies can indeed, as we describe in **Chapter 8**, increase the risk of transplacental ZIKV transmission. This could provide important information to take into account in decision-making for (future) DENV vaccination strategies. To study this, pregnant women in regions with active ZIKV circulation need to be observed throughout their pregnancy for ZIKV infection and related pregnancy complications and pregnancy outcomes. Early in pregnancy, the DENV (and other flavivirus) serostatus must be determined, preferably with a VNT. These studies can give us a better understanding of the risk factors associated with transplacental ZIKV transmission, ultimately leading to better counselling and even preventive or therapeutic intervention strategies.

Surveillance and diagnostics

To be able to respond rapidly to a new ZIKV outbreak, early surveillance and response are essential. Several strategies can be used for the early detection of ZIKV. One of these is syndromic surveillance in persons that present with arbovirus-related symptoms. The symptoms of a ZIKV infection are quite similar to infections with other arboviruses, especially DENV. Therefore, this syndromic surveillance should be performed on a (random) subset of patients presenting with arbovirus-related symptoms, either in a public-health setting or in a

hospital. Ideally, the obtained samples should also be tested for DENV and chikungunya virus, in order to also monitor the circulation of these viruses. Even better, the samples that are tested negative for ZIKV, DENV and CHIKV should be sequenced to see whether other arboviruses with emergence potential, such as Mayaro virus, are starting to circulate (343). Alternatives to syndromic surveillance are surveillance of a random subgroup of people (since arbovirus infections are often asymptomatic) or a one-health approach in which vector populations are structurally tested for arboviruses and once increased virus circulation is detected in these populations, sampling of the human population will be intensified. Surveillance programmes like these are essential for ZIKV-infection control, which should be a global effort. The economic burden of a ZIKV outbreak, along with the associated complications like GBS and birth defects, is significant (344). Therefore, targeted arbovirus surveillance in regions that are at risk of arbovirus epidemics might be cost-effective and it could significantly lower the morbidity associated with these outbreaks (345).

Syndromic ZIKV surveillance should be performed with RT-PCR on blood or urine and not with serology. This is because discriminating flavivirus serology, especially in the acute phase of infection, is very challenging, if not impossible, at the moment (46, 346). Diagnosing ZIKV with RT-PCR is, however, also not without its challenges. The short period of several days of viremia during a ZIKV infection gives just a small window for the molecular detection of the virus (347). This window can be extended by several days if urine is used to detect ZIKV (347, 348). Another challenge is the quality assurance associated with the molecular tests to detect ZIKV (349). In an external quality assessment in Brazil, it was found that 73% of participating laboratories showed limited specificity or sensitivity (350). For several reasons, current ZIKV-surveillance strategies in ZIKV-endemic countries are mainly based on clinical-disease detection. These reasons are the limited availability for ZIKV molecular tests, the often-inadequate performance of these tests and the lack of collaboration between the different sites and laboratories that are involved in ZIKV diagnoses and surveillance (351). A possible consequence of all this is that if ZIKV does re-emerge, its detection will be hampered and delayed. This will make it more difficult to stop new outbreaks with simple strategies, such as mosquito control and personal mosquito-protection measures.

Given that syndromic ZIKV surveillance in humans currently seems to be inadequate in many ZIKV-endemic regions, other ZIKV-surveillance strategies should be employed. One of these could be entomological surveillance for ZIKV (and other arboviruses) in mosquitoes (352). For this, Aedes mosquito pools should be tested with RT-PCR for ZIKV, and preferably DENV, CHIKV and other arboviruses. Regular testing of mosquito pools, in urban as well

as rural areas, might be an adequate method for the early detection of increased ZIKV circulation, in both the sylvatic and urban cycle.

