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DEVELOPMENT OF MOUSE MODELS FOR THE STUDY OF ZIKA VIRUS PATHOGENESIS AND ANTIBODY RESPONSE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Pathobiological Sciences

by
Anna Beatriz Kawiecki
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ABSTRACT

After the emergence of Zika virus (ZIKV) in the Americas in 2015, ZIKV infection was associated for the first time since its discovery with severe symptoms in both adults and congenital cases, including neurological, ocular, and developmental manifestations. Previous ZIKV circulation in Africa and Southeast Asia has been characterized by mild symptoms and small-scale case-counts. It is unclear whether the unprecedented size and severity of the ZIKV outbreak in the Americas are the consequence of a change in the virus, different background flaviviral immunity in the population, or a reporting issue. In addition, ZIKV has been shown to be transmitted through sexual contact, and the shedding of ZIKV from various bodily fluids in both humans and *in vivo* models suggests that other potential routes of transmission exist. We present here two mouse models that can be used to further investigate ZIKV pathogenesis, transmission, and immune response. Mice lacking interferon regulatory factors 3 and 7 (IRF 3/7 DKO) supported robust infection with the prototype MR766 strain from Uganda, while maintaining a 72% survival rate, and recapitulated symptoms and tissue lesions associated to infection with American ZIKV isolates in humans and other *in vivo* models. The MR766 strain was capable of causing retinal lesions and viral RNA shedding from the conjunctival fluid, hitherto unreported to be caused by an African strain. Further, ZIKV was visualized in the seminal fluid co-localized with infected epithelial epididymal cells, suggesting a possible cellular component of sexual transmission. Immunocompetent C57BL/6 mice, although not susceptible to ZIKV, were capable of mounting a robust antibody response that strongly neutralized ZIKV and was also able to cross-neutralize DENV2. We further report that homologous re-exposure with ZIKV in C57BL/6 mice reduced the DENV2

cross-neutralizing capability of the antibody population, while at the same time increasing enhancement of DENV2 infection. We conclude that ZIKV strains of the African and Asian lineages share a similar pathogenesis, suggesting that the increased severity of symptoms is unlikely to be due to a change in the virus. We also show that re-exposure to ZIKV can alter the antibody response to increase the risk of heterologous infection.

CHAPTER 1: INTRODUCTION AND REVIEW OF ZIKA VIRUS DISEASE AND ANTIBODY RESPONSE

1.1 INTRODUCTION

1.1.1 Background

Zika virus (ZIKV) is an arbovirus and a member of the *Flaviviridae* family, related to other flaviviruses such as West Nile virus (WNV), yellow fever virus (YFV), and dengue virus (DENV)[1]. ZIKV was originally isolated from a rhesus macaque (*Macaca mulatta*) in the Zika Forest in Uganda in 1947[2]. It was consecutively isolated from *A. africanus* mosquitoes in the same forest in 1948 [2], and the first isolation in humans was confirmed to have occurred in Uganda in 1962-1963[3, 4].

Data from serosurveys dating back to the 1950s suggest circulation of ZIKV in Africa, the Indian subcontinent, and Southeast Asia[5]. Although these reports should be interpreted with caution due to antibody cross-reactivity with other flaviviruses, this evidence, together with sporadic isolations of ZIKV from humans, mosquitoes, and non-human primates, indicates that ZIKV is endemic in Africa and several countries in Asia[1, 5]. ZIKV was not reported to cause any major outbreaks until 2007 on the Island of Yap in Micronesia[6]. Up to this point ZIKV had been characterized by largely asymptomatic infections or inducing mild symptoms that generally resolved within 1-2 weeks of onset, some of the most frequent being maculopapular rash, fever, arthritis, non-purulent conjunctivitis, myalgia, and headache[5, 6]. This clinical presentation can be confounded with that of other diseases that often circulate in the same areas, such as chikungunya virus (CHIKV) or DENV. However, in ZIKV infections, limb oedema and

conjunctivitis seem to be more frequent, while hepatomegaly, leukopenia, and thrombocytopenia are less so[7].

The next substantial outbreak of ZIKV occurred in French Polynesia in 2013, where the first evidence of ZIKV association with more severe symptoms made its appearance: the incidence of Guillain- Barré syndrome was 20 times higher during the ZIKV outbreak than in non-outbreak periods[8, 9], and an association between ZIKV infection and microcephaly was found retrospectively[10]. After the outbreak in French Polynesia, ZIKV disseminated through the South Pacific, with outbreaks in the Cook Islands and Easter Island. Finally, in late 2014 it arrived in Brazil, from whence it spread explosively throughout the Americas[5, 11], accompanied by a dramatic increase in incidence microcephaly and other congenital anomalies in babies born to ZIKV-infected mothers that were preliminarily associated to ZIKV infection[10, 12]. These events caused the WHO to declare the ZIKV outbreak as a Public Health Emergency of International Concern in 2016. Since then, the birth defects caused by ZIKV have been defined as the congenital Zika Syndrome (CZS), and the link of causality between CZS and ZIKV has been confirmed by the CDC[13, 14], supported by the detection of ZIKV in amniotic fluid, fetal brain tissue, placentas, and proof of concept in mouse models[12, 15, 16]. CZS is characterized by five features: microcephaly with partially collapsed skull, decreased brain tissue with a characteristic pattern of damage (such as brain calcifications), damage to the back of the eye, joints with limited range of motion, and an elevated muscle tone[14]. In adults, other symptoms, neurological and otherwise, have also been associated with ZIKV infection, such as Guillain Barré, meningoencephalitis[17], acute myelitis[18], hearing loss[19, 20], and ocular

pathology[21, 22]. Although many studies have contributed to strengthening the link of causality between ZIKV and these other neurological an congenital symptoms, they have yet to be confirmed[5, 8, 12, 23-26]. Deaths due to ZIKV are rare and are usually accompanied by co-morbidities or immunosuppression[27, 28].

Little is known of the infection kinetics of ZIKV. *Aedes* mosquitos have been shown to be competent vectors, and are likely the main form of transmission (see below). After the bite of an infected mosquito, ZIKV likely replicates in the skin cells[29] and from there enters the bloodstream. ZIKV can be detected in blood from 3.5 to 10 days after infection[1], and is usually present at low levels[30]. It appears ZIKV is associated with cells in the blood, given that ZIKV RNA levels are found at higher titers and for a longer time in whole blood than in serum, in addition to the fact that ZIKV has been shown to infect monocyte- derived dendritic cells[31, 32]. ZIKV is shed from various bodily fluids for variably prolonged periods of time. For example: in urine, the median time of shedding is 8 days; in semen, although the longest persistence of RNA was reported to be 188 days after symptom onset[33], a recent report indicates that 95 % of males stop shedding the virus after 3 months[34]. ZIKV infection and persistence in different tissues is still under study.

ZIKV infection induces an innate and adaptive immune response. IgM antibodies appear around 9 days post infection[5] and can be detected for 2-3 months[35]. IgG antibodies appear later and are thought to remain present for life[5, 35].

Genetically, ZIKV is has evolved into two lineages: the African lineage, which includes strains circulating in Central and Western Africa, and the Asian lineage, which includes

strains circulating in Southeast Asia, the Pacific, and the strains participating in the current outbreak in the Americas[35-37]. The strains circulating in the Americas have more than a 99% nucleotide identity with the French Polynesian strain[1].

1.1.2 Replication

ZIKV has a single strand of positive sense RNA, which is a common feature of flaviviruses. It has one open reading frame (ORF) which encodes a single polyprotein that is later cleaved into the structural and non- structural proteins, as well as a 5' untranslated region (UTR) with a type I cap structure, and a 3' UTR that lacks a polyadenylate tail[1, 35]. The structural proteins are the capsid (C), that is associated with the RNA, the membrane (prM), that must be cleaved into M protein in order to produce a mature virus particle, and the envelope protein (E)[35]. Cryoelectron microscopy has provided information on how these proteins are organized in a virus particle: the C proteins associated to the RNA strand form the nucleocapsid, that is surrounded by a lipid bilayer acquired during cell egress, in which the E and M proteins are anchored forming an icosahedral surface[38]. The surface of the virus is spiky in immature virions where the E and uncleaved prM proteins form heterodimers, but is smooth in mature virions where the E proteins form homodimers and the cleaved M protein lies flat[39, 40]. The E protein has three domains (DI-III), one of which, DII, contains the fusion loop (FL) which is responsible for fusion with the endosome upon viral entry. The structure of the ZIKV E protein is similar to the DENV E protein, with some differences in the glycosylation sites as well as a positively charged patch adjacent to the FL, which could determine the virus' cellular attachment or neurovirulence[40, 41]. The non-structural proteins participate in viral replication,

processing of polyproteins and host immune response modulation[35]. There are seven non-structural proteins in the ZIKV virus genome: NS1, which in ZIKV has some unique electrostatic features in the interface with host factors[35]; NS2A and NS2B; NS3, which in ZIKV has a different conformation than in other flaviviruses, with the protease portion forming an homodimer and the helicase portion forming a monomer [42, 43], NS4A and NS4B, which participate in forming the replication complex, and NS5 which is the RNA polymerase[35].

The replication of ZIKV virus has not been fully explored, but is thought to be similar to that of other flaviviruses[44]. The cellular receptor that allows internalization of the virus is still unknown, although several candidates have been proposed, including DC-SIGN, TIM-1, and AXL. After endocytosis, changes in the pH of the endosome lead to a conformational change in the E protein to a trimeric form, exposing the FL portion of the E protein[45]. This allows the FL portion to fuse the endosomal membrane and the viral envelope, resulting in the release of the viral genome into the cytoplasm. The viral RNA is immediately translated in association with cellular membranes, and the resulting non-structural proteins commence replication of the genome in membrane vesicles in the endoplasmatic reticulum[45]. The new genomes are packaged in the ER, and the immature virions are transported through the cellular secretory pathways, where they acquire the lipid membrane. The E protein is glycosylated and the prM protein is cleaved to produce mature virions, which are then released by exocytosis[35].

1.1.3 Transmission

ZIKV is mainly transmitted by *Aedes* mosquitoes[7]. Different members of the *Aedes* species have been associated with ZIKV outbreaks, such as *A. hensili* in Yap state, *A. aegypti* in New Caledonia and *A. polynesiensis* in French Polynesia[46], although *A. aegypti* and *A. albopictus* pose the greatest threat due to their broad distribution and anthropophilic behavior[47]. *A. aegypti* has been confirmed as a competent vector for ZIKV, being able to transmit ZIKV after feeding from an infected human volunteer to a susceptible mouse[48]. Field-caught specimens of *A.albopictus* in Mexico[49] and *A. aegypti* in Brazil[50] have been found to be infected with ZIKV, therefore confirming the probable role of these two mosquito species in transmission in the Americas. ZIKV has also been isolated from other *Aedes* mosquitos such as *A. africanus*, *A. luteocephalus* and *A. furcifer*, which are forest dwelling mosquitos in Africa probably related to the sylvatic cycle of ZIKV[51]. In addition, ZIKV has been isolated from non- *Aedes* genera of mosquitos such as *Mansonia*, *Culex* and *Anopheles*[51]. However, isolation of virus from a mosquito does not equate to vector competence, and the role of these mosquitoes in ZIKV transmission is unclear.

The ability of a mosquito to get infected will depend in part on the duration and the magnitude of the host's viremia[5]. The incubation period in human patients ranges from 3.5 to 10 days[1] after which point the virus can be detected in blood. Viremia peaks upon symptom onset[5, 35], and is generally of low titer[30, 52]. ZIKV can induce intermittent viremia, as has been shown to occur in 10% of the study subjects in a recent report, where the positive samples were separated from each other from 14 to 62 days[34]. This is supported by reports of secondary episodes of symptomology that

occur after initial remission[1, 21, 53], and by experimental infections of rhesus macaques, where recurrence of low-level viremia was seen for up to 17 days post infection[54]. ZIKV RNA has been found in whole blood for as long as 58 days after symptom onset[32], implying that ZIKV may persist in the cellular component of the blood. In addition, pregnant women and non-human primates infected with ZIKV have been shown to have prolonged viremia, possibly due to viral dissemination from the infected fetus, which indicates that pregnant women may play an important role in transmission[34, 54, 55].

Once a competent mosquito ingests an infected blood-meal, it is able to transmit the virus to susceptible humans after an extrinsic incubation period (EIP). The EIP was found to be about 10 days in *A. aegypti*[56]. One study found that *A. aegypti* infected with the Brazilian ZIKV isolate BRPE243/2015 had levels between 4 and 7 log 10 of ZIKV in saliva[57], and another study showed that high levels of virus were maintained in the mosquito for up to 60 days post infection, although mosquitoes do not live that long outside of a laboratory setting[7, 56, 58].

Sexual transmission has been reported from male to female, female to male, and male to male[59-62]. This is supported by the isolation of virus from the vaginal mucosa[63] and semen[64]. In males, ZIKV is able to persist for long periods of time in the semen[34] and seems to be able to cause lesions in the reproductive organs evidenced by hematospermia and prostatitis[59, 65]. ZIKV has also been visualized in the spermatozoa in an infected male[66], supporting the involvement of sperm cells in transmission. However, the exact route of male to female transmission during intercourse remains unclear and may not be restricted to the sperm cells, as there have

been reports of transmission either orally or by pre-ejaculate[67, 68], in addition to transmission from a vasectomized male, where the involvement of spermatozoa is unlikely[69]. In a mouse model, infection of the vagina of a pregnant female mouse led to fetal brain infection, indicating that the sexual transmission route can cause the same repercussions to the fetus as transmission by mosquito bite[70]. ZIKV sexual transmission could contribute to increasing the size and spread of epidemics as well as their duration by sustaining transmission even at times when mosquito populations are low[71, 72].

Epidemiological evidence and the isolation of virus from various bodily sources indicate that other transmission routes are possible. The first perinatal cases of ZIKV reported in 2013 were suspected to result from contamination occurring during delivery, breastfeeding, or close maternal contact rather than from transplacental transmission, due to the lag in viremia appearance in the neonates[73]. The perinatal transmission route is supported by detection of ZIKV RNA in the milk[74] and the saliva of ZIKV infected mothers[75], although neither of these routes have been confirmed. ZIKV has also been isolated from saliva in infants[75] and non-human primates[76]. Transplacental transmission has later been confirmed in congenital cases in Brazil, through detection of either ZIKV RNA, proteins or infectious virus particles in amniotic fluid, blood and tissue of fetuses and in the placenta[15, 55, 77-79]. The ability of ZIKV to cause congenital disease is reminiscent of other TORCH pathogens (*Toxoplasma gondii*, other, rubella, cytomegalovirus and herpes simplex virus) and could merit the inclusion of ZIKV in this group[79].

Transmission by transfusion and organ donors is also a legitimate concern due to the high rate of asymptomatic infections of ZIKV. In French Polynesia 2.8% of the blood donors tested had ZIKV RNA[80], and in Puerto Rico a total of 0.5% of donors had detectable ZIKV in a 5 month period, the incidence increasing over time[81]. Infection through platelet transfusion has been reported to occur in Brazil[82].

Other possible routes of ZIKV infection have been suggested, but more evidence is needed to confirm their role in transmission. In the case of a terminal ZIKV patient in Utah, transmission to a care-giver occurred where the vector-borne and sexual routes were ruled out, and the most likely explanation was infection while the care-giver wiped the patient's eyes without wearing gloves[83]. This case, together with previous ZIKV isolation from the conjunctiva in adult patients[84], opens the possibility of transmission through eye-exudate. Another possible route that has been suggested is through monkey-bite in Indonesia, although in that case mosquito infection could not be ruled out[85]. These reports, together with ZIKV isolation at high levels from saliva and urine in both humans and non-human primates, and a case where transmission during oral intercourse could not be ruled out[67], suggest that transmission from glandular secretions and other bodily fluids could be a potential additional transmission route of ZIKV, and should be further investigated.

1.1.4 ZIKV outbreak in the Americas

The recent explosive outbreak of ZIKV in the Americas has raised several questions, mainly: why has the virus spread so efficiently in the region, and more importantly, why have severe neurological and congenital symptoms only become evident after the

outbreak in French Polynesia, in spite of the ongoing circulation of ZIKV for decades before in other regions?[86].

Transmission of ZIKV in the Americas could have been facilitated by environmental factors such as the warmer temperatures and flooding in 2015-2016, which could have increased the efficiency of transmission or the range of distribution of competent vectors. The increasing urbanization in the Americas that provides an apt environment for mosquito proliferation could also have played a role[5]. Another explanation could be regional genetic variations in the mosquito populations that improve their vector competence, allowing for increased transmission in certain areas. Large regional differences in vector competence inside the same species of mosquito has been previously noted for other viruses[87]. However, a more likely explanation for the large number of cases is that the virus was introduced into a completely susceptible population, and therefore was able to spread explosively as had occurred previously with chikungunya virus[88].