ZIKV vaccine

Another, and possibly the most important, tool that will prevent or mitigate (the complications of) future ZIKV outbreaks, is an effective ZIKV vaccine. Modelling studies have shown that if an effective ZIKV vaccine were to be available and administered to girls and women between 15-30 and living in ZIKV-endemic countries, it could significantly reduce ZIKV-associated birth defects during ZIKV outbreaks and even reduce the likelihood of new ZIKV outbreaks (175). Several ZIKV vaccine candidates are currently under evaluation in phase I and phase II studies (353). These include DNA-, mRNA-, live attenuated- and inactivated vaccines. However, the low incidence of ZIKV means that testing the efficacy of these vaccines in the target population is currently not feasible. A possible strategy that might overcome this problem is to carry out human challenge trials (HCT), in which volunteers are intentionally exposed to a pathogen in a controlled medical setting. HCT can be controversial, and it raises complex, ethical questions. However, when conducted ethically, they can contribute significantly to medical advances and are recently performed for SARS-CoV-2 (354-357). ZIKV infections are generally mild or even asymptomatic, which is why the use of HCT has been suggested for ZIKV. In 2017, a multidisciplinary expert panel was assembled to decide whether their use could be ethically justified (358). This panel concluded that, provided several important conditions were met, using HCT for ZIKV could be ethically justified. However, at the time it was deemed premature because there was insufficient evidence that HCT would accelerate vaccine development and that field trials would be 'prohibitively difficult to conduct' (359). To date, more than four years after HCT were considered premature, little progress has been made in testing the efficacy of these vaccine candidates. Perhaps, therefore, the time is ripe to re-evaluate whether it is still premature to use HCT and if the results of these trials will help accelerate ZIKV vaccine development and registration. If ZIKV HCT are to be used, only the most promising vaccine(s) should be tested. This will keep the number of volunteers that will be exposed to ZIKV to an absolute minimum. Furthermore, to reduce the risk of complications, the trials must be done with healthy, non-pregnant women volunteers that do not have a medical history of GBS. And to limit the risk of ZIKV transmission to other individuals, trial participants should remain in a clinical facility during viremia and abstain from sexual intercourse, or use a condom, for four weeks after ZIKV exposure. Given that ADE of a ZIKV infection does not seem to occur in non-pregnant individuals, there is no need to perform these studies with flavivirus-naïve individuals (115).

Aside from the difficulty in testing the efficacy of ZIKV vaccination candidates, another major challenge for vaccine-development is that the antibody repertoire induced by the vaccine must not increase the risk of subsequent severe DENV infections through ADE. One approach to address this concern might be to create a vaccine that not only induces antibodies against the surface proteins of ZIKV but also induces a strong T-cell response against ZIKV non-structural proteins. This could be achieved by making an attenuated Zika virus that can be used as a vaccine, comparable to the successful yellow-fever virus vaccine. Another approach is a ZIKV NS1 vaccine. It has been shown that this kind of vaccine can protect against ZIKV infection but will not increase the risk of ADE because ZIKV NS1 is not expressed on the surface of the virion (360-362). The NS1 antibodies induced by this vaccine will not be able to neutralise ZIKV, but they will be able to aid the immune response against ZIKV through FcγR-mediated functions of these antibodies; opsonisation, complement recruitment and the activation of NK cells (antibody dependent cellular cytotoxicity). Furthermore, the NS1 antibodies will be able to neutralise soluble ZIKV NS1, which has been shown to induce endothelial dysfunction in several tissues (42). A third approach to making a ZIKV vaccine with low risks of causing DENV ADE, is to make a vaccine that consists of or codes for immunogenic epitopes that are less conserved between flaviviruses and therefore ZIKV-specific. An example of such an epitope is domain 3 of the ZIKV E-protein, which is highly immunogenic and flavivirus-specific, in contrast with domain 2, in which the conserved fusion loop is present and to which many cross-reactive antibodies are targeted (363). For the development of a vaccine that contains or codes for multiple ZIKV-specific epitopes, or even ZIKV and DENV or other flavivirus epitopes, the mRNA platform might be well suited. As was seen during the SARS-CoV-2 pandemic, mRNA vaccines can be highly immunogenic and multiple different types of mRNA can be added to one vaccine, potentially making it possible to produce a combined ZIKV/DENV vaccine or pan-flavivirus vaccine (364).

Concluding remarks

Since the 2015-2016 outbreak in the Americas, rapid progress has been made in understanding ZIKV and its complications. Unfortunately, as a result of the sharp decrease in ZIKV incidence during these past few years, research interest and public awareness for ZIKV has also decreased. And this, while many tools to combat or prevent a new ZIKV outbreak, such as a vaccine and an international surveillance network, are not yet in place. This brings with it the risk that in the coming years, the policy for our preparedness for ZIKV will be of a wait-and-see nature, until the virus re-emerges, as has been the case with many other viruses. Right now, would be the time to invest in and persevere in continuing research efforts that were

initiated during previous years. And to create the tools we will need for predicting, detecting, or preventing future ZIKV outbreaks and their associated complications.

CHAPTER 10

Appendix

10.1 Nederlandse samenvatting

Het zikavirus (ZIKV) is een virus dat wordt overgedragen door muggen en behoort tot de virusfamilie waartoe ook het dengue virus (knokkelkoorts), het gelekoortsvirus en het westnijlvirus behoren. ZIKV is in 1947 voor het eerst beschreven in het Zikawoud in Oeganda. Sindsdien is er maar weinig onderzoek naar dit virus verricht omdat een ZIKV-infectie in mensen vaak asymptomatisch is en het virus daardoor als niet klinisch relevant werd beschouwd. Deze kijk op ZIKV veranderde eind 2015 toen bleek dat een infectie met ZIKV, dat zich inmiddels snel verspreidde in Brazilië, gepaard kon gaan met ernstige complicaties. Een van deze complicaties was het Guillain-Barré syndroom (GBS). GBS is een aandoening van het zenuwstelsel dat zich uit door een reversibele, snel toenemende spierzwakte met daarbij soms ook gevoelsverlies of juist pijn. Andere complicaties van ZIKV die voor het eerst gemeld werden in Brazilië in 2015 waren aangeboren aandoeningen bij kinderen van wie de moeder geïnfecteerd was met dit virus tijdens de zwangerschap. De meest gerapporteerde aangeboren aandoening die geassocieerd werd met ZIKV was microcefalie, een afwijking aan het centrale zenuwstelsel waardoor de hoofdomvang te klein is. Inmiddels is duidelijk dat een congenitale ZIKV-infectie een spectrum aan aangeboren afwijkingen kan veroorzaken zoals aandoeningen aan het oog, microcefalie, een verhoogde spierspanning (hypertonie) en calcificaties in het brein. Al deze afwijkingen behoren tot het congenitaal Zika syndroom.

De snelle verspreiding van ZIKV in Midden- en Zuid-Amerika, met de daarbij toenemende incidentie van GBS en aangeboren afwijkingen, leidde ertoe dat de Wereldgezondheidsorganisatie de ZIKV uitbraak tot een 'Public Health Emergency of International Concern' uitriep op 1 februari 2016. Sindsdien is er veel onderzoek verricht naar ZIKV en is er significant meer kennis opgedaan over dit virus en de complicaties die het kan veroorzaken. Echter is er ook nog veel onbekend over onder andere de mechanismes achter de schade die ZIKV kan aanrichten, de werkelijke omvang van de ZIKV uitbraak in 2015-2016 en hoe ZIKV de placenta kan passeren.

Dit proefschrift beschrijft onderzoek naar de associatie tussen GBS en ZIKV, de epidemiologie en antistof respons na een ZIKV-infectie en de rol van kruisreagerende antistoffen op een ZIKV infectie gedurende zwangerschap en op ZIKV transmissie over de placenta.

Deel I: Zika virus en het Guillain-Barré syndroom

In **hoofdstuk 2** en **hoofdstuk 3** worden de resultaten van een observationeel onderzoek naar GBS in Suriname weergegeven. Dit onderzoek is gestart in 2016 tijdens de piek van de ZIKV uitbraak in Suriname waar het door gezondheidsmedewerkers opviel dat het aantal GBS gevallen significant steeg tijdens deze uitbraak. In dit onderzoek hebben we gekeken