The cases of neurological symptoms such as Guillain- Barré and CZS reported to date have all been associated with strains of ZIKV circulating in the Americas[89]. These strains have been shown to belong to the Asian lineage of ZIKV that had been circulating in Southeast Asia for decades previously[1]. However there are no reports tying ZIKV to these symptoms in Southeast Asia, or in Africa where serosurveys have shown that ZIKV is endemic. This has led some researchers to speculate that ZIKV underwent a genetic mutation around the time of the French Polynesian outbreak, allowing it to cause neurological lesions and be transmitted through the placenta[86, 89]. To investigate this possibility, recent studies have contrasted the genetic

differences between African and Asian strains, as well as differences between the Asian strains belonging to the epidemic and previous strains not associated with severe symptoms. The most significant changes have been found in the prM, NS3, NS5, C, and E proteins, as well as the 5' end of the UTR, that can lead to the formation of differently shaped RNA loops (the 3' UTR seems to be more conserved among all ZIKV strains)[89, 90]. Although there is variation among the amino acid changes found in the different studies, some changes are consistent, namely: the S139N mutation of the prM protein that appeared in the French Polynesian strain and is conserved in the strains circulating in the American epidemics[89, 91, 92]; the Y2086H mutation in the NS3 protein, that was found by Faria et al. (2016) to influence the physicochemical properties of the protein environment, and was also found, like S139N, to be conserved among the epidemic strains from the French Polynesian outbreak onward[89, 91]; N25S, L27F, R101K, I110V, and I113V of the capsid protein were also common to the epidemic strains[92, 93]; M2634V of the methyl-transferase domain of NS5 is present in all American strains, and whether it is present in the French Polynesian strain as well is a matter of divergence between two studies[89, 91]. Further studies are needed to establish whether these mutations have any effect on the tropisms, pathogenicity, or physicochemical and conformational properties of the virus.

In addition to the amino acid changes in the epidemic strains, the evolutionary rates of the two lineages have been compared. While there is no consensus among the studies of when certain strains appeared, there is agreement that the evolution rates are higher in the isolates from the current outbreak, having higher rates than the African or even the French Polynesian strains[90, 93, 94].

However, the sudden appearance of these neurological and congenital symptoms could also be due to the fact that the sheer magnitude of the outbreak in the Americas, with a large number of people infected, has allowed significant differences to be noticed and reported in what are otherwise rare disease outcomes. Before the outbreak on the island of Yap, only 14 human cases of ZIKV had been reported in countries where other infectious diseases could have confounded the diagnostics[1, 95], and the subsequent reported outbreaks occurred on islands with small populations.

1.2 PATHOGENESIS OF ZIKV

1.2.1 Animal models of ZIKV pathogenesis

1.2.1.1 Mouse models

Several mouse models of ZIKV infection have been developed, allowing further exploration of pathogenesis. Wild type (WT) mice have been shown not to be readily susceptible to the related flavivirus DENV[96], and similar results have been shown with ZIKV. ZIKV infection of C57BL/6 WT mice has been shown to be age dependent: in several studies, adult mice do not allow effective replication of ZIKV[97, 98]; however, young C57BL/6 WT mice can become infected and even present representative ZIKV lesions. Direct intracranial infection with the ZIKV SZ01 isolate of 13.5 day old fetuses in 10-week old C57BL/6 pregnant mothers resulted in the infection of brain cells and neurological developmental anomalies[99], although a similar experiment where C57BL/6 pregnant females were injected with a high dose of the Paraiba Brazil 2015 isolate subcutaneously did not cause major body alterations in the fetuses, possibly because the virus was unable to cross the placenta[16]. Postnatal infection of WT

C57BL/6 mice that were 8 days old resulted in moribund animals that presented with ZIKV RNA in the spleen, brain, and eyes, as well as apoptosis in the CNS, including ocular processing regions[100]. Finally, 1 week old C57BL/6 old WT mice infected with the H/PF/2013 isolate presented a 70 % mortality[97].

Another WT mouse with a different background, 129Sv/Ev, survived and did not show signs of disease upon infection with African isolate ZIKV MP1751 but presented low levels of ZIKV RNA in the blood, ovary, and spleen at 3 days post infection (dpi), with persistence of RNA in tissues until 7 dpi, indicating that these mice become subclinically infected[101]. In addition, Swiss Jim Lambert (SJL) WT pregnant female mice infected with high titers of the Paraiba Brazil 2015 isolate of ZIKV resulted in whole-body growth delay or in intrauterine growth restriction (IUGR), mimicking the effects congenital ZIKV infection has on human fetuses[16]. Intracraneal injection of ICR WT fetuses with the ZIKV SZ01 isolate also resulted in infection of brain cells and neurodevelopmental anomalies[102]. Neonatal Swiss Webster mice were highly susceptible to intracranial ZIKV infection with both Asian and African isolates[103].

In adult mice, knock-out models lacking elements of the interferon (IFN) response have been shown to be more susceptible to strais from either lineage of ZIKV, and present similarities in the kinetics of infection and lesions induced. Adult mice ranging in age from 5 weeks to 6 months old of the Ifnar11 -/- strain, that lack the IFN receptor and are therefore unable to respond to either IFN alpha or IFN beta, are highly susceptible to ZIKV, presenting a high mortality rate[97]. The interferon regulatory factor (IRF) 3/5/7 triple knock-out (TKO) strain, that produce almost no type I IFN, present similarly high susceptibility and mortality as the Ifnar1 -/- strain[97]. 0% of the IRF 3/5/7 TKO mice

survived, independent of the route of infection or the ZIKV strain used. On the other hand, the Ifnar11 -/- mice presented 100% lethality upon H/PF/2013 infection, but had varying rates of survival depending on the age and infection route upon MR766 infection[97]. This seems to indicate that in these two strains of mice, ZIKV H/PF/2013 is more pathogenic than the MR766 strain. In addition, infection with both strains caused neurological signs in both mouse models, such as paralysis and hindlimb weakness. Ifnar11 -/- mice also presented with high viral loads in the liver, kidney, spleen, serum, CNS tissues, and testes after infection with ZIKV H/PF/2013. Interestingly, in this mouse model, ZIKV RNA persisted in the brain and testes until 28 dpi, paralleling findings in humans in whom ZIKV can still be detected in the semen long after infection[34].

Other knock-out mouse models have shown susceptibility to both lineages of ZIKV. AG129 mice, with C57BL/6 background but lacking IFN alpha, beta, and gamma receptors, were extremely susceptible to Asian isolates of ZIKV, presenting severe signs of disease and weight loss even after infection with 1 plaque forming unit (PFU) of ZIKV H/PF/2013[104]. ZIKV infection in these mice was 100% lethal by 6 dpi independently of the age or dose administered[98, 104]. Viremia peaked at 2 dpi and high levels of ZIKV RNA were also found in brain and testes[98, 104]. Histologically, ZIKV infection resulted in inflammatory cell infiltration and neuron necrosis in the brain, as well as myofiber degeneration and necrosis of the muscles in the posterior limbs[104]. A129 mice, which are background 129 mice lacking IFN alpha and beta receptors, presented similar kinetics as the AG129 mice after infection with either an African isolate (ZIKV MP1751) or an Asian one (FSS13025)[98, 101]. A129 mice,

however, have shown age dependence in disease severity, with full recovery of 11-week-old mice as opposed to the 100% mortality of 3-and-5-week-old mice. Viremia also peaked at 2 dpi, and interestingly, as virus levels decreased in the serum, they increased in the testes and brain, reaching high levels at 6 dpi[98, 101]. One important difference between the AG129 model and the A129 model is that in the AG129 model mice developed neurological signs after infection, while the A129 model did not. It was therefore suggested that IFN gamma could play an important role in protection of the central nervous system (CNS)[98]. Histologically these mice also developed lesions in the brain, presenting perivascular cuffing in the meninges and parenchyma and degenerate nuclei in the hippocampus. These mice also developed lesions in the spleen, which showed large and poorly-defined germinal centers and a depletion of mature lymphocytes, although the ovaries were found to be normal[101]. However, in some cases the deletion of individual elements of the antiviral response was not sufficient to permit ZIKV infection. IRF 3 -/- mice, in which a single IRF that induces the expression of type I IFN is knocked-out, and MAVS-/- mice, that lack an intermediate element in the RIG-I pathway, are not susceptible to ZIKV[97].

A defective type I IFN response can also be achieved in immunocompetent mice by blocking IFN receptors with specific monoclonal antibodies. Five-week-old C57BL/6 mice pre-treated with several doses of MAb-5A3, a monoclonal antibody that targets the Ifnar11 subunit of the IFN alpha and beta receptors, infected with African ZIKV isolate DAK AR D 41525 developed severe disease that was dependent on the infection route[105]. The advantage of these mice is that the immunosuppression occurs only at the time of infection, but the lesions and subsequent immune response develop in an

immunocompetent mouse. This model presented acute to subacute encephalitis/encephalomyelitis including neuronal death and inflammatory cell infiltration, with the hippocampus being the most affected, followed by the thalamus and cerebrum. In addition, ZIKV RNA was visualized in these regions by *in situ* hybridization (ISH), confirming the causality of the lesions. The spleen and skeletal muscle of the head and skeletal column were also affected, similarly to what had been described earlier in AG129 and A129 mice by Dowall et al. and Aliota et al.[101, 104, 105]. Similar pre-treatment with anti-IFN antibodies allowed the sequential infection with both African and Asian strains of ZIKV to generate a panel of monoclonal antibodies from immunocompetent C57BL/6 mice[106].

1.2.1.2 Non-human primate models

Female and male rhesus macaques (*Macaca mulatta*) and cynomolgous macaques (*Macaca fascicularis*) presented similar infection kinetics after infection with either the African isolate MR766 or virus isolates from the Asian lineage. Viremia peaked between 2- 6 dpi[54, 107-109]. In both rhesus and cynomolgus macaques infected with Asian strains, viremia could be detected after initial clearance. In rhesus macaques this intermittent detection was in the form of low-level “blips” of RNA detection in plasma until 17 dpi[54], while in cynomolgus macaques it was a moderate viremic load that rebound at 30 dpi[109]. However, the African strain IBH30656 was unable to mount a productive infection in cynomolgus macaques, resulting in very low viral RNA levels in blood and other tissues[109].

In addition, ZIKV RNA was detected in other bodily fluids for variably prolonged periods of time after viremia clearance. In rhesus macaques infected with strains from either lineage, ZIKV RNA could be detected in the cerebrospinal fluid (CSF) as late as 14-21 dpi[54, 107, 108]. In urine and saliva, ZIKV RNA shedding lagged several days behind viremia in both rhesus and cynomolgus macaques, with detection in some animals as late as between 9-28 dpi[54, 107-109]. In one model, the detection of virus in urine coincided with the intermittent “blips” of viremia in plasma[54]. Another study found the levels of virus in blood to be correlated with those in urine[107]. ZIKV RNA was also detected sporadically in the vagina after peak viremia[54, 107] and could be detected up to 28 dpi in the semen[107]. Ocular fluid has also been shown to present ZIKV RNA[110]. Further, pregnant rhesus macaques infected with an Asian strain maintained plasma viremia between 29 and 57+ days, coinciding with the persistent viremia detected in human mothers[34, 55].

The intermittent detection of ZIKV RNA in various body fluids and tissues after initial viremia clearance has led several of these studies to suggest that certain tissues could be acting as reservoirs for viral dissemination, such as the male gonads, the oral mucosa, or the infected fetus[54, 107, 109]

ZIKV infection with either MR766 or Asian strains in rhesus macaques induces the activation of an innate and adaptive immune response that peaks between 5 and 10 dpi, coinciding with reduction of viremia. This indicates that the immune response could be influential in clearing the virus[54, 108]. The immune activation was evidenced in both pregnant and non-pregnant subjects by expansion of CD8+ T cells, CD4+ T cells by 6 dpi, expansion of NK cells after 6 dpi, plasmablast expansion that peaked between 7-10

dpi, and high neutralizing antibody titers as early as 14 dpi. This immune response protected from homologous and heterologous challenge in the non-pregnant subjects, but, interestingly, it was not sufficient to inhibit persistent viremia in pregnant subjects[54, 107, 108].

1.2.2 Skin pathogenesis

PF-25013-18 strain, a French Polynesian strain of ZIKV, was able to infect fibroblasts, keratinocytes, and immature dendritic cells and produce infectious virions, indicating that ZIKV can productively infect the skin[44]. This is an important step in the ZIKV cycle, as primary transmission route is by mosquito bite into the skin. Several cell receptors have been shown to allow ZIKV entry into the cell, including DC-SIGN, TYRO3, and AXL. TIM-1 seems to play a supplementary role by aiding AXL-mediated entry[29]. TYRO3 is an immune regulating tyrosine kinase that is expressed mainly in the central nervous system, while AXL belongs to the same family but is more widely expressed [44]. AXL was shown to be an important entry factor, as neither cutaneous fibroblasts nor cutaneous keratinocytes express DC-SIGN but they do express AXL.

Hamel et al. (2015) explored the localization of ZIKV replication inside the cell by electron microscopy[29]. Infected primary skin fibroblasts showed membrane vesicles closely associated to the endoplasmic reticulum, hinting at membrane-associated ZIKV replication inside cells. Infected fibroblasts also presented with autophagosome-like vesicles. Although autophagy can form part of an antiviral mechanism to degrade virus particles, it is used by other flaviviruses such as DENV or JEV to promote replication[111-113]. This seems to be also the case with ZIKV, as inducing

autophagosomes increased viral replication, while blocking them decreased the amount of virus. ZIKV also co-localizes with LC3, a cytosolic microtubule-associated protein, indicating that autophagosomes are likely another replication site for the virus[29]. In addition, infected epidermal keratinocytes developed vacuoles in the cytoplasm, which, together with the picnotic nuclei in the stratum granulosum, indicates that ZIKV induced cellular apoptosis. It is speculated that this could be a strategy to increase viral dissemination[29].

1.2.3 Ocular pathogenesis

During the current ZIKV outbreak in the Americas, there have been increasing reports of ocular involvement in both adult and congenital cases of ZIKV. Conjunctivitis is one of the signature symptoms of ZIKV infection that distinguish it from other arboviral infections and occurs in a high percentage of infected adults[6, 100, 114]. More severe cases of ocular lesions have been reported adults as well: a ZIKV-infected 26-year old man developed photopsia (light flashes) associated with bilateral posterior uveitis and chorioretinal lesions two weeks after symptom onset[21]; another man in his forties developed bilateral uveitis 8 days after ZIKV symptom onset and presented ZIKV RNA in the anterior chamber of the eye[22]; in other cases in China, infectious ZIKV, confirmed to be an Asian strain, was isolated from conjunctival swabs at high viral loads[84]. In infants born to ZIKV-infected mothers, ocular anomalies including macular alterations (pigment mottling and/or chorioretinal atrophy), optic nerve abnormalities, lens subluxation, and bilateral iris coloboma, present themselves both with and without concomitant microcephaly[115-118]. In one study, at least one of these lesions was found in 34.5% of the congenital ZIKV cases, although lesions were not always

bilateral[119]. All these cases either occurred in Brazil or were travel-related cases returning from Latin America.

ZIKV involvement in the eye was confirmed by Miner et. al. (2016) in *in vivo* experiments infecting ZIKV-susceptible mice with ZIKV strains form the recent outbreak in the Americas[100]. Adult WT mice pre-treated with anti-Infnar1 antibody and infected with both the Brazil Paraiba 2015 and the H/PF/2013 strains of ZIKV resulted in ZIKV RNA presence in the eye that incremented from 2 to 6 dpi[100]. Ifnar11 -/- mice infected with Brazil Paraiba 2015 ZIKV strain resulted in ZIKV RNA presence in tear fluid and lacrimal gland at 7 and 28 dpi[100]. The presence of infectious ZIKV at 7 dpi was confirmed by inoculation of another susceptible mouse, AG129, with ocular homogenates from these Ifnar11 -/- infected mice, causing the death of the AG129 mice[100]. However, samples from 28 dpi were not found to be infectious, which indicates clearing of infectious virus in spite of RNA persistence. In Ifnar11-/- immunodeficient adult mice, ZIKV was shown to infect the cornea, iris, lens, retina, choroid complex, and optic nerve, while causing conjunctivitis, panuveitis, and neuroretinitis, although these lesions did not cause global photoreceptor abnormalities[100]. In addition, ZIKV was able to infect and cause lesions in the optic tract, geniculate nucleus, and visual cortex of WT C57BL/6 8-day-old mice with intact interferon pathways[100]. ZIKA RNA has also been detected from the ocular fluid of rhesus macaques[110].

The ocular pathogenesis of ZIKV is currently under investigation. AXL, a cellular receptor important in ZIKV entry [29], was found to be highly expressed along the outer margin of the neural retina and in the adjacent cells of the ciliar marginal zone[120],

which could explain the ocular tropism and lesions caused by ZIKV. However, Miner et al. (2016) performed infections with ZIKV Paraiba 2015 strain on AXL-/- and Mertk -/- mice and found no difference in either brain or ocular ZIKV FFU levels with infected mice sporting intact AXL and Mertk. This suggested that neither AXL nor Mertk receptors are essential for either brain or ocular infection[100]. It was speculated that the ZIKV-induced ocular lesions could also be a result of inflammation caused by cell death or the activation of intra- and/or extracellular PAMPS by ZIKV RNA[100], but further studies must be undertaken to elucidate the pathways of ZIKV pathogenesis in the eye. In addition, all the cases of severe ocular pathology induced by ZIKV to this point have been associated with the Asian strain of ZIKV circulating in Americas. The question of whether the African strain of ZIKV is similarly capable of ocular lesions has not yet been answered.