naar de mogelijke associatie tussen ZIKV en GBS maar ook naar de klinische presentatie en behandeling van GBS in Suriname en hebben we onderzocht welke andere infecties geassocieerd zijn met GBS in Suriname. In **hoofdstuk 2** worden de eerste drie deelnemers aan dit onderzoek beschreven die een mogelijke ZIKV geassocieerde GBS hebben. In **hoofdstuk 3** worden de resultaten gepresenteerd van het volledige GBS-onderzoek dat we in Suriname hebben gedaan tussen 2016 en 2018. Hierbij hebben we behalve naar ZIKV, ook naar veel andere pathogenen gekeken die mogelijk GBS kunnen veroorzaken. Bij één deelnemer vonden we bewijs voor een recente infectie met het dengue virus (DENV). GBS is geen algemeen bekende complicatie van een infectie met DENV maar onze observatie, in combinatie met enkele andere observaties de afgelopen jaren, impliceren dat GBS ook door een DENV-infectie uitgelokt kan worden. Een andere belangrijke bevinding van dit onderzoek is dat intraveneuze immunoglobulinen en plasma uitwisseling, twee behandelingen die het herstel van GBS kunnen bespoedigen, vaak niet gedaan worden in Suriname vanwege gelimiteerde beschikbaarheid. Meer beschikbaarheid van deze therapieën kan de behandeling van GBS in Suriname verbeteren.

Deel II: Zika virus epidemiologie en immuunrespons

De ZIKV uitbraak, die erg snel begon in Midden- en Zuid-Amerika in 2015 en waarbij naar schatting miljoenen mensen zijn geïnfecteerd, doofde in 2016 ook erg snel weer uit. Een mogelijke oorzaak voor de snelle uitdoving van deze ZIKV uitbraak is groepsimmuniteit; als genoeg mensen in een bepaald gebied een virusinfectie hebben doorgemaakt en daarna immuun zijn voor dit virus, dooft de circulatie van dit virus uit vanwege een gebrek aan beschikbare gastheren. Het totaal aantal mensen dat tijdens deze uitbraak geïnfecteerd raakte met ZIKV was moeilijk in te schatten omdat diagnostiek naar ZIKV in veel landen niet (voldoende) mogelijk was en omdat veel ZIKV infecties asymptomatisch verlopen en dus niet gediagnosticeerd worden. In **hoofdstuk 4** worden de resultaten gepresenteerd van een ZIKV seroprevalentie onderzoek dat we in 2017 hebben uitgevoerd in Suriname. Dit onderzoek hebben we verricht om een betere inschatting te krijgen van de grootte van de ZIKV uitbraak in 2015-2016 en om een indicatie te krijgen van de kans op nieuwe ZIKV uitbraken in de aangedane gebieden. Voor dit onderzoek hebben we bloed afgenomen van 770 inwoners van Suriname en hebben hierin de aanwezigheid van antistoffen tegen ZIKV gemeten. Aanwezigheid van antistoffen tegen ZIKV toont aan dat iemand deze infectie heeft doorgemaakt en omdat ZIKV nog niet aanwezig was in Zuid-Amerika vóór 2015, is het aannemelijk dat deze infectie heeft plaatsgevonden tijdens de ZIKV uitbraak van 2015-2016. Om dit te bevestigen hebben we ook 44 bloedmonsters getest van inwoners uit Suriname die vóór 2015 waren afgenomen.

Zoals verwacht waren er in al deze bloedmonsters geen antistoffen tegen ZIKV te vinden. Opmerkelijk was dat de ZIKV seroprevalentie, behalve in de hoofdstad Paramaribo, ook vrij hoog was in het afgelegen dorp Laduani en het zeer afgelegen dorp Kwamalasamutu (36.7% en 24.5%, respectievelijk). Dit toont aan dat ZIKV zich over heel Suriname verspreid heeft tijdens de 2015-2016 uitbraak en dat hierbij ruim ⅓ van de Surinaamse bevolking met ZIKV geïnfecteerd is geraakt.

In het onderzoek beschreven in **hoofdstuk 5** hebben we opnieuw bloed afgenomen van inwoners van Suriname om te testen op aanwezigheid van ZIKV-antistoffen. Alle studiedeelnemers van dit onderzoek hadden een PCR bevestigde ZIKV-infectie doorgemaakt drie jaar voordat ze meededen aan dit onderzoek. Een van de doelen van dit onderzoek was om te kijken of we de antistoffen tegen ZIKV nog steeds konden meten drie jaar na een doorgemaakte infectie. Zoals recent beschreven door andere onderzoeksgroepen vonden wij dat in een groot aantal deelnemers deze antistoffen niet meer te detecteren zijn. Deze relatief snelle daling van de hoeveelheid ZIKV-antistoffen tot onmeetbare waardes kan het lastig maken om ZIKV seroprevalentie onderzoeken uit te voeren met deelnemers die mogelijk meerdere jaren geleden ZIKV hebben gehad. In dit onderzoek hebben we tevens gekeken of deelnemers die meerdere dengue virus infecties hebben gehad, lagere antistof titers tegen ZIKV hebben vanwege een mechanisme genaamd original antigenic sin (OAS). We vonden hiervoor geen aanwijzingen in dit onderzoek. Het is momenteel nog onduidelijk of lage antistofwaardes tegen ZIKV ook een risico op herinfectie met ZIKV geven en of lage ZIKV-antistof titers het risico op transmissie van ZIKV over de placenta (moederkoek) wanneer dit virus wordt opgelopen tijdens de zwangerschap doet toenemen, dit zal verder onderzocht moeten worden.

Deel III: Antistof-gemedieerde verergering van infectie

Antistof-gemedieerde verergering van infectie (ADE, naar het Engelse antibody-dependent enhancement) is een mechanisme dat vooral voor DENV beschreven is. DENV is erg verwant aan ZIKV en bestaat uit vier serotypes (DENV-1 t/m DENV-4). Ernstige DENV-infecties komen vaker voor in personen die voor een tweede keer met DENV geïnfecteerd raken ten opzichte van personen die voor een eerste keer met dit virus geïnfecteerd raken. Het veronderstelde mechanisme achter ADE van een DENV-infectie, is dat als iemand eerst geïnfecteerd wordt met bijvoorbeeld DENV-1 en daarna met DENV-2, de antistoffen die gemaakt zijn tijdens de eerste infectie, ook kunnen binden aan DENV-2 terwijl ze dit virus niet volledig onschadelijk kunnen maken (neutraliseren). Dit complex van DENV-2 met daaraan gebonden antistoffen tegen DENV-1, wordt vervolgens efficiënt opgenomen

door bepaalde cellen van het afweersysteem die dit soort complexen normaal gesproken wegruimen. Echter, omdat DENV-2 niet geneutraliseerd wordt door de DENV-1 antistoffen, kan het virus zich gaan repliceren zodra het opgenomen is door deze afweercellen. Daardoor is DENV-2, met behulp van de DENV-1 antistoffen als een soort Trojaans paard efficiënt de afweercellen binnengedrongen en gebruikt het deze cellen om zich te repliceren.

Vanwege kruisreactiviteit van ZIKV-antistoffen met DENV en vice versa en omdat ZIKV en DENV in dezelfde gebieden circuleren, werd al vroeg tijdens de 2015-2016 ZIKV uitbraak in Midden- en Zuid-Amerika gedacht dat ADE van een ZIKV-infectie wellicht de verklaring was van de complicaties die bij sommige mensen optreden tijdens een ZIKV-infectie. In **hoofdstuk 6** geven we een overzicht van de literatuur die tot 2019 beschikbaar was omtrent de risico's voor ADE van een ZIKV-infectie door DENV-antistoffen en voor ADE van een DENV infectie door ZIKV-antistoffen. Hierbij concluderen we dat er tot 2019 te weinig bewijs was dat DENV antistoffen een risico vormen voor een vergerering van ZIKV-infectie en andersom maar dat dit in mensen onvoldoende onderzocht is.

Uit onderzoek uitgevoerd na 2019 blijkt echter dat DENV-antistoffen geen risico vormen voor een verergering van symptomen van een ZIKV-infectie. De mogelijke rol die DENV antistoffen hebben op het verergeren van een ZIKV-infectie tijdens zwangerschap en op het risico van transmissie over de placenta (transplacentale transmissie) van ZIKV is echter minder duidelijk en nog niet voldoende onderzocht. Daarom hebben we in **hoofdstuk 7** en **hoofdstuk 8** onderzoek verricht naar de rol van kruisreagerende DENV-antistoffen op ZIKV-infectie van witte bloedcellen tijdens zwangerschap en op het risico van ZIKV transmissie over de placenta.