1.2.4 Neurological pathogenesis

Both the Asian and African strains of ZIKV have been shown to infect human neural progenitor cells, neurons, and astrocytes in culture *in vitro.* These infections are productive, resulting in an increase of viable infectious viral particles, in addition to a dysregulation of the cell cycle and a modulation of the anti-viral response of the infected cells[121-123]. ZIKV infection of neuronal cells resulted in apoptosis, necrosis, or decreased proliferation[16, 123]. The same effects were reproduced in neurospheres and organoids, which are more complex *in vitro* models of the human developing brain. In neurospheres, both the MR766 strain and the Asian strain were able to reduce the size of the neurospheres by inducing apoptosis and reduced proliferation, although this effect was more dramatic after infection with the Asian strain[16, 124]. Organoids are

3D models that recreate the architecture of a fetal cortex, including the lamination and distribution of the progenitor cells[16]. In organoids, infection with strains from both lineages of ZIKV resulted in a decrease in overall size, thinner neuronal layers, and enlarged lumen of the ventricles[16, 124-126]. Dang et al. (2016) implicated TLR-3 activated genes involved in cell proliferation, apoptotic pathways, and axongenesis in causing these changes[126]. Nowakowski et al. (2016) found that the candidate ZIKV receptor AXL is strongly expressed in radial glia neural stem cells, cortical astrocytes, blood microcapillaries, and microglia in human-derived organoids, indicating it may determine susceptibility of cells to ZIKV[120]. However, experiments *in vivo* in which ZIKV-infected AXL -/- mice did not have a decreased amount of ZIKV in the brain suggest that AXL is not essential for ZIKV infection[100].

Some difference in the kinetics of infection of neural cells have been observed between the African and Asian strains. Simonin et al.(2016) found that an African strain modulated the cell cycle progression and anti-viral response of neural progenitor cells more strongly than an Asian strain, and another study found differences in the antiviral response induced by strains from the two lineages in astrocytes as well[121, 122]. In organoids, which permit the study of the specific cortical layers affected by ZIKV infection, studies do not have uniform results on the differential infection of cellular subtypes by the two lineages. Quian et al. (2016) found that both African and Asian strains of ZIKV (MR766 and FSS13025) preferentially infected SOX+ neural progenitor cells in the ventricular zone and in the outer subventricular zone, although ZIKV was also detected in CTIP2+ neurons and in some GFAP+ astrocytes[125]. On the other hand, Cugola et al. (2016) found that infection with the Asian ZIKV reduced the number

and cortical plate thickness of a broader spectrum of cortical neuron subtypes, including SOX+, TBR1+, CTIP1+, PAX6+, and Ki67, while the MR766 strain affected only CTIP2 and PAX6+, not including SOX+[16], therefore countering what was found by Qian et al. (2016). Cugola et al. (2016) also found an increase in the number of caspase-3+ apoptotic cells in the organoids after infection with the Brazilian strain of ZIKV but not the MR766 strain, although in other studies using human neural progenitor cells MR766 was shown to induce caspase-3 mediated apoptosis as well[16, 123].

Mouse models of congenital infection with the Asian strain of ZIKV parallel many of the findings seen in the *in vitro* models of human neural cells in the brains of the infected fetuses, including the induction of apoptosis and the downregulation of genes involved in proliferation, differentiation, and organ development[16, 99, 102]. Therefore ZIKV infection in mice seemed to suppress neural progenitor cell proliferation and differentiation while replicating in these cells at high efficiency, similarly to what occurs in human cells[102]. Intracraneal infection of ICR fetal mice in the mother's womb with ZIKV SZ01 resulted in smaller brains with enlarged ventricles, a thinner cortical plate and ventricular and subventricular zones, as well as thinner cortical layers although without disturbance of the lamination[102]. Neural progenitor cells were established as the main target for ZIKV, although post-mitotic cells were also seen to be infected after 5 days of infection[102]. Intraperitoneal infection of pregnant C57BL/6 mice mothers with SZ01 strain of ZIKV caused lesions in the brain of the pups, with infected radial glia cells and reduced number of cortical neural progenitors. This model presented some differences with the human *in vitro* models, because although the outer perimeters of the cortex were shorter, there was no change in the relative thickness of the individual

cortical layers and the cavities of the ventricles were reduced instead of being enlarged as seen in the human organoids[99]. In addition, infection with the Paraiba Brazil 2015 ZIKV isolate in SJL WT mouse pregnant mice induced whole-body growth delay or intrauterine growth restriction of the fetuses, although the same experiment in C57BL/6 mice resulted in normal pups[16].

In addition to models of congenital infection, ZIKV infection in older mice also has resulted in brain lesions. 5-week-old immunocompetent C57BL/6 mice were infected with African ZIKV strain DAK AR D 41525 after pre-administration with anti-interferon antibody, resulting in acute to subacute encephalitis and encephalomyelitis presented by neuronal death, astrogliosis, microgliosis, and mild mononuclear inflammatory cell infiltrate. These lesions were most prominent in the hippocampus, especially in the pyramidal and granule cell layers but were also present in the thalamus and cerebellum[105].

There are two ways ZIKV could likely be inducing microcephaly: by decreasing the number of neuronal progenitor cells (NPCs) through the induction of apoptosis and dysregulation of their replication, or by targeting cells that are important for the correct development of the cortex, such as radial glial cells that serve as a scaffold for the migration of cortical neurons to other areas during the corticogenesis[127].

Although in the *in vivo* experiments, microcephaly was achieved after infection with the Asian strain, the *in vitro* experiments with human organoids and neurospheres indicate that the African MR766 strain is able to cause similar defects in humans.

1.2.5 Placental pathogenesis

Strains from both the Asian and African lineages of ZIKV have been shown to infect placental cells derived from both humans and mice. The African MR766 and the Asian FSS13025 strains of ZIKV were both able to infect trophoblast-derived cell lines BeWo, JEG-3, JAR choriocarcinoma cells and the extravillous trophoblast cell line HTR8/SVneo, although BeWo cells were the least susceptible to infection[128]. Primary human cells isolated from mid to late-gestation placentas and infected with either MR766 and or a Nicaraguan isolate, Nic1-16, were able to mount productive infections, including amniotic epithelial cells (AmEpCs), cytotrophoblasts (CTBs), trophoblast progenitor cells (TBPCs), human placental fibroblasts (HPF), and human umbilical vein endothelial cells (HUVECs)[129]. More viral progeny was produced in AmEpCs by the low-passage Nicaraguan isolates than by MR766. In explants of chorionic villi from 1st-trimester placentas, ZIKV infected and replicated in CTBs and invasive CTBs, which then ceased to proliferate, as well as Hofbauer cells in the villus core. The receptor TIM1 was found to be consistently expressed in many of these systems, particularly in the basal decidua, parietal decidua, amniotic membranes, primary AmEpCs, and chorionic villi, while the expression of Axl and Tyro3 was variable by cell line and stage of differentiation. TIM-1 binds to phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the envelope of flaviviruses such as DENV and WNV [130]. Duramycin, which binds PE, blocked the infection with either strain of ZIKV in primary placental CTBs, AmEpCs, and chorionic villus explants, indicating that TIM-1 might be an important cofactor for ZIKV infection in the placenta. On the other hand, an AXL inhibitor only modestly reduced infection[129].

A study by Bayer et al. (2016) using primary human trophoblasts isolated from full-term placentas found these trophoblasts to be refractory to infection by both the African MR766 strain and the French Polynesian strain FSS13025. In addition, syncytiotrophoblasts were found to constitutively release type III IFN (IFNlambda1) that not only is likely responsible for protecting the trophoblasts from infection, but was also shown to protect non-placental cells in a paracrine manner[128]. However, Quicke et al. (2016) showed that Hofbauer cells, and to a lesser extent syncytiotrophoblasts, also isolated from full-term placentas, resulted in productive infections after infection with the PRVABC59 isolate of ZIKV[131]. The infection induced an antiviral response in these cells, in this case not accompanied by cell death. The fact that Hofbauer cells were efficiently infected by ZIKV but not killed is suggestive that these cells might play an important role in viral dissemination[35]. Type I IFN and cytokine production were induced by the infection, but there was no evidence of type III IFN release. The difference between the two studies may be due to the different ZIKV isolates used or to a difference in the time points measured: Bayer et al. (2016) measured only at 24 hours post infection, and although Quicke et al. (2016) also did not observe replication in the syncytiotrophoblasts during the early stages of infection, by 96 hours post infection a low level of replication did occur[131]. Interestingly, Tabata et al. also did not find ZIKV infection in syncytiotrophoblasts[129].

In experiments *in vivo*, immunodeficent Ifnar11 -/- mice females crossed with WT males and infected with H/PF/2013 strain of ZIKV resulted in severe intrauterine growth restriction (IUGR), placental ischemia, and resorption of the fetuses[132]. Presence of ZIKV was confirmed in different trophoblast cells, including glycogen trophoblasts,

spongiotrophoblasts, syncytiotrophoblasts, and also mononuclear trophoblasts, with electron microscopy showing dense bodies consistent with ZIKV virions. ZIKV virions were also observed by electron microscopy in the trophoblasts and fetal endothelium lining damaged capillaries, and apoptotic trophoblasts were present in the infected placentas. In addition, the fetal brains presented apoptotic cells in the mid and hind-brain, indicating that ZIKV vertically transmitted through the placenta can reach the fetal brain[132].

These findings point to several possible mechanisms of placental infection that may vary at different pregnancy stages. It is possible that ZIKV initially infects numerous cell types in the placenta, including trophoblasts and fetal endothelial cells, producing a large number of infectious particles which disseminate through the different placental membranes and infect Hofbauer cells, which in turn are able to maintain the infection[78]. ZIKV infection of these tissues could lead to severe vascular damage of the placenta accompanied by reduced blood flow, and the dissemination of ZIKV into the fetal circulation and to the fetal brain[129, 131, 133]. Tabata et al. (2016) found that AmEpCs from mid-gestation placentas produce higher virus titers than those from late-gestation placentas, indicating that this mechanism may be more likely in early-term infections[129]. On the other hand, in late-gestation infections, in which the innate immune responses of the placental trophoblasts seem to block productive infections[128, 129], ZIKV may rely on forming complexes with IgG non-neutralizing or cross-reactive antibodies to infect Fc-receptor-expressing Hofbauer cells that are capable of efficient replication[129, 131].

1.2.6 Testicular pathogenesis

Reports of ZIKV sexual transmission, of lesions in the reproductive masculine organs, and of prolonged ZIKV detection in the semen indicate that ZIKV is able to infect, replicate, and possibly persist in the male gonads[34, 59-62]. ZIKV antigen has been visualized in spermatozoa in the semen of an infected male[66]. However the mechanism of ZIKV infection in the male gonads is not fully characterized.

AXL has been shown to be an important receptor involved in ZIKV cellular entry[120, 134]. AXL is expressed in human testicular cells such as spermatogonia (SG), tubular-myoid cells, Leydig cells, and epididymal epithelial cells, but not in the cells of the prostate or the seminal vesicle [135]. The same pattern of expression is observed in testicular cells derived from WT C57BL/6 mice. In mice, infection with an Asian strain of ZIKV induced inflammation and damage in the testis and epididymis but not in the prostate or seminal vesicle, indicating that AXL expression may play a role in susceptibility to ZIKV[135]. However, in other *in vivo* experiments, AXL -/- mice pre-treated with anti-Ifnar11 antibody and infected with either an Asian or African strain of ZIKV presented high levels of virus in both the epididymis and testis, indicating that AXL is not essential for ZIKV infection in these tissues[136].

Both Asian and African strains of ZIKV were able to infect and replicate in specific cells in the testis in *in vivo* mouse models, at the same time altering the inflammatory cytokine response and hormone production of these cells[136]. ZIKV RNA was detected by ISH in spermatogonia and Sertoli cells, and to a lesser extent in Leydig cells[136]. Similarly, another study used an immunofluorescence assay to detect ZIKV in

spermatogonia and in Leydig cells, the antigen labeling being weaker in the latter[135]. This study visualized ZIKV in other cell types as well, including in testicular peritubular-myoid cells, that are stem-like progenitors of Leydig cells, and epididymal epithelial cells, as well as in cells inside the lumen of the epidydimis. In addition, infectious virus has been isolated from mature sperm and fluid collected from the lumen of the epididymis in mice[135].

Infection with an Asian ZIKV strain induced increased inflammatory cytokine and type I IFN mRNA expression and secretion in Sertoli cells, Leydig cells, and epididymal epithelial cells. Levels of TNF- alpha, IL-6, IFN b, and CXCL10 were elevated at 24 hours post infection, although levels of IFNa were decreased. However, no cytokines were secreted from peritubular-myoid cells or germ cells. ZIKV infection of progenitor Leydig cells could induce a lack of regeneration in these cells, and therefore lead to a decrease in testosterone, as well as serving as a potential cellular reservoir for ZIKV replication in the testes[135]. Indeed, in Ifnar1 -/- mice, the serum testosterone levels were decreased at 8 dpi [135], while in C57BL/6 pre-treated with anti-Ifnar11 antibody, the testosterone and inhibin levels in the testes decreased and remained low until 21 dpi after an initial increase at 7dpi[136]. These findings indicate that ZIKV infection can alter hormone secretion of Leydig and Sertoli cells.

ZIKV involvement in the male reproductive organs has been evaluated in three different mouse models: C57BL/6 mice pre-treated with anti-Ifnar1 antibody, Ifnar1 -/- knock-out mice, and WT C57Bl/6 mice[135, 136]. Of these, C57Bl/6 mice pre-treated with anti-Ifnar1 antibody and the Ifnar1 -/- mice present similar persistent lesions in both the testis and the epididymis[135, 136]. Both models present degeneration and depletion of

testicular and epididymal cells by approximately 14 dpi, with a progressive loss of structure of the seminiferous tubules and germinal epithelium accompanied by leukocyte infiltration, which is still present at the endpoint of the two studies; that is, 21-60 dpi[135, 136].

In the Ifnar1 -/- mice, the levels of ZIKV detection in the cells of both the testis and epididymis by immunohistochemistry, although strong at 8 dpi, decreased over time until they were no longer detected at 30 dpi[135]. However, at 60 dpi these mice presented testicular atrophy, indicating that the tissue lesions initiated by ZIKV persist in these mice despite reduction of ZIKV presence as detected by antibody. Interestingly, in the C57BL/6 mice pre-treated with anti-Ifnar1 antibody, the levels of ZIKV RNA detected by ISH remained high to 21 dpi in the remaining testicular cells and in the mature sperm in the lumen[136]. Therefore, it is possible that, similarly to what has been suggested to occur in the eye, the presence of ZIKV RNA and the subsequent activation of PAMPs that induces an inflammatory response may be responsible for the lesions in the genital organs[100].

In these two mouse models, ZIKV-induced lesions resulted in a decrease in size and weight of the testicles and the seminal vesicle[135, 136]. In addition, in the C57BL/6 mice pre-treated with anti-Ifnar1 antibody, fertility was demonstrably decreased after ZIKV infection: the total and motile sperm counts were diminished from 14 dpi until around 42 dpi, and the pregnancy rate and number of viable fetuses in females crossed with ZIKV-infected males was also decreased[136].

WT C57Bl/6 mice did not present testicular lesions after intraperitoneal injection with ZIKV but did develop similar disease progression as the Infar -/- after intratesticular injection[135]. WT mice intratesticullarly injected with DENV and phosphate-buffered saline (PBS) served as control, showing that the infection-induced lesions were not due exclusively to the injection route. Ten percent of the DENV-infected mice developed orchitis and testicular atrophy at 8 dpi with full recovery by 30 dpi, compared to the same development in 100 % of the ZIKV-infected mice, that continued progressing after 30 dpi[135]. Therefore, ZIKV is demonstrably capable of causing lesions in the gonads in immunocompetent mice. This indicates that once the infection is established in the gonads, an intact immune system is incapable of preventing, and perhaps even plays a role in causing, severe lesions in the tissues.

Intratesticullar infection induced lesions in the testicle, whereas intraperitoneal infection in the same mouse model did not. These findings indicate that crossing the blood-testicle-barrier could be a limiting factor for ZIKV infection. Both lack of type I IFN response and direct inoculation seem to allow ZIKV to surmount this barrier. However, in the case of C57BL/6 mice pre-treated with anti-Ifnar1 antibody, the blood-testicle-barrier was shown to be intact at 7 dpi, while ZIKV RNA could already be detected in spermatogonias and Sertoli cells. This would imply that destruction of the blood-testicle-barrier is not required for infection of the testicular tissue. More studies are needed to elucidate the mechanisms of infection and pathogenesis of ZIKV in the gonads. In addition, fertility was observed to be reduced in mice; whether this could be a problem in the human population has yet to be reported.

1.2.7 Innate immune response to ZIKV

In a study by Hamel et al. (2015), infection with the PF-25013-18 isolate of ZIKV in human skin fibroblasts induced the expression of several antiviral genes[134]. TLR3 expression was induced strongly and rapidly, possibly priming the expression of RIG-I and MDA-5 that followed at a later stage. siRNA inhibition of TLR3 expression resulted in increased ZIKV replication, demonstrating the importance of TLR3. However, the type I IFN expression was not modified, indicating that TLR3 does not control ZIKV replication through the IFN pathway. Interestingly TLR7 expression was not induced at all. Transcription of IRF7 was also increased upon infection, and this corresponded to the increase of IFN-alpha and IFN-beta production, although the expression of IRF3 remained unchanged. IFN-stimulated genes were also upregulated, including OAS2, ISG15, and MX1, as were certain chemokines involved in innate immunity, such as CXCR3 ligands, CXCL10, CXCL11 and CCL5. The inflammosome pathway also seemed induced by ZIKV infection. In addition, ZIKV replication was inhibited by both type I and type II IFNs[44].