In **hoofdstuk 7** hebben we een vergelijking gemaakt tussen verschillende cellijnen en primaire witte bloedcellen van gezonde donoren wat betreft gevoeligheid voor een ZIKV infectie en voor ADE van ZIKV-infectie. Dit hebben we gedaan omdat tot nu toe niet duidelijk was wat de rol van de keuze voor een bepaalde cellijn of primaire cel is op de uitkomt van deze zogenaamde *in vitro* ADE-assays. Hierbij vonden we dat voornamelijk macrofagen gevoelig zijn voor zowel ZIKV-infectie als ADE van ZIKV-infectie en dat de cellijnen U937, THP-1 en K562 allen gevoelig zijn voor ADE van ZIKV-infectie, hoewel er verschillen bestaan in de mate van gevoeligheid. Vervolgens hebben we een vergelijking gemaakt tussen de gevoeligheid van monocyten en macrofagen van zwangere vrouwen ten opzichte van niet-zwangere vrouwen voor zowel ZIKV-infectie als ADE van ZIKV-infectie. Hierbij vonden we dat, ondanks de vele immunologische veranderingen die plaatsvinden tijdens zwangerschap, er geen verschil is tussen zwangere en niet zwangere vrouwen wat betreft de gevoeligheid van monocyten en macrofagen voor ZIKV-infectie en ADE van ZIKV-infectie.

In **hoofdstuk 8** hebben we ten slotte onderzocht wat de rol van DENV-antistoffen is op het faciliteren van transport van ZIKV over de placenta. De is een belangrijk feto-maternaal orgaan dat tijdens de zwangerschap onder andere voor gas- en nutriënten transport zorgt en een belangrijke immunologische barrière tegen pathogenen vormt. Voordat ZIKV tijdens de zwangerschap het ongeboren kind kan infecteren, moet het eerst de placenta passeren. De centrale hypothese van het onderzoek uitgevoerd in **hoofdstuk 8** is dat wanneer niet-neutraliserende kruisreagerende DENV-antistoffen gebonden zijn aan ZIKV, dit immuuncomplex actief over de placenta getransporteerd kan worden net zoals vrije maternale antistoffen (IgG) actief over de placenta naar het kind worden getransporteerd. In dit onderzoek hebben we gebruik kunnen maken van een placenta transfusie model. In dit zeer natuurgetrouwe model wordt een placenta direct na de geboorte aangesloten op een perfusiemachine waarbij een maternale en foetale circulatie nagebootst kan worden en de placenta voor enkele uren in leven gehouden kan worden. Door gebruik te maken van dit model zagen we dat geïnactiveerd ZIKV veel efficiënter wordt opgenomen door de placenta wanneer het gebonden is aan DENV-antistoffen ten opzichte van ongebonden ZIKV. Deze bevindingen hebben we ten slotte bevestigd door stukjes placenta weefsel en cellen geïsoleerd uit de placenta te infecteren met ZIKV in aan- of afwezigheid van DENV-antistoffen waarbij we een duidelijk efficiëntere infectie detecteerde in de aanwezigheid van DENV-antistoffen. De resultaten van dit onderzoek geven aan dat de aanwezigheid van kruisreagerende DENV antistoffen mogelijk een risicofactor vormen voor transplacentale ZIKV transmissie.

Afsluitende opmerkingen

Sinds de uitbraak van ZIKV in Midden- en Zuid-Amerika in 2015-2016 is er door onderzoek veel meer bekend geworden over dit virus. Echter is er nog steeds veel onbekend over ZIKV, met name de beschermingsduur na infectie en de mechanismes achter de schade die het virus in ongeboren kinderen kan aanrichten. Tevens heeft de korte periode van wereldwijd intensief onderzoek naar ZIKV nog geen nieuwe preventieve of behandelmogelijkheden, zoals een vaccin, opgeleverd. Hierdoor bestaat er het risico dat wanneer er een nieuwe ZIKV uitbraak plaatsvindt, we hier niet veel beter op voorbereid zijn dan in 2015. Daarom is het belangrijk dat er onderzoek verricht blijft worden naar ZIKV en andere opkomende virusinfectie die door muggen verspreid kunnen worden. Hierbij zal met name ingezet moeten worden op detectie en preventie van deze virussen, bijvoorbeeld door surveillance programma's, en de ontwikkeling van vaccins.