Bowen et al. (2016) showed that primary dendritic cells (DCs) derived from human serum were productively infected by four different strains of ZIKV, from the currently circulating PR-2015 isolate to ancestral Asian (P6-1966) and African (MR-1947 and Dak-1984) strains[31]. The viruses from the African lineage replicated faster and created more progeny than the Asian viruses and also differed from the Asian lineage in that they caused cell death. ZIKV infection of DCs strongly upregulated the production of antiviral components like RLR proteins (RIG-1, MDA5, LGP2), STAT proteins (STAT1 and STAT2) and antiviral effectors (IFIT1, IFIT3, and viperin). Although type I IFN

mRNA was upregulated, there was minimal translation of either type I or type III IFN proteins, indicating that ZIKV antagonizes downstream type I IFN translation. However, an antiviral state was induced similar to that achieved by RIG-I agonist treatment, indicating that the antiviral response induced by ZIKV is type I IFN-independent[31]. This was confirmed when treatment with RIG-I agonist was able to effectively block ZIKV replication in DCs, whereas type I IFN treatment had minimal effects. The weak effect of type I IFN can be explained by the downstream blockade of STAT1 and STAT2 phosphorylation performed by all four isolates of ZIKV. The ZIKV NS5 protein has been shown to induce the degradation of human STAT2 (but not mouse STAT2) in the proteasome[137, 138]. Although Bowen et al. (2016) found a similar antagonism of type I IFN response, STAT2 was not found to be degraded in their study; the differences in the reports could possibly be due to the difference in cell types used. In mice, ZIKV NS5 has been shown not to induce the degradation of mouse STAT2, which may partially explain the resistance of WT mice to ZIKV [35]. This may explain why knock-out mice with a deficient IFN response are more susceptible to ZIKV than immunocompetent mice[139].

Although ZIKV induces an antiviral state in DCs, it does not induce their activation, producing a minimal amount of co-stimulatory and major histocompatibility complex (MHC) molecules and of inflammatory cytokines[31]. This indicates that DCs are unlikely to be responsible for the increase in cytokines in human serum at the beginning of ZIKV infection[140]. The role of monocytes in ZIKV infection is unclear[139]. As opposed to DENV infection, no “cytokine storm” effects have been observed in the acute phase of ZIKV infection, which lacks a significant increase of TNF-alpha of IFN-

gamma levels, as well as thrombocytopenia. There seems to be a higher level of chemokines than cytokines, and the cytokine profile suggests a Th2 bias compared to the immune response induced by DENV infection[140]. After ZIKV infection, Hofbauer cells have also been found to be poorly immunogenic[131, 141]; on the other hand human embryonic cranial neural crest cells and fibroblasts secrete a high quantity of cytokines[142]. Therefore, the capacity to induce pro-inflammatory cytokines secretion in response to ZIKV infeciton seems to depend on the cell type[31].

1.3 ANTIBODY RESPONSE TO ZIKV

1.3.1 Background of antibody neutralization and enhancement:

Members of the *Flaviviridae* family induce antibodies that have varying degrees of cross-reactivity to other flaviviruses, demonstrated by their abilities to either bind, neutralize, or enhance a heterologous infection[143]. Therefore, previous flaviviral immunity can a have a protective effect, or can increase the severity of disease in the case of an infection with a related flavivirus. For instance, immunity to members of the Japanese Encephalitis serogroup provide protection against other viruses of this group in various animal models[144], and Japanese Encephalitis Virus (JEV) has been shown to provide protection against DENV in mice[145]. On the other hand, enhancement of heterologous flaviviral infection by immunity to another flavivirus has been observed *in vitro* and even *in vivo* among different combinations of flaviviruses[146-148]. The phenomenon of enhancement has been observed to cause increased disease severity in human populations. A well-researched example is the case of infection with heterologous DENV serotypes, in which previous immunity to one serotype of DENV

has been shown to increase disease severity upon infection with a heterologous DENV serotype. The mechanism by which this occurs is hypothesized to be antibody-dependent enhancement (ADE), whereby sub-neutralizing antibodies against the primary infecting serotype aid the entry of a secondary infecting serotype into antigen-presenting cells through the Fc-receptor[143].

There is considerable evidence of ADE among DENV serotypes. Secondary infection with a heterologous DENV serotype has been associated with severe forms of DENV disease in various cohorts in Thailand. A secondary serologic response was present in more than 90% of children with symptomatic DENV in one study[149], while in another study it was twice as likely in patients with dengue hemorraghic fever (DHF), and was associated with all dengue shock syndrome (DSS) cases[150]. Furthermore, DHF cases had 100-1000-fold higher peak viremia titer than classic dengue fever cases[150]. This aspect of ADE has been reproduced in various animal models, in which passive transfer of antibodies can achieve higher viremia titers in rhesus macaques compared to infection in absence of antibodies[151, 152]. In addition, experiments in mouse models were also able to replicate the increased disease severity and lethality of heterologous DENV infection in the presence of previously administered antibody[153-155]. It was also demonstrated that this enhancement is mediated through attachment of antibodies to cellular Fc-receptors, as the addition of modified antibodies unable to bind to Fc receptors inhibited the effects of enhancement upon passive antibody transfer[152, 153]. This confirms observations from *in vitro* studies achieving higher titers of virus in enhancement assays using different Fc-receptor-bearing cell types, such as Thp-1, K562, and U937 cells[152, 156, 157], although extrapolation of results from *in vitro* to *in*

vivo must be performed with caution. Passive transfer of either monoclonal or polyclonal antibodies are enough to induce enhancement in *in vivo* models. Therefore, although other elements of the immune response, such as complement and antiviral elements can also participate in enhancement[143], antibodies play an essential role in the increase of disease severity.

Whether antibodies have an enhancing or protective effect depends on a series of characteristics, such as the ability to neutralize the infecting virus, the binding affinity and avidity to the virus, and the concentration. For antibodies to neutralize a flavivirus effectively, a certain critical number must be attached to the virus surface[143]. Therefore, neutralization will depend on the number of epitopes occupied by antibodies and the strength of the attachment, or affinity[158, 159]. Enhancement, on the other hand, occurs at sub-neutralizing antibody concentrations between an upper limit whereby the number of antibodies bound to a virion is sufficient to cause neutralization and a lower limit whereby there are not enough antibodies bound to the virion to attach it to the Fc-receptors of cells[143]. Strongly neutralizing antibodies can induce enhancement at low concentrations when the quantity of antibodies is insufficient to block viral entry to the cell, and is therefore sub-neutralizing[143, 157]. On the other hand, antibodies that are weakly neutralizing can enhance infection at a wide range of concentrations, due to the need of a large number of these antibodies to achieve neutralization[143]. Correlation between antibody affinity and enhancement has also been shown with monoclonal DENV antibodies isolated from human patients: strongly binding antibodies enhanced at lower concentrations than weakly binding antibodies[160]. Concentration-dependent differences in enhancement were also shown

in the previously discussed *in vivo* models[152, 153, 161]. The epitope to which antibodies bind can also determine enhancement capability. Antibodies binding domain III of the DENV E protein (EDIII) are highly serotype-specific and among the most strongly neutralizing, while antibodies specific to domains I and II of the E protein (EDI and EDII) are cross-reactive and enhancing. After a primary DENV infection, a large component of the antibodies target the E protein, while after a secondary DENV infection the response is broader and includes prM and NS1- specific antibodies[162]. prM-binding antibodies are highly cross-reactive, poorly neutralizing, and strongly enhancing, as well as numerous[163]. In conclusion, a combination of the neutralizing ability, epitope specificity, binding affinity, and concentration determine if and how much an antibody population will enhance heterologous infection.

These antibody characteristics change over time, indicating that the timing of secondary heterologous infection is fundamental in the consequences it will have. In DENV, immunity against one serotype seems to confer protection against homologous and heterologous serotypes from between 3 months to up to 3 years[164-166], after which time risk of severe disease upon infection with a heterologous serotype is increased. A similar phenomenon is observed in infants from DENV-immune mothers upon primary DENV infection. These infants are under increased risk of DHF between the 4^{th} and the 9^{th} months of age, presumably due to a waning in the DENV immunity passed on from mother to child in the placenta that makes it acquire sub-neutralizing characteristics[167-169]. What determines the change from cross-protection to cross-enhancement in an individual is as yet unconfirmed, but it is thought to be influenced by a change in the neutralization properties of the antibodies. Over time, antibodies seem

to suffer decay in their overall neutralizing ability and avidity and to become less broadly serotype cross- neutralizing and more serotype-specific[170-172]. A clear example of monotypic antibodies causing increased severity of diseased was observed in Cuba, where a DENV1 epidemic in 1977 was followed 4 years later by a DENV2 epidemic in 1981, and 20 years later by another DENV2 epidemic in 1997 with no intervening DENV transmission. The homologous neutralizing antibodies had increased significantly from samples collected 4-8 years after the DENV1 epidemic to those collected 22 years later, and this increase was paralleled by a case fatality rate for DENV1-DENV2 secondary cases 3-4 times higher in 1997 than in 1981. In addition, the phenomenon of increased disease in infants from DENV-immune mothers was accurately reproduced in the AG129 mouse model and was associated to a decrease in the neutralization of DENV over time. Mice born to DENV-1 immune mothers had enhanced disease severity and lethality upon DENV-2 infection in an age-dependent manner, where 2-week-old mice with strongly neutralizing antibodies were protected from infection, but 5-and 8-week old mice with poorly neutralizing antibodies suffered from enhancement[173].

However, a recent study shows that the antibody neutralization of primary infected subjects in a cohort in Nicaragua, instead of becoming more serotype-specific and less neutralizing, tends to increase in overall mean neutralizing titer and in number of serotypes neutralized over time. A possible explanation for this could be re-exposure to DENV that would boost the immune response and prevent a decline in neutralization[174]. Re-exposure to homologous DENV serotypes has been reported on several occasions[149, 175, 176] and is likely to occur in areas with very intense transmission. In one of these reports homologous re-exposure was able to induce up to

6.44 log10 viremia[177], which is a sufficient amount of virus in blood to be taken up by a mosquito and continue the transmission chain[178]. This not only indicates that homologous protection is incomplete, but also that re-exposure should be taken into account in transmission models. However, the effect re-exposure might have on the antibody response and the dynamics of protection or enhancement has not been studied.

1.3.2 ZIKV cross-neutralization and enhancement with DENV and other arboviruses.

Due to the recent emergence of ZIKV in areas in the Americas where DENV is endemic, there is concern of possible interactions between pre-existing DENV immunity and a subsequent ZIKV infection. ZIKV and DENV are closely related both genetically and antigenically [179]. The ZIKV E protein has a 54% total homology with the DENV E protein, although the homology varies among the different domains: the fusion loop (FL) domain is 100% conserved[180], while the DI, DII, and DIII domains are 35, 51, and 29% conserved, respectively[181]. In addition, the existence of conserved quaternary epitopes providing strong cross-neutralization between DENV and ZIKV suggests that these two viruses can be clustered in a super serogroup based on their antigenic relatedness[179].

1.3.2.1 Effect of DENV immunity on ZIKV infection

Due to these similarities, DENV antibodies specific to these epitopes can cross-react, cross-neutralize, or cross-enhance ZIKV to different degrees. The commercial and broadly flavivirus cross-reactive DENV-derived monoclonal antibody 4G2, that binds to the FL portion of the E protein [182], was able to enhance ZIKV infection *in vitro* in Thp-

1 cells[183]. Several studies have explored the properties of monoclonal antibodies derived from DENV patients in their reactivity to ZIKV. Antibodies specific to the FL portion of the DENV E protein are highly cross-reactive to ZIKV by ELISA, likely due to the high homology of this portion between the two viruses. Most of the monoclonal antibodies with FL recognition studied were shown to be unable to neutralize ZIKV, although they were able to induce 39-91-fold enhancement of ZIKV in U937 and K562 cells[179, 184, 185]. In addition, enhancement was shown to be Fc-receptor II-dependent. However, one broadly neutralizing FL-specific monoclonal murine antibody developed by Dai et al. (2016) was able to neutralize ZIKV and even protect from infection *in vivo* in A129 mice[41]. Interestingly, in spite of the 100% homology of FL between ZIKV and DENV and the strong binding of DENV FL-specific antibodies to ZIKV, these antibodies have a differential ability to neutralize the two viruses. While these antibodies are able to neutralize DENV to some extent, although poorly, they do not at all neutralize ZIKV. This may be because the FL is exposed on the E protein more often in mature DENV than in mature ZIKV. The reduced exposure of FL on mature ZIKV could be a result of the lesser variability of the surface epitopes or "breathability" of ZIKV, that in turn could be consequence of the higher thermal stability of mature ZIKV virions[39, 179]. On the other hand, DENV antibodies specific to other domains of the E protein showed different reactivity to ZIKV: DENV monoclonal antibodies with EDI/II specificity showed cross-reactivity to ZIKV, however EDIII specific antibodies did not cross-react and were specific to DENV[181]. The cross-reactivity to ZIKV of highly DENV neutralizing monoclonal antibodies that bind to quaternary epitopes and that were isolated from DENV patients was also explored. These

antibodies bind the dimer structure of two E proteins, named the “envelope dimer epitope” (EDE)[186]. They are subdivided into EDE1 and EDE2 based on their dependence on the N-linked glycan Asn153 to bind to the E protein (EDE2 needs this glycan to bind). These antibodies are able to broadly neutralize all four serotypes of DENV at very low concentrations. The majority of the EDE1 antibodies, but less than half of the analyzed EDE2 antibodies, were able to cross-react with ZIKV, and these antibodies were also able to induce enhancement of ZIKV in U937 cells, although at a lower concentration than FL-specific antibodies. In addition, EDE1 antibodies with high avidity to ZIKV were able to inhibit ADE of ZIKV by DENV-immune plasma at high concentrations, whereas FL-specific antibodies were unable to inhibit this enhancement[184]. Another study analyzed a panel of monoclonal antibodies isolated from the serum of DENV patients and found that approximately half of the monoclonal antibodies cross-reacted with ZIKV[180]. Of those, highly ZIKV-neutralizing antibodies were able to induce ADE at low concentrations[180]. Half of these neutralizing antibodies bound to conformational epitopes of ZIKV[180]. Antibodies with intermediate levels of neutralization induced ZIKV enhancement at high concentrations, and those that didn’t neutralize at all induced low level enhancement at low concentrations[180]. Non-cross-reactive antibodies did not neutralize or enhance ZIKV[180].

The ZIKV reactivity of DENV-immune serum or plasma with a polyclonal population of antibodies has also been analyzed. One study found DENV serum to be highly cross-reactive and cross-neutralizing to ZIKV[180]. In another study, DENV-immune plasma was found to be poorly ZIKV-neutralizing but highly ZIKV-enhancing, although a small portion of the samples was indeed able to neutralize ZIKV. In addition, this study found

differential enhancement capability depending on the amount of time after recovery from DENV infection the serum was collected, with serum collected 1-2 weeks post-DENV recovery inducing a median of 12-fold higher ZIKV infection in U937 cells compared to the 100 fold increase induced by serum collected over 6 months post-recovery[184]. DENV-immune serum, therefore, has varying degrees of ZIKV neutralization and can enhance ZIKV infection at different concentrations depending on the cross-neutralization[185]. The sera used in these studies were from patients from Southeast Asia or Jamaica with primary or secondary DENV infections. However, the serum of DENV-immune pregnant women from Recife, Brazil, which was the epicenter of the ZIKV outbreak in the Americas, has also been shown to enhance ZIKV infection in K562 cells, indicating the relevance of the previous work to the current outbreak. In this study, DENV-immune sera of women with monotypic and multitypic antibody responses were evaluated, but no significant difference was found in the subsequent ZIKV enhancement between the sera of women that had had only one or multiple previous DENV exposures[187]. These results, together with the information learned by studying monoclonal antibodies of different epitope specificities, indicate that the mechanisms of neutralization and ADE of ZIKV are likely very similar to those of DENV, varying mostly in the increased stability of the ZIKV mature virus particle that leads to a different exposure of epitopes on the surface.