10.2 About the author

Thomas Langerak was born on November 3rd 1990 in Eindhoven, the Netherlands. In 2009 he graduated from the Van Maerlant Lyceum in Eindhoven and started to study medicine at the Erasmus University Medical Center in Rotterdam, the Netherland. He finished his masters with an internship in Internal Medicine at the Franciscus Gasthuis in Rotterdam and received his medical degree in December 2015. In 2016 he started to work as a PhD candidate at the department of Viroscience at Erasmus Medical Center under supervision of Prof. Dr. Eric van Gorp, Prof. Dr. Marion Koopmans and Dr. Barry Rockx. His PhD research mainly focused on Zika virus, in part the spread and related neurological complications of this virus in Suriname but also the effects that cross-reactive antibodies can have on a Zika virus infection during pregnancy and on transplacental Zika virus transmission. In January 2022 he started working as a medical doctor at the Internal Medicine department of Ikazia hospital in Rotterdam, the Netherlands.

In January 2023 he will start his Internal Medicine Residency at the Ikazia Hospital in Rotterdam.

10.3 PhD portfolio

Courses	Year	Workload
OpenClinica	2016	0.7 ECTS
BROK (Good Clinical Practice)	2016	1.5 ECTS
Biomedical English Writing course	2016	2.0 ECTS
Course in Virology	2016	1.8 ECTS
USMLE Step 1 Preparation Course	2016	3.0 ECTS
Basismodule reizigersadvisering en immunisatie voor artsen	2016	3.0 ECTS
Biomedical Research Techniques	2017	1.5 ECTS
Scientific Integrity Course	2020	0.3 ECTS
BROK (Good Clinical Practice)	2021	1.5 ECTS

National and international conferences	
LabTechnology, Utrecht, the Netherlands (oral presentation)	2016
Interne Geneeskunde wetenschapsdagen, Antwerp, Belgium	2016
International Meeting on Emerging Diseases and Surveillance (IMED), Vienna, Austria (poster presentation)	2016
The Annual Course on Molecular Medicine	2017
Havensymposium	2017
Dutch Annual Virology Symposium (oral presentation 2021)	2018, 2019, 2021
NCOH Annual Scientific Meeting (elevator pitch)	2018
International Symposium of Zika virus Research, Marseille, France (oral presentation)	2018
Dutch Arbovirus Network Meeting	2018, 2019, 2021
European Congress of Virology, Rotterdam, the Netherlands (poster presentation)	2019
American Society for Virology Meeting, online (oral presentation)	2021

Lecturing	
Viruskenner kick-off meeting and masterclasses (the Netherlands and Suriname)	2016-2021
Infection and Immunity research master	2020, 2021
NSPOH modules for nurses and physicians	2016-2021
Allemaal Beestjes	2016-2020
MEDTalks	2020-2022
Museum Jeugd Universiteit	2020

Supervising, tutoring	
Viruskenner Coach (the Netherlands and Suriname)	2016-2021

Supervising Master Thesis	
Tom Brinkman (medicine), Irene van Rooij (medicine) Rimke de Kroon (biomedicine) Nadia van der Meijs (biomedicine)	2017-2020

Other	
Peer review *Viruses*	2018
Peer review *Genome Medicine*	2019
Peer review *Frontiers in Microbiology*	2020
Peer review *Frontiers in Neurology*	2020
Peer review *Vaccine*	2021
Peer review *American journal of tropical medicine and hygiene*	2021

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11. J. Spikman, H. van der Horn, M. Scheenen, M. de Koning, M. Savas, **T. Langerak**, E. van Rossum, J. van der Naalt. Coping with stress before and after mild traumatic brain injury: a pilot hair cortisol study. Brain Injury, 2021.
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Mathis, my fellow placenta enthusiast. Thanks for sharing all the useful and especially less-useful knowledge that is stored in this massive brain of yours.

Ron en Pieter, ik was altijd blij als ik in mijn agenda keek en het bleek dat het de eerste dinsdag van de week was en dus tijd voor een klein borreltje (min of meer) na werktijd. Behalve de gezelligheid heb ik ook best nog af en toe eens wat opgepikt van jullie immer genuanceerde meningen.

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Lisa, mijn stoere grote zus. Als er één iemand is op wie ik blind kan vertrouwen dan ben jij het. Nog geen 10 minuten nadat ik mijn promotiedatum had doorgegeven had je al een ticket naar Nederland geboekt! Dank dat je er voor mij bent als het nodig is en weet dat ik er ook altijd voor jou zal zijn.

Maria; ¡Eres la leche!

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