1.3.2.2 Effect of ZIKV immunity on DENV infection

In addition to the effect previous DENV immunity can have on a subsequent ZIKV infection, the reverse scenario has been explored by observing the effect of ZIKV antibodies on a subsequent DENV infection, although not to the same extent. By using

monoclonal antibodies isolated from ZIKV infected donors, the role of antibodies specific to different domains of the ZIKV E protein was evaluated[181]. EDI/II-specific ZIKV monoclonal antibodies cross-reacted to all 4 DENV serotypes but were either poorly neutralizing to ZIKV or not at all. However, the poorly EDI/II-specific ZIKV-neutralizing antibodies were able to enhance both ZIKV and DENV-1 infection in K562 cells. On the other hand, EDIII specific ZIKV antibodies did not cross-react to DENV and were specific to the ZIKV E protein, mirroring the behavior of DENV EDIII-specific antibodies. These antibodies were able to neutralize ZIKV, and interestingly, also able to enhance ZIKV infection in K562 cells at a broad range of concentrations, including those at which they neutralized infection. A group of antibodies was isolated that was able to neutralize ZIKV but did not bind to the E protein, and was therefore named neutralizing-non-E-binding (NNE). These antibodies did not cross-react to either the ZIKV or DENV E protein by enzyme-linked immunosorbent assay (ELISA), but were highly ZIKV-neutralizing, and also enhanced ZIKV at a broad range of concentrations including neutralizing concentrations. Plasma from ZIKV-infected patients was able to enhance both ZIKV and DENV-1 at a similar level to the DENV-1 enhancement caused by DENV-3 reactive serum. To investigate the mechanism of this enhancement, LALA mutant antibodies were created of several ZIKV and DENV monoclonal antibodies specific to different domains of the E protein. LALA mutant antibodies have been modified in such a way that they cannot attach to the Fc-γ-receptor of cells or to complement. Enhancement of ZIKV by ZIKV-immune plasma was completely inhibited by the addition of ZIKV EDIII-specific LALA mutant monoclonal antibodies, and partially inhibited by adding cross-reactive DENV EDI/II specific LALA monoclonal antibodies.

This cross-reactive DENV EDI/II specific LALA monoclonal antibody was also able to completely block the enhancement of DENV-1 by both ZIKV and DENV-3-immune plasma. However, the enhancement of DENV-1 by ZIKV and DENV-3-immune plasma was not blocked at all by ZIKV EDIII-specific LALA mutant antibodies. This is probably due to the fact that ZIKV EDIII-specific antibodies do not bind to DENV-1. LALA mutants may block ADE either by competing for surface epitopes on the virus with the polyclonal plasma antibodies, and therefore reducing the virus' ability to attach to the cellular Fc-receptors, or by neutralizing the virus in the endosomes once it has been internalized[181].

In addition to these human-derived antibodies, the ZIKV-induced antibody response has also been explored in animal models. ZIKV-infected rabbit antiserum was shown to enhance DENV-2 *in vitro*[188]. In mice, a panel of monoclonal antibodies resulting from serial infection of MR766 and H/PF/2013 in the IRF 3-/- mouse model produced both EDIII and FL-specific antibodies[106]. The EDIII- specific antibodies only bound to ZIKV EDIII by ELISA and did not cross-react to either DENV or JEV and were able to neutralize 4 different strains of ZIKV to different degrees. The FL-specific antibody cross-reacted to all 4 serotypes of DENV and was weakly ZIKV-neutralizing. Two of the EDIII-specific strongly neutralizing monoclonal antibodies were able to protect from infection with an African strain of ZIKV *in vivo.* C57BL/6 WT mice pre-treated with anti-interferon antibodies are susceptible to ZIKV infection, but addition of EDIII-ZIKV antibodies inhibited their susceptibility. In this model there was also a strong correlation between the neutralization ability of the antibodies and their affinity and avidity for their corresponding epitopes (EDIII and FL). All the antibodies were able to enhance ZIKV

infection in K562 cells at different concentrations in relation to their binding affinity, with weakly binding antibodies enhancing at high concentrations and strongly binding ones at sub-neutralizing concentrations. In contrast, the only antibody capable of enhancing DENV was the cross-reactive FL-specific antibody. Interestingly, 3 of the monoclonal antibodies were specific to cryptic epitopes that are hidden on the surface of the mature ZIKV virion. The most likely explanation for this is that the ZIKV virus particle “breathes”, exposing different epitopes during maturity, although exposure to partially immature viruses or soluble envelope proteins is also possible. However, as seen above, it is likely that ZIKV has a lesser “breathability” than DENV, possibly due to its higher thermal stability.

These *in vitro* studies are of great value to investigate the interactions between DENV or ZIKV immunity and heterologous infection, as well as informing on the characteristics of the immune response that drive enhancement. However, *in vivo* studies are needed to demonstrate the relevance of these phenomena in living organisms. To our knowledge, only one study, by Stettler et al. (2016), has performed any enhancement experiments with ZIKV *in vivo*[181]. This group was able to demonstrate enhancement of DENV-2 infection by the pre-administration of highly cross-reactive monoclonal ZIKV EDI/II-specific antibodies to AG129 mice. The enhancement of DENV disease was evidenced by an increase in disease severity and 100% lethality at 5 dpi, compared to control mice to which no antibodies were administered that maintained 100% survival rate for the duration of the experiment[181]. This shows that ZIKV immunity can enhance DENV infection *in vivo*. However, pre-administration of DENV cross-reactive monoclonal antibodies to immunocompetent 129v/ev mice failed to enhance disease or

lethality upon ZIKV infection. An immunocompetent mouse model was chosen for this experiment due to the extreme lethality of ZIKV infection in other immunocompromised mouse models, which would make detection of enhanced disease difficult[97, 101, 189]. WT 129v/ev appear able to acquire a subclinical ZIKV infection, as after ZIKV infection they do have detectable ZIKV RNA in serum and later in tissues but do not evidence any signs of disease or histological lesions[101]. However, it is possible that this mouse model is not susceptible enough to ZIKV to serve as an adequate model for enhancement. Therefore, experiments in a more susceptible yet non-lethal mouse model are needed to demonstrate ZIKV enhancement by DENV immunity *in vivo.*

1.3.2.3 Consequences of flaviviral immunity on ZIKV in the Americas

The duration of the neutralizing characteristics of ZIKV-induced antibodies will determine the epidemic dynamics in ZIKV-affected territories. A recent study by Ferguson et al. (2016) calculated that the current outbreak will likely last 3 years, whereupon the development of sufficient herd immunity will avoid another large-scale outbreak in the next 10 years, although the possibility remains that a permanent low-level endemicity or sporadic small outbreaks will occur. However, in their analysis, both ADE and cross-immunity increased the likelihood of endemicity and shortened the time between epidemics[190]. Previous DENV epidemic modeling efforts have shown how cross-neutralization and ADE work together to explain DENV epidemic dynamics in Southeast Asia[191-194]. Although it is likely that ZIKV provides life-long immunity[5, 35] the effect of immunity to other circulating arboviruses in the area must be taken into account to predict future ZIKV epidemics and disease outcomes accurately.

Due to the common vectors and geographical distributions of ZIKV and other flaviviruses, including the four serotypes of DENV, the co-circulation of these closely related viruses is and will continue to be a common occurrence in many areas of the world. Recent studies show that DENV antibodies are able to cross-neutralize and cause ZIKV enhancement *in vitro*[180, 181, 184]. On the other hand, previous arboviral immunity might have a protective effect: one study suggests that women in areas with high yellow fever vaccination pose a lower risk of microcephaly due to ZIKV in Brazil[195]. Another study found that certain monoclonal DENV antibodies are able to protect against ZIKV *in vivo*[196]. Older studies support these findings of interaction between flaviviral immunity and ZIKV infection, although they must be interpreted with caution due to the very small sample sizes used. One study showed that immune ascitic fluid of mice infected with West Nile Virus (WNV) or YFV was able to enhance ZIKV infection *in vitro*[188]. In another study, a ZIKV-immunized vervet monkey had a reduced viremia upon YFV challenge, although this was not reproducible in rhesus monkeys[197]. In a different study, a ZIKV-immunized rhesus monkey had reduced liver pathology after YFV challenge[198]. Other data seem to indicate that heterologous immunity does not confer protection, as ZIKV has been able to infect people with the YFV 17D vaccine[59], and neither ZIKV or DENV immunity prevented a YFV outbreak in Nigeria in 1970[199] nor has previous DENV immunity prevented the spread of ZIKV through DENV endemic areas[139].

Although both WNV and YFV circulate in the Americas, the possible consequences of previous DENV immunity have been the deepest cause for concern during the ZIKV outbreak. To date, there has been no epidemiological evidence of ZIKV enhancement

by DENV immunity, but it is speculated that enhancement of ZIKV infection could be at least partly responsible for the extreme severity of the outbreak in the Americas, which has been characterized by neurological and congenital outcomes previously unrelated to ZIKV infection[200].

DENV antibodies might be inducing more efficient transmission to mosquitoes, transplacental transmission, or neuropathogenicity[184, 200]. The circulation of ZIKV in other areas of the world where DENV is prevalent, such as Southeast Asia or Africa, and where these ZIKV-associated syndromes have not been reported, seems to cast doubt on this hypothesis[199, 201], especially in the case of Southeast Asia, where the circulating strain is the same as in the Americas. However, it has been suggested that the differences in DENV background immunity between Southeast Asia and the Americas could be the cause of this divergence in presentation: in Southeast Asia, all four serotypes of DENV have co-circulated for decades, and individuals can acquire multiple infections and therefore a broadly DENV-neutralizing immunity by an early age[200]. Meanwhile, in the Americas DENV has not been hyperendemic as long, and it is possible that the DENV immunity in American individuals is less broad than in Southeast Asian individuals[200]. Among DENV serotypes, enhancement is induced in secondary heterologous infections, after which the DENV immunity becomes broadly neutralizing, and post-secondary infections are thought to be milder or asymptomatic[202]. It may be the case that the number of previous DENV infections can influence ZIKV enhancement, although no difference was found in the ZIKV enhancement by DENV monotypic and multitypic sera from pregnant women in Brazil[187]. However, antibody characteristics change over time, indicating that the

timing of ZIKV infection after DENV infection could be an important factor in enhancement.

The interactions between DENV and ZIKV could determine the progression of epidemics in areas where both viruses co-circulate, and many questions remain. Enhancement of ZIKV by previous DENV immunity has yet to be studied *in vivo*. On the other hand, enhancement of DENV by ZIKV immunity has been achieved *in vivo* with monoclonal antibodies [203]. This scenario is bound to occur, as the ZIKV outbreak will have induced ZIKV-immunity in a portion of the population that was DENV-naïve, especially younger children or in children born from ZIKV-infected mothers, but that is at risk of acquiring DENV infection due to the continued circulation of the 4 serotypes of DENV in the Americas. The consequences, if any, that this could have in the human population are unknown. To understand and predict this event, a more complete understanding of the polyclonal antibody population induced by ZIKV infection and its change over time is necessary. Lanciotti et al. (2008) explored some of these characteristics using sera from the Yap island ZIKV outbreak in Micronesia, finding that while secondary ZIKV infection was associated with high cross-neutralization to other flaviviruses, primary ZIKV infection was highly specific[204]. However, further studies into the polyclonal antibody population induced by ZIKV-infection, especially of the epitope-specificity of the antibodies after primary or secondary infections, as well as the epitope exposure and frequency of this exposure on the virion surface, are needed to increase our understanding of ZIKV neutralization. In addition, due to the recent reports of DENV homologous re-exposure, the effects of re-exposure to ZIKV on the antibody population should be studied. Although ZIKV-immunity provides protection from

challenge with heterologous strains of ZIKV[205], how such a re-exposure modifies the antibody population and how these changes could affect a subsequent DENV infection must be elucidated to predict the possible consequences of DENV enhancement by ZIKV immunity. Answers to these questions are especially relevant in the light of the likely roll- out of a ZIKV vaccine. Concerns that enhancement in vaccinees after subsequent DENV infection will occur, as has been the case with the DENV Sanofi-Pasteur CYD-TDV vaccine trials[206], must be assuaged before such a roll- out can safely go forward.

1.4 AIMS

For this project, my aims were to further explore the presence of ZIKV in the Americas, from the origin of the severe symptoms characteristic of this outbreak to the consequences that ZIKV-immunity in the population could have on subsequent flaviviral infections. To determine whether the increase in severity of ZIKV-induced symptoms is due to a change in the virus upon its arrival to the Americas, I characterized the pathogenesis of the prototypical MR766 strain from the African lineage compared to that of the currently-circulating ZIKV strains. For this purpose, I infected IRF 3/7 DKO mice with the MR766 strain and evaluated the infection kinetics, tissue lesions, and antibody response. To explore the ability of ZIKV-induced antibodies to enhance heterologous infection, especially in the context of intense transmission where re-exposure is bound to occur, I performed several boosts of ZIKV infection on immunocompetent C57BL/6 mice and evaluated the neutralizing and enhancing capability of the polyclonal antibody population. I hypothesize that homologous re-exposure will increase the specificity of

the antibodies to ZIKV and that this change will affect how the antibody population is able to enhance heterologous DENV infection.

CHAPTER 2: CHARACTERIZATION OF A MURINE MODEL OF ZIKA VIRUS INFECTION: INFECTION KINETICS, TISSUE TROPISMS, AND ANTIBODY RESPONSE

2.1 INTRODUCTION

Zika virus (ZIKV) has recently emerged in the Western Hemisphere where to date 48 countries have reported intense transmission of the virus since 2015[207, 208]. ZIKV was first isolated in Uganda in 1947[2], and since then it has been classified into two lineages: African and Asian, according to the areas where it has been found to circulate[1, 36]. In the decades after its discovery, ZIKV was thought to cause a mild febrile illness[5]. However, during the outbreak in the Americas ZIKV infection was associated with an upsurge in incidence of Guillain- Barré and most importantly microcephaly, as well as other congenital malformations and neurological symptoms in adults[12, 25]. Neither the African strains circulating in Africa, nor the Asian strains circulating in Southeast Asia, have been previously reported to cause any of the severe manifestations prevalent in the American outbreak, despite the fact that the strains circulating in the Americas belong to the same Asian lineage of ZIKV[200]. Therefore, it has been speculated that a mutation in the virus may be responsible for the unprecedented severity and size of the current outbreak[86, 89].

The main transmission route of ZIKV is by mosquito bite, but it has also been shown to be transmitted sexually. ZIKV has been detected in semen for prolonged periods of time, the median being 34 days post symptom onset[34]. In addition, ZIKV has been detected for variably prolonged periods of time in other bodily fluids such as urine, saliva, and conjunctival fluid[32, 34]. These findings, together with reports of ZIKV

transmission that cannot be explained by mosquito bite or contact with sperm cells[69, 83], point at the existence of other potential routes of transmission. In addition, while the CDC has confirmed the link between microcephaly and ZIKV[13, 14], the association between ZIKV and neurological symptoms in adults has yet to be established. Further studies are needed to explain the mechanisms of ZIKV persistence and shedding from different organs, as well as the pathogenesis of neurological disease in adults.

Several recently developed mouse models have provided insight into the dynamics and pathogenesis of ZIKV infection[16, 97, 98, 100, 101, 104, 132]. In adult mice, increased susceptibility to ZIKV has been observed in knock-out models with a deficient type I and/or II interferon (IFN) response. However, in many cases infection in these knock-out mice induces a high mortality rate, rendering these models unsuitable for long-term studies[97, 98, 101, 104].

We present in this study a model of ZIKV infection in mice lacking interferon regulatory factors (IRF) 3 and 7, that retain blunted production of type I INF as well as the IFN receptors[209]. Infection of these mice with the MR766 African isolate of ZIKV presented similar infection outcomes to those observed in other knock-out mouse models infected with Asian lineage isolates, while maintaining a 70% survival rate. These mice also mounted a strongly neutralizing antibody response to ZIKV. Therefore, this is an apt model for long-term studies, and further emphasizes the similarity between the infection outcomes induced by the two ZIKV lineages. This would suggest that the increase in severe symptoms during the outbreak in the Americas may not be caused by a change in the virus, as the African strain is able to induce similar pathogenesis as the Asian strain.

2.2 MATERIALS AND METHODS

2.2.1 Ethics Statement

All experiments involving mice were approved by the Louisiana State University (LSU) Institutional Animal Care and Use Committee (IACUC protocol 15-078) in adherence with policies of the American Veterinary Medical Association and in compliance with the guidelines laid out by the National Institutes of Health's Guide for Care and Use of Laboratory Animals, 2011.

2.2.2 Virus

The ZIKV strain MR766 was originally isolated from the serum of a sentinel Rhesus monkey. We obtained it from Dr. Robert Tesh at the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch as lyophilized stock and passaged it once in C6/36 cells and once in Vero cells prior to use in these studies. ZIKV was determined to have a titer of ~10^7 plaque forming units (PFU)/mL via plaque assay prior to beginning the experiments.

2.2.3 Mouse experiments

The IRF3/7 DKO mice were originally provided by Dr. Michael Diamond. To characterize ZIKV infection kinetics, one group of male mice (n=5) and one group of female mice (n=6) were challenged with ZIKV MR766. These groups of 6- to 10-week-old mice were inoculated with 10^6 PFU in 100 µl of ZIKV MR766 subcutaneously. The mice were first anesthetized and bled via cheek bleed as in [210] on days 1, 2, 4, and 6 post infection. Mice were weighed on days 0, 4, 6, and 8-12 and percent reduction

weight was calculated. If an individual lost more than 20% of the initial body weight, it was euthanized as per the approved IACUC protocol. Two individuals presented with eye pathology in the form of mucous or crusty discharge; in these cases the discharge was collected with a sterile swab.

To characterize the tissue lesions induced by ZIKV, a separate group of 8-10 week old female mice (n=5) was infected with 10^6 PFU in 100 µl of ZIKV MR766 subcutaneously, and after confirmation of infection by qPCR, euthanized at 7, 10, and 12 dpi (1, 2 and 2 mice, respectively) and processed for histology and immunohistochemistry. In addition, two mice from the infection kinetics study that died or were euthanized (one male on 7 dpi and one female on 8 dpi) were included in this group and processed in the same way. Following a complete multisystemic gross examination immediately after euthanasia at 7, 10, and 12 dpi (1, 2, and 2 mice, respectively), tissues were collected and fixed in 10% neutral buffered formalin. Tissues were progressively dehydrated in alcoholic solutions and xylene, were embedded in paraffin. Five µm thick tissue sections were obtained for slide preparation, were stained with hematoxylin and eosin and coverslipped. For IHC, the slides were prepared as in[211], with the specific reagents as follows: mouse-on-mouse (MOM) kit for mouse primary antibody detection (Vector Labs Cat # PK-2200), control mouse IgG (Biocare, NC494H), and primary antibody 4G2 (Anti-Flavivirus group antigen antibody, EMD Millipore) at a working dilution of 1:100. The slides were examined via light microscopy and pathologic changes recorded by ACVP board certified pathologists at the LSU School of Veterinary Medicine.

2.2.4 Viral RNA detection

Blood samples were allowed to clot at room temperature for 30 minutes and were then centrifuged for 4 minutes at 4°C and 6000 relative centrifugal force (rcf). Serum was separated from the clot and stored in a sterile microcentrifuge tube at -80°C until further processing. Eye-swab samples were processed by adding 250 µl of M199E media to the swab cotton in a centrifuge tube, vortexing and centrifuging for 2 minutes at 4°C and 6000 rcf. The supernatant was extracted and stored in a separate microcentrifuge tube at -80°C. RNA extraction was performed using the Kingfisher™ (Thermo-Fisher) automated extraction platform and the Ambion MagMax™ viral isolation kit (Thermo-Fisher), as per manufacturer's instructions. Viral RNA was detected by qRT-PCR using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies) on a Roche Lightcycler® 480 (Roche). ZIKV-specific primers and probe were previously designed by Faye et al.[212]. This assay was shown to be highly specific and sensitive. Under our optimization protocol it was able to detect up to 7.55 strands of RNA/ml (data not shown).

2.2.5 Plaque Reduction Neutralization Test (PRNT)

PRNTs were performed following the WHO PRNT protocol for DENV and previously shown to work just as well with ZIKV[213] on serum collected from surviving ZIKV-infected male mice one month post-infection (n=4). Briefly, virus was standardized to 25 virions in 50 µl per well. Mouse serum was complement inactivated for 30 minutes at 56°C and then serially diluted 1:2 in M199E media from 1:10 to 1:1280 and mixed with 50 µl of the standardized virus solution and allowed to incubate at 37°C for 1 hour. The

mixture was then inoculated onto ~80% confluent Vero cell monolayers in 12-well plates. Also included were a negative control well (media only) and a positive control (virus only). The first overlay was added immediately after incubation, and the second overlay was added 3 dpi for ZIKV. Plaques were visible and counted on the following day (4 dpi). The percent reduction in plaques per dilution was calculated and results are expressed as the reciprocal of the dilution in which the desired percentage of plaque reduction was achieved; we report both PRNT50 and PRNT80.

2.2.6 Statistics

All statistics were performed using SAS 9.4 (Cary, NC). The changes in weight were reported as the percent lost compared to initial weight. Viremia was transformed logarithmically transformed and reported as log titer. We evaluated the differences in weight and viremia between sexes and dpi with a repeated measures ANOVA analysis, using a mixed model to evaluate the effect of sex, day post-infection, and their interaction on either the percent weight reduction or the log viremia titer (PROC MIXED). For this, we specified a spatial power covariance structure to account for the uneven sampling intervals. Finally, percent reduction of neutralization resulting from the PRNTs is presented as means with binomial 95% confidence intervals. Figures were created with R version 3.2.2 or images compiled in Microsoft PowerPoint (Seattle, WA).

2.3 RESULTS

2.3.1 Infection kinetics of ZIKV in IRF3/7 DKO mice

To test the susceptibility of IRF 3/7 DKO mice to ZIKV, we inoculated 10^6 PFU/mouse of MR766 ZIKV Uganda strain subcutaneously in mice between 6-10 weeks of age, one group of males and one group of females. Viral RNA was detected in the serum of all mice, peaking at 2 dpi for both groups. The female mice had on average higher serum viral titers each day, with a peak average log titer of 6.2 compared to 4.49 in males, although this difference was not found to be significant (Figure 2.1A, $p>.05$). Both groups of mice began to lose weight within 2 days of infection. The mice had a maximum average weight loss of 7.6% at 9 dpi for the males and 18.4% at 8 dpi for the females. Weight loss was not significantly different between sexes (Figure 2.1B, $p<.05$). One male died at 7 dpi and two females had to be euthanized on 8 dpi because they lost over 21% of their initial body weight. This translates to an approximate 72% survival rate. The male mouse that died had a higher log titer than the average of the surviving male mice (log titer of 6.1 versus 4.39 of survivors). The two euthanized females had the two highest peak viremias of the group (log titer of 7.24 and 6.58 versus the average of 5.84 of the other 4 females), although another female of the group had log titer of 6.53 but survived infection. This may indicate that higher viremia titers correlate with disease severity in IRF 3/7 DKO mice.

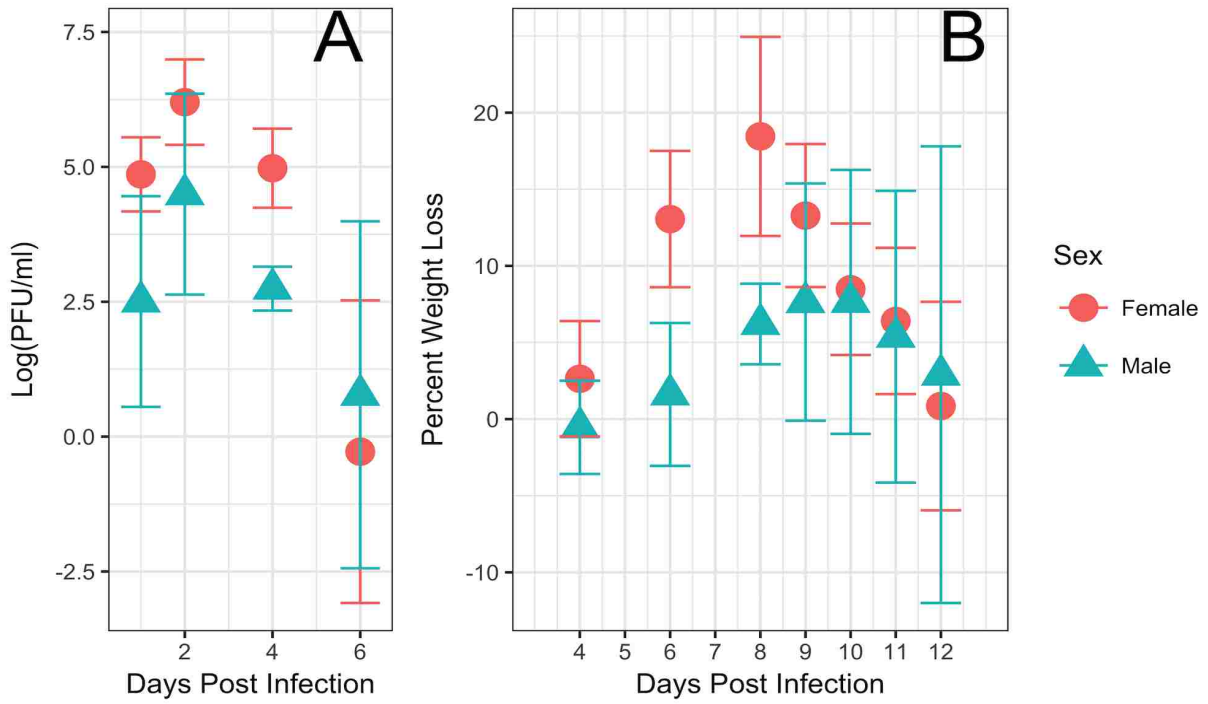

Figure 2.1: Repeated measures ANOVA analysis of ZIKV infected females (red dots) and males (green triangles) showing the average measurements for each group and time point, together with the 95% confidence interval: A) log viral titer (RNA copies/ml) and B) percent weight loss after infection with ZIKV. No significant difference was found.

Signs of overt disease observed in all infected mice included reduced activity, hunched posture, and ruffled fur. These signs began around 5 dpi and increased in severity concurrently with weight lost, reaching a maximum at 8-9 dpi, at which point they began to regain weight and recover. In addition to the non-specific signs, two mice developed ocular disease in the form of crusty discharge in the eye: one male mouse in the right eye at 11 dpi, and one female mouse in the left eye at 6 dpi. The male mouse fully recovered as of 8 months post infection (at the time of writing this manuscript). The female mouse was euthanized at 8 dpi due to excessive weight loss. The ocular discharge was collected and tested for presence of ZIKV RNA, and found in the case of

the female mouse to contain 2.280x10^3 genome copies/mL of ZIKV NS5 as per the qPCR assay. Attempts to isolate infectious virus from the supernatant were unsuccessful.

2.3.2 Organ and tissue lesions

To explore the lesions produced by acute infection with ZIKV in IRF 3/7 DKO mice, we infected a second group of IRF3/7 DKO females for the purpose of analyzing their tissues histologically. The viremia in this second group was not statistically different from the other groups (Figure 2.2, p>.05).

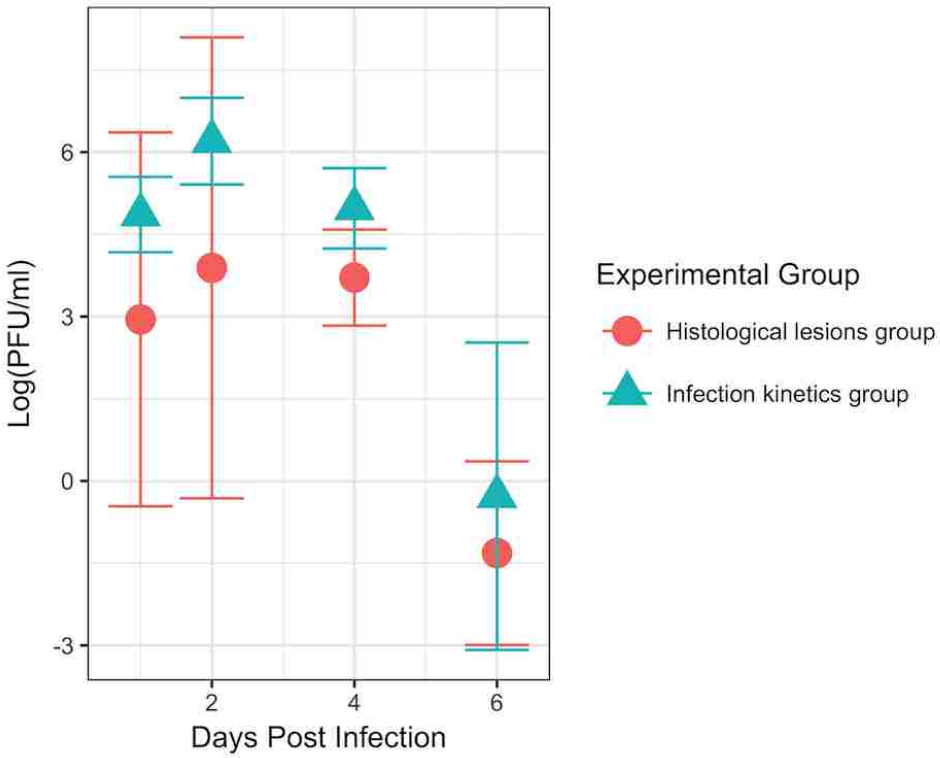

Figure 2.2: Repeated measures ANOVA analysis of females from different experimental groups 1) infection kinetics group (green triangles) and 2) histological lesions group (red dots). Points represent the average daily log PFU/ml for each group and the 95% confidence interval. No significant difference was found.

We observed multifocal mild to moderate histologic lesions in the brain, spinal cord, and eye of the sacrificed females. The brains and spinal cords of two out of five female mice had encephalomyelitis with lymphocyte perivascular cuffing, gliosis, and neuronal necrosis (Figure 2.3A). Using IHC, we were able to visualize viral antigen in the hippocampus (Figure 2.4A-B) of one of these females. Another female mouse from this group had bilateral epiphora and crusting at 7 dpi; at the time of euthanasia at 12 dpi, it had retinal ganglion cell necrosis and vitreitis, presenting inflammatory cell invasion of the vitreous humor where in normal circumstances there is none (Figure 2.3B).

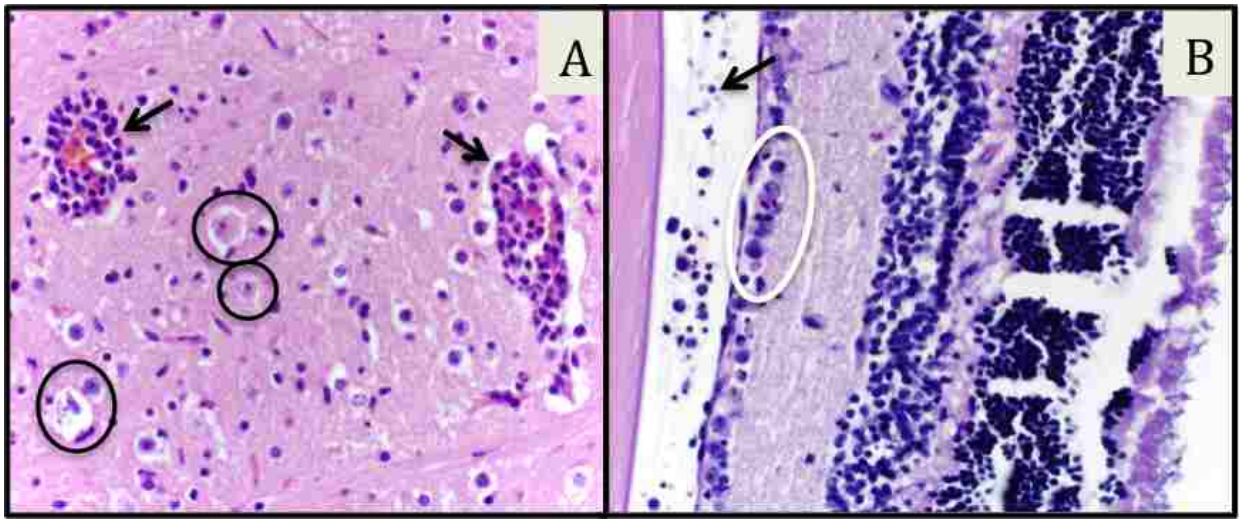

Figure 2.3: A) Encephalitis, with perivascular cuffing as the result of recruitment of leukocytes into the brain (arrows) and neuronal degeneration and necrosis (circled) in a female ZIKV-infected mouse (400X magnification). B) Ocular histology of a ZIKV-infected female mouse with ocular discharge and crusting around the eye that revealed retinal ganglionar cell necrosis (circled) and vitreitis with cellular infiltrate into what is normally clear vitreous humor (arrow) at (400X magnification).

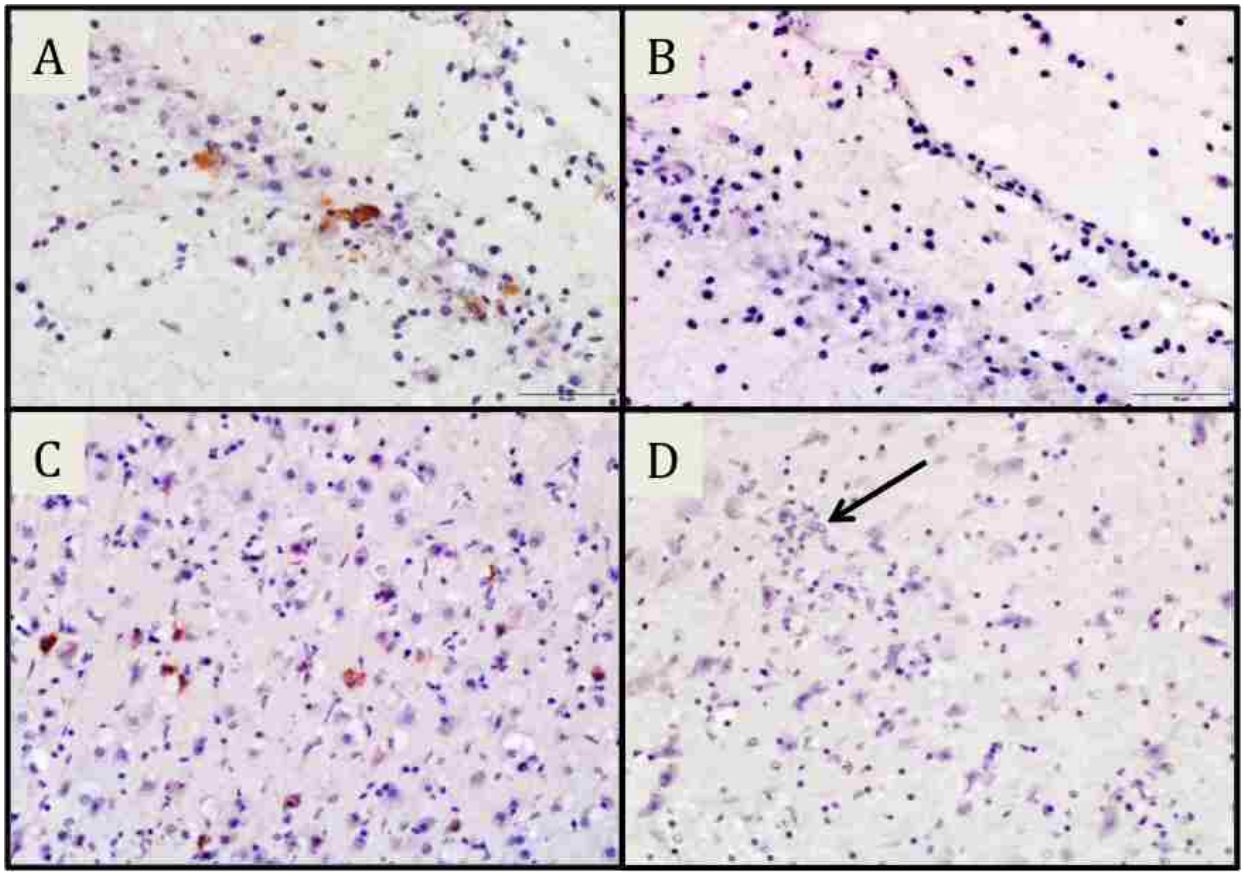

Figure 2.4: IHC labeling positive for ZIKV antigen in the brain: A) hippocampus of a ZIKV- infected female mouse and C) cerebral cortex of the ZIKV- infected male mouse that died versus negative controls of B) hippocampus and D) cerebral cortex with evidence of encephalitis (arrow) (all at 400X magnification).

In addition to these females, we also studied the organs of two mice from the infection kinetics groups: the one male mouse that died and the eyes of the female with ocular discharge that had to be euthanized. In spite of finding ZIKV RNA in the ocular discharge from this female, no histological lesions were found in the eyes.

The male mouse presented viral antigen visualized by IHC in the cerebral cortex (Figure 2.4C-D), as well as in the masculine reproductive organs.

The reproductive organs of the male mouse presented severe necrosuppurative epididymitis associated with abundant viral antigen within the epididymal lining and sloughed off epithelial cells in the lumen (Figure 2.5 A and B).

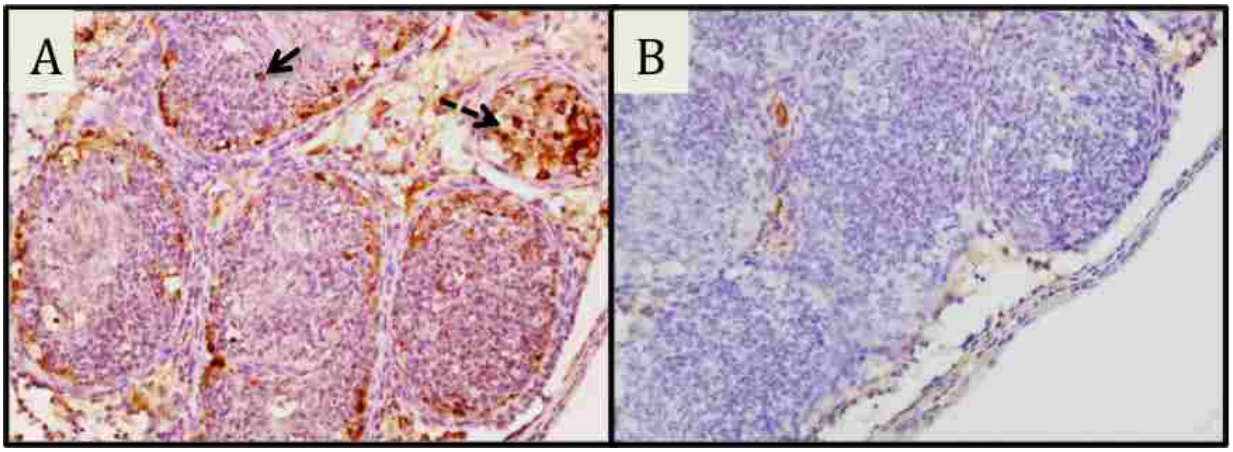

Figure 2.5: A) IHC and hematoxylin of ZIKV- infected male mouse that died wtih necrosuppurative epididymitis with ZIKV antigen within the cytoplasm of lining epithelial cells (solid arrow) and sloughed intraluminal degenerate and necrotic epithelial cells (dashed arrow). B) Negative control of the epididymis with necrosuppurative epididymitis (400X).

The affected portion of the epididymis was in stark contrast to other portions of the epididymis with normal architecture and no viral antigen (Figure 2.6). We were also able to visualize viral antigen in the testicular tissues and seminiferous tubules, including in the germ cells and spermatogonias themselves (Figure 2.7). In addition, we observed abundant ZIKV antigen staining in the seminal fluid inside the lumen of the ductus deferens, mostly concentrated in what were interpreted as sloughed off epithelial cells (Figure 2.8). Thus, we present here a potential clarification of the mechanism of sexual transmission from males to females of ZIKV through infected epithelial cells and spermatozoa in seminal fluid.

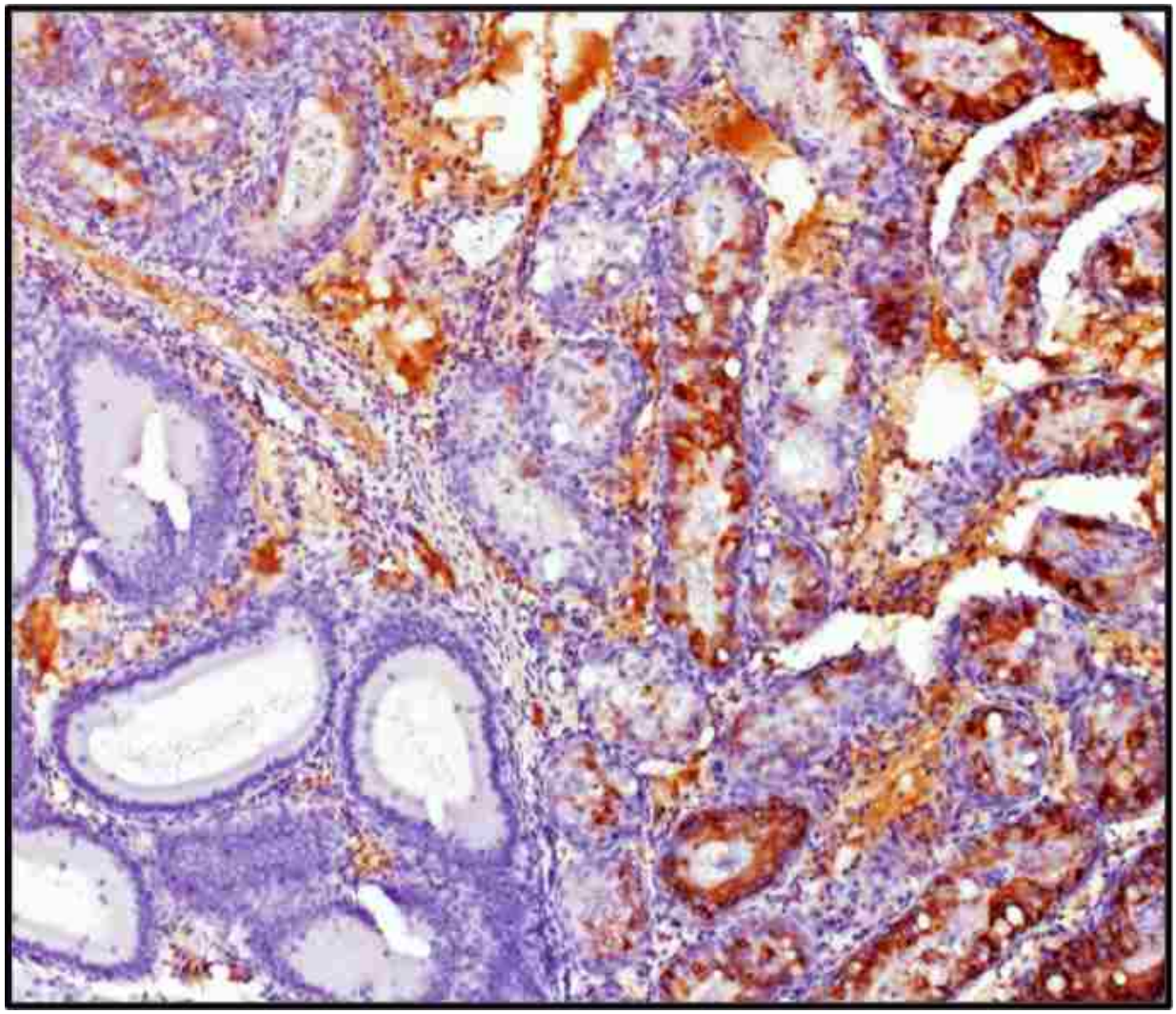

Figure 2.6: IHC from infected male that died of ZIKV shows extensive degeneration and necrosis of the epithelial lining associated with abundant viral antigen in the right-hand portion of the image compared to the internal negative control that shows a lack of viral antigen and intact normal tissue on the left (100X magnification).

The lesions were examined independently by three ACVP board certified pathologists at the LSU School of Veterinary Medicine and confirmed to deviate from the presentation of healthy mouse tissues.

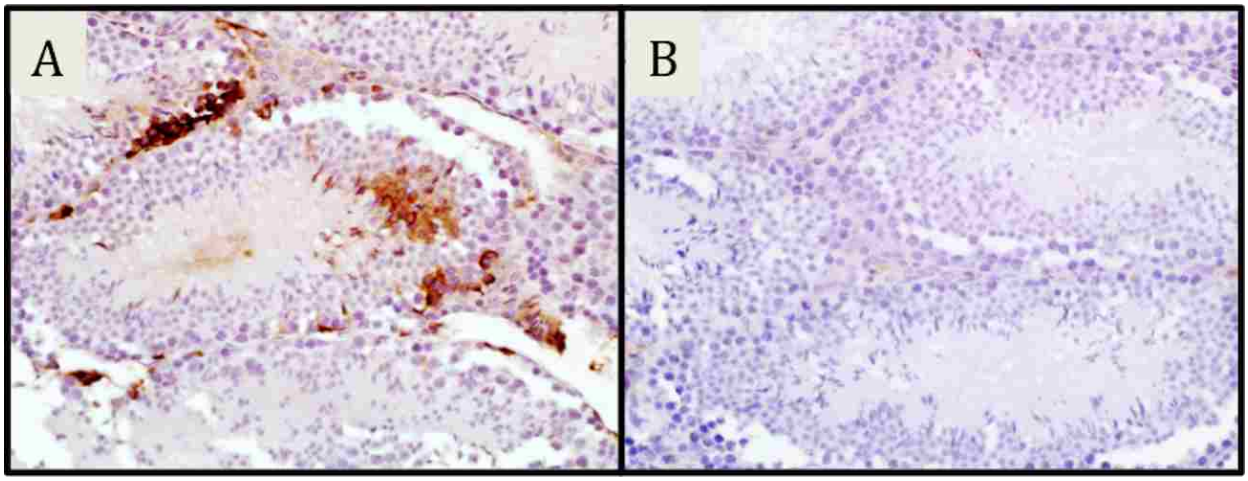

Figure 2.7: IHC shows labeling for ZIKV antigen in the germ cells of the seminiferous tubules in the testes (400X) (A) as opposed to the negative control (400X) (B).

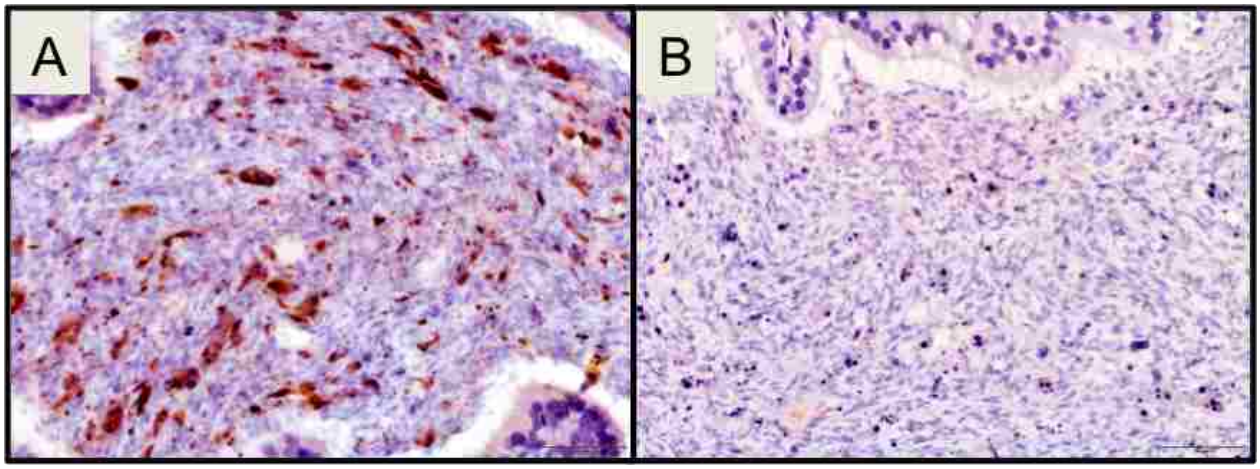

Figure 2.8: IHC shows ZIKV antigen within the seminal fluid in the lumen of the ductus deferens (400X) (A) versus negative control (400X) (B).

2.3.3 Antibody response to ZIKV

Since this is a predominantly non-lethal model, we explored the utility of this model for studies in which the antibody response would be an important endpoint. We observed that serum collected from the male group one-month post-infection with ZIKV mounted

a strongly neutralizing antibody response against ZIKV, with an average PRNT80 titer of 320 and PRNT50 titer of 640 consistent among all mice in the group (Figure 2.9).

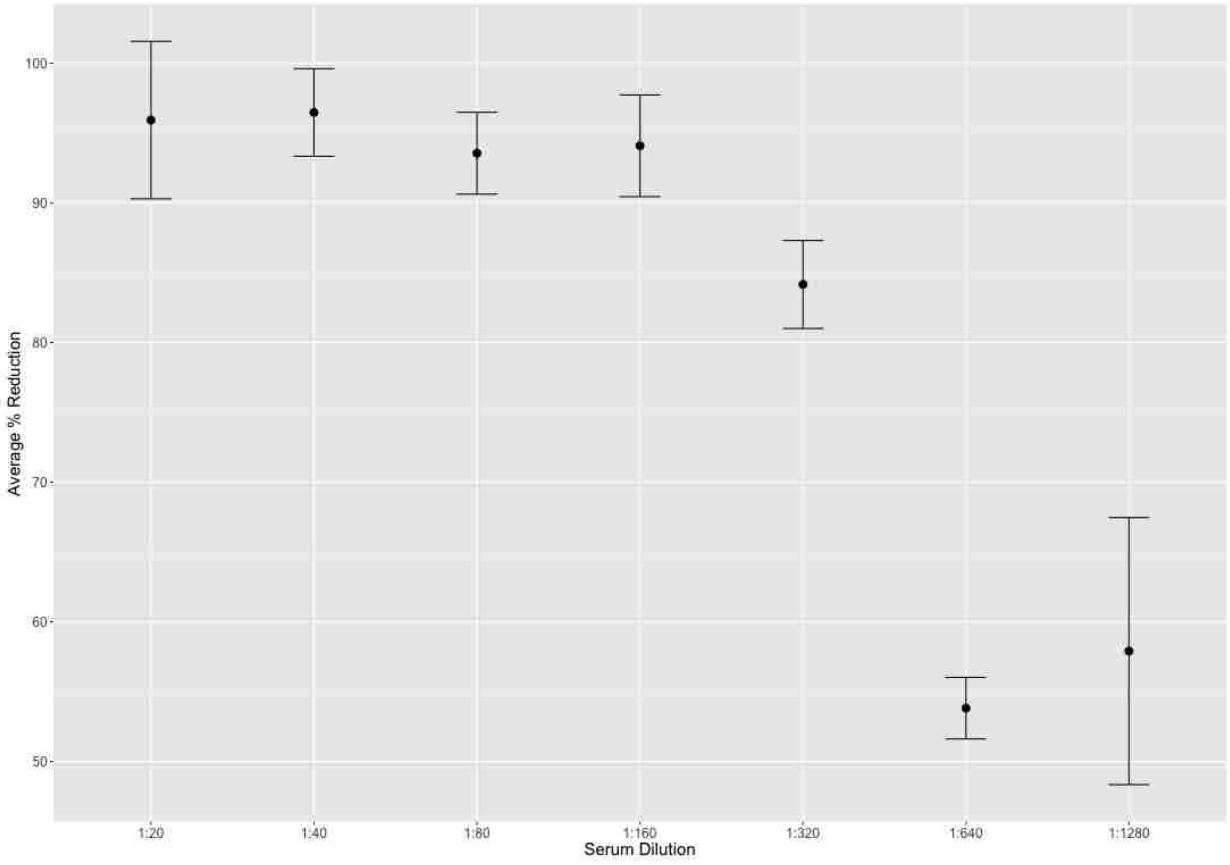

Figure 2.9: Average neutralizing titers and 95% confidence intervals of the male mice infected with ZIKV show strong neutralization one month post infection.

2.4 DISCUSSION

Our findings confirm that the IRF3/7 DKO mouse strain is susceptible to ZIKV infection, and that the MR766 ZIKV strain is capable of inducing similar tissue lesions and clinical presentation *in vivo* as the Asian strains of ZIKV in other mouse models[97, 98, 104]. Weight loss began at 2 dpi, coinciding with peak viremia. However signs of infection (hunched posture, inactivity) started 3 days after this peak and were most severe at 8-9

dpi. By 6 dpi, the clearance of systemic viremia was well underway, with only 3 mice presenting detectable quantities of viral RNA in serum. It is likely that at 8-9 dpi the virus was practically absent from the serum[101], and this is supported by observations in other mouse models where over the first 6 days of infection the amount of viral RNA decreases in serum, but increases in the testes and brain[98]. Upon sacrifice at 7-12 dpi, mice showed signs of infection-induced lesions in several organs. In a rhesus macaque model, ZIKV RNA was detected intermittently in plasma, urine, and saliva for up to 17 days, while in humans ZIKV RNA can be persistently or intermittently detected in serum, urine or semen for variably prolonged periods of time[34, 54]. This suggests that ZIKV may continue to replicate and disseminate from certain select organs, and the timing of the ZIKV-induced symptoms implies that this may be the cause of the clinical presentation, rather than an inflammatory reaction induced by an increase of viremia levels. This is supported by the fact that ZIKV does not induce a “cytokine storm” effect in acute infection such as what is seen in DENV infection[140].

On average mice lost under 20% of their initial body weight, not necessitating humane euthanasia in the majority of cases, and therefore presenting only a 72 % mortality rate. The mice that either died or had to be euthanized had some of the highest peak viremia titers in their respective groups. This may indicate that higher viremia titers correlate with disease severity in IRF 3/7 DKO mice. A similar correlation has been observed in humans infected by the closely-related flavivirus DENV, though this relationship between viremia and disease outcome remains unclear[214-216].

The fact that type I interferon deficient mice support robust viral replication, while WT mice with intact type I interferon responses do not, reinforces the importance of type I

IFN in ZIKV protection. Other mouse models lacking type I and/or II IFN receptors, such as the AG129, A129 and Ifnar1 1 -/- models have shown high morbidity and mortality after ZIKV infection[97, 98, 101, 104]. Interestingly, the IRF3/5/7 triple knock-out (TKO) mouse model inoculated with a similar dose of the MR766 ZIKV strain, had 0% survival[97], while our IRF 3/7 DKO mice had a high survival rate. The difference between these two models is the presence of IRF5, which may be responsible for an IRF3/7 independent signaling pathway[209, 217] and has been shown to produce enough IFN-beta to establish an anti-WNV response[209]. This suggests that the blunted type I IFN response in the IRF 3/7 DKO mice is sufficient to protect from severe ZIKV disease.

During the ZIKV outbreaks in French Polynesia and the Americas there has been an increased incidence of neurological symptoms in adults and of congenital malformations. It is unclear whether this increase is due to a change in the virus, or if it is an issue of the unprecedented size of the outbreak that allows rare manifestations to become apparent, or of the heightened reporting due to social concern about microcephaly[86]. After infection of the IRF 3/7 DKO mice with the MR766 strain, the patterns of infection and tropism mirror what has been observed in other murine models utilizing the Brazilian and French Polynesian strains (both of the Asian genotype), in which the mice also presented ocular discharge containing viral RNA, as well as infection in neurological and male reproductive tissues[97, 98, 100]. While we were unable to isolate viable virus from the ocular discharge of our infected mice, we did detect viral RNA, which has also been found in the conjunctival fluid of imported cases infected with the Asian strain of ZIKV[84]. In another report, a terminal ZIKV patient

transmitted ZIKV to a care-giver when neither mosquito nor sexual contact were possible, but the care-giver had wiped the patient's eyes without wearing gloves[83]. This, together with the detection of ZIKV RNA from the conjunctival fluid suggest that contact with eye-exudate might serve as an additional transmission route. There have been increasing reports of ocular lesions during the outbreak in the Americas, both in adults and congenitally infected cases, most frequently affecting the retina[17, 22, 116, 218-220]. Our results show that the African prototype strain of ZIKV can also cause ocular lesions in this mouse model, and that IRF3/7 DKO mice are well suited to study the transmission route that is possibly associated with these lesions.

We visualized the localization of ZIKV antigen in the male reproductive organs through IHC, observing labeling in the germ cells, in the seminiferous tubules, and in the epithelial lining of the epididymis. In the epididymis, labeling was co-localized with abundant tissue destruction and inflammation in the form of necrosuppurative epididymitis, which could account for reports of hematospermia in some ZIKV-infected men, associated with the Asian lineage in French Polynesia[221]. ZIKV antigen labeling was detected in the seminal fluid inside the lumen of the ductus deferens, where it was predominantly found in what are interpreted to be epithelial cells. These infected epithelial cells are thought to have sloughed off the wall of the epididimys and ductus deferens into the seminal fluid. Visualization of ZIKV in seminal spermatozoa has been previously reported[222]. The finding of epithelial cells containing ZIKV antigen in the seminal fluid of the mouse suggests an alternative transmission mechanism through the seminal fluid, that may also serve to explain the case of sexually transmitted ZIKV in a vasectomized male in which sperm cells would not have comprised a potential source

for viral transfer during intercourse[69]. ZIKV antigen labeling in the seminal fluid of the male IRF 3/7 DKO mouse in both epithelial and sperm cells in the seminal fluid presents a possible mechanism of sexual transmission from male to female and opens the possibility of sexual transmission studies using this mouse model.

Therefore, we find that the IRF 3/7 DKO mouse model, while infected with the prototype MR766 ZIKV strain belonging to the African genotype, presents similar signs and lesions as other mouse models and humans infected with strains circulating in the Americas that belong to the Asian lineage. This would indicate that, although the two genotypes have been shown to have some differences in their pathogenesis[16, 121, 122], both are capable of similar lesions *in vivo*. Thus, it is unlikely that the increase in severe symptoms is due only to a mutation of the virus.

The IRF3/7 DKO mouse mounted a strong neutralizing antibody response to ZIKV, comparable to the antibody response observed in a rhesus macaque model[76]. These mice also allow productive ZIKV replication and acquire characteristic ZIKV-induced lesions, while maintaining a high survival rate. Thus, this small animal model offers unique opportunities for therapeutic evaluation, as antibody responses are critical for quantifying the efficacy of vaccine and other anti-viral candidates, and long-term studies, thanks to the low mortality rate, as well as transmission studies.

CHAPTER 3: BOOSTING ALTERS THE CROSS-NEUTRALIZATION AND ENHANCEMENT CAPACITY OF THE ANTIBODY-RESPONSE FOLLOWING ZIKV EXPOSURE IN C57BL/6 MICE

3.1 INTRODUCTION

Zika virus (ZIVK) recently emerged as a public health threat in the Americas where currently 48 countries have reported local transmission[208]. In the majority of these countries, dengue virus (DENV) has circulated or continues to circulate. ZIKV is a flavivirus of the Japanese Encephalitis Virus (JEV) group, related to other viruses including DENV and Yellow Fever virus (YFV)[204, 223]. ZIKV was first identified from a sentinel monkey in the Zika Forest of Uganda and has been associated with sporadic outbreaks in Africa and Asia[2, 224-226]. In 2007, ZIKV was implicated and later confirmed to be the cause of an outbreak of febrile illness in Micronesia[6, 204]. It was then detected in Cambodia in 2010, French Polynesia in 2013, and on Easter Island in 2014[227-232]. In 2015, ZIKV was first identified in Bahia, Brazil, and has since spread throughout South and Central America [11, 233]. Conditions that support DENV transmission also support ZIKV transmission, as they have in common their primary vectors: *Aedes aegypti* and *Aedes albopictus* [234]. In addition, similarities in clinical presentation to DENV make symptomatic diagnosis unreliable. Further, serological diagnostics are confounded by the high degree of cross-reactive antibodies between ZIKV and those pre-existing to DENV[204, 226, 235-237].

Pre-existing immunity against one flavivirus can affect not only the diagnosis of disease produced by infection with a heterologous flavivirus, but potentially the clinical outcomes. The existence of antibodies to a previous flavivirus infection can result in

increased severity of disease upon infection with a secondary, heterologous flavivirus infection, as has been observed in secondary (and higher order) DENV infections of different serotypes[238]. This phenomenon is explained by the hypothesis of antibody dependent enhancement (ADE). ADE suggests that antibodies against a primary infecting flavivirus assist the subsequent, heterologous flavivirus to enter Fc-receptor-presenting cells, leading to increased viremia and enhanced disease. ADE has also been observed between different viruses of the flavivirus family in *in vivo* and *in vitro* assays[147, 188, 239]. Recent studies have shown that, *in vitro,* DENV monoclonal antibodies and DENV-immune serum are capable of enhancing ZIKV infection, and ZIKV monoclonal antibodies and ZIKV-immune plasma are capable of enhancing DENV infection[179, 180, 184, 185, 203]. In addition, pre-administration of a ZIKV monoclonal antibody to AG129 mice was able to enhance disease severity and accelerate mortality *in vivo*[203]. While no specific cases of enhancement of DENV due to pre-existing ZIKV antibodies have been reported in humans, it remains an important question when considering that DENV will likely not be displaced in areas of intense ZIKV transmission.

In this study we investigate the capability of antibodies raised to a primary ZIKV exposure to neutralize and/or enhance infection of DENV-2 and the effect of repeated exposure to ZIKV on this capability.

3.2 MATERIALS AND METHODS

3.2.1 Ethics Statement

All experiments involving mice were approved by the LSU Institutional Animal Care and use Committee (protocol 15-078) in adherence with policies of the American Veterinary

Medical Association and in compliance with the guidelines laid out by the National Institutes of Health's Guide for Care and Use of Laboratory Animals, 2011.

3.2.2 Virus

ZIKV strain MR766 and DENV2 strain 16803 were generously provided by Dr. Robert Tesh at the Center for Biodefense and Emerging Infectious Diseases at the University of Texas Medical Branch. Virus titers were initially determined via plaque assays on Vero cells prior to use in plaque reduction neutralization test; both ZIKV and DENV2 had titers of 10^7 PFU/ml. For the *in vitro* assay, concentrated DENV2 had an initial titer of 10^9 PFU/ml but was subsequently diluted to achieve specific multiplicity of infection (see methods below).

3.2.3 Mouse exposures

Five female 8-10-weeks-old C57BL/6 mice were injected subcutaneously with approximately 10^6 plaque forming units (PFU) of ZIKV in a volume of 100 µl. Mice were first anesthetized and then bled via cheek bleed as in[210] for 7 days. Following this primary exposure, mice were then boosted three times with the same volume and titer of virus as in[240]. The first boost was approximately 60 days post-exposure and the second and third boosts were at one-month intervals.

3.2.4 Viral RNA detection

Blood samples were allowed to clot at room temperature for 30 minutes and were then centrifuged for 4 minutes at 4°C and 6000 rcf. Serum was separated from the clot and stored at -80°C until further processing. RNA was extracted from serum using the

QIamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Viral RNA was detected by qRT-PCR using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies, Carlsbad, CA) on a Roche Lightcycler 480 (Roche). ZIKV-specific primers and probe were designed by Faye et al.[212].

3.2.5 Plaque Assays and Plaque Reduction Neutralization Test (PRNT)

PRNTs were performed following the WHO PRNT protocol for DENV[213]. Briefly, the number of virus particles was standardized to 50 virions in 100µl per well for both ZIKV and DENV2. Mouse serum was complement inactivated for 30 minutes at 56°C and then serially diluted 1:2 in M199E media from 1:10 to 1:2560. Equal volumes of 100µl of the standardized virus (50 pfu/100µl) was mixed with each dilution of serum for each mouse and allowed to incubate at 37°C for 1 hour, before inoculation onto confluent Vero cell sheets in 6-well plates and first overlay. Also included were a negative control well (media only) and a positive control (virus only). The timing of the second overlay was associated with the growth kinetics of the virus and was done at 3 days post inoculation (dpi) for ZIKV and 4 dpi for DENV2. Plaques were visible and counted the following day (4 dpi for ZIKV and 5 dpi for DENV2). The percent reduction in plaques per dilution was calculated and results are expressed as the reciprocal of the dilution in which the desired percentage of plaque reduction was achieved; we report both PRNT50 and PRNT80.

3.2.6. *In vitro* ADE assay

Previous studies have demonstrated low DENV infectivity of THP-1 cells except in the presence of anti-DENV antibody[241]. To determine whether anti-ZIKV antibodies could

also cause *in vitro* enhancement to DENV2, we exposed THP-1 cells to DENV2 both in the presence and in the absence of polyclonal serum from the ZIKV-exposed mice, where possible. THP-1 cells were generously provided by Dr. Juan Martinez at Louisiana State University. For two mice in time point 1, we did not have serum available for the enhancement assay. Thus, for the ADE analysis, only three mice were used for both time points. The ADE assay was performed largely as in[241], with minor modification. Briefly, THP-1 cells were counted to a density of approximately 2.5 x 10^5 cells per tube and then exposed to infection at an MOI of 10 with DENV2 16803 either 1) alone (virus only control) or 2) with ZIKV-exposed, heat inactivated mouse serum at a dilution 1:320. This was performed for time point 1 (following initial exposure) and time point 2 (following boosts). The final volume of THP-1 cells and treatment suspension was 200 µl. Samples were incubated for 2 hours at 37°C and gently shaken every 20 minutes to prevent premature sedimentation. Cells were centrifuged (900 rcf for 3 minutes) and the pellet was washed six times followed by the addition of clean media to remove residual virus and antibody. After the 6^{th} wash, the mixture was re-suspended in clean media and incubated for 3 days at 37°C. Lastly, we used this mixture (as opposed to [241] which separated supernatant and used the pellet for additional testing) to detect DENV2 infection of THP-1 cells via plaqueing on Vero cells as above. Additional negative controls of THP-1 cells only were processed in the same way without virus or serum. DENV2 plaque assays were performed at the time of the ADE treatment plaque assays to ensure that viable DENV2 was present.

3.2.7 Statistics

Statistical significance of differences in percent reduction by time-point and virus in the PRNTs was determined using a two-way analysis of variance (ANOVA), and of the average titers by time-point in the ADE assay using a one-way ANOVA. P-values below 0.05 were considered significant. Analyses were performed in R version 3.2.3 software.

3.3 RESULTS

3.3.1 Mouse Exposure to ZIKV

Of the five mice, only four had detectable viral RNA. In four of them, there was detectable RNA on only the first day post inoculation and these were very low levels (less than 1000 pfu/ml), indicating that the mice likely did not produce viremia but cleared the virus within 24-48 hours (Table 3.1). Thus, C57BL/6 mice are not good infection models for ZIKV.

Table 3.1: Mice exposed to ZIKV did not develop significant viremia with ZIKV being detected only 24 hours post exposure.

Mouse ID	DPI	Viremia (PFU/mL)
1	1	9.76E2
2	1	2.17E2
3	1	0
4	1	2.75E2
5	1	1.15E2

3.3.2 Neutralization of ZIKV and DENV2

Even in the absence of robust viremia, all five mice produced highly neutralizing antibodies to ZIKV (Table 3.2) and moderately cross-neutralizing antibodies to DENV2 (Table 3.3). After the primary exposure, highly neutralizing antibody to ZIKV, on average, reduced the formation of plaques by 80% with a titer of 40 and by 50% at dilution with a titer of 320 (Figure 3.1A).

Table 3.2**:** 50% and 80% neutralizing titers for each mouse at both time points to ZIKV following ZIKV exposure(s).

Mouse ID	Time point	PRNT50 titer	PRNT80 titer
1	1	1280	40
2	1	640	20
3	1	320	20
4	1	320	40
5	1	160	40
1	2	640	160
2	2	160	80
3	2	160	40
4	2	160	20
5	2	80	80

In addition, there was considerable cross-neutralization of DENV2, with an average PRNT50 titer of 20. Neutralization for DENV2 did not reach 80%.

Antibodies also highly neutralized ZIKV at time point 2. This average neutralization was not significantly different from time point 1 (Figure 3.1B). However, cross-neutralization of DENV2 decreased and, in some mice, was negligible with titers <10 (Table 3.3), indicating that homologous boosting drives the specificity of the antibody response towards ZIKV-specificity.

Table 3.3**:** 50% neutralizing titers for each mouse (ID) at both time points to DENV2 following ZIKV exposure(s).

Mouse ID	Time point	PRNT50 titer
1	1	160
2	1	20
3	1	20
4	1	40
5	1	10
1	2	10
2	2	<10
3	2	10
4	2	<10
5	2	<10

3.3.3 Antibody-dependent enhancement of DENV2 infection *in vitro*

Enhancement of DENV2 was induced by polyclonal serum collected at time point 2, as indicated by the significantly greater average DENV2 titer in THP-1 cells ($p<.05$)

compared to the DENV2 titer induced by control and the serum collected at time point 1. No significant enhancement of the average DENV2 titer was observed at time point 1 ($p>.05$), though this may be due to the small sample size. The level of enhancement at time point 2 is similar to what has been seen in previous studies[152, 241, 242], indicating that this model is appropriate for assessing the cross-enhancement capabilities of ZIKV-induced polyclonal antibodies to at least DENV2.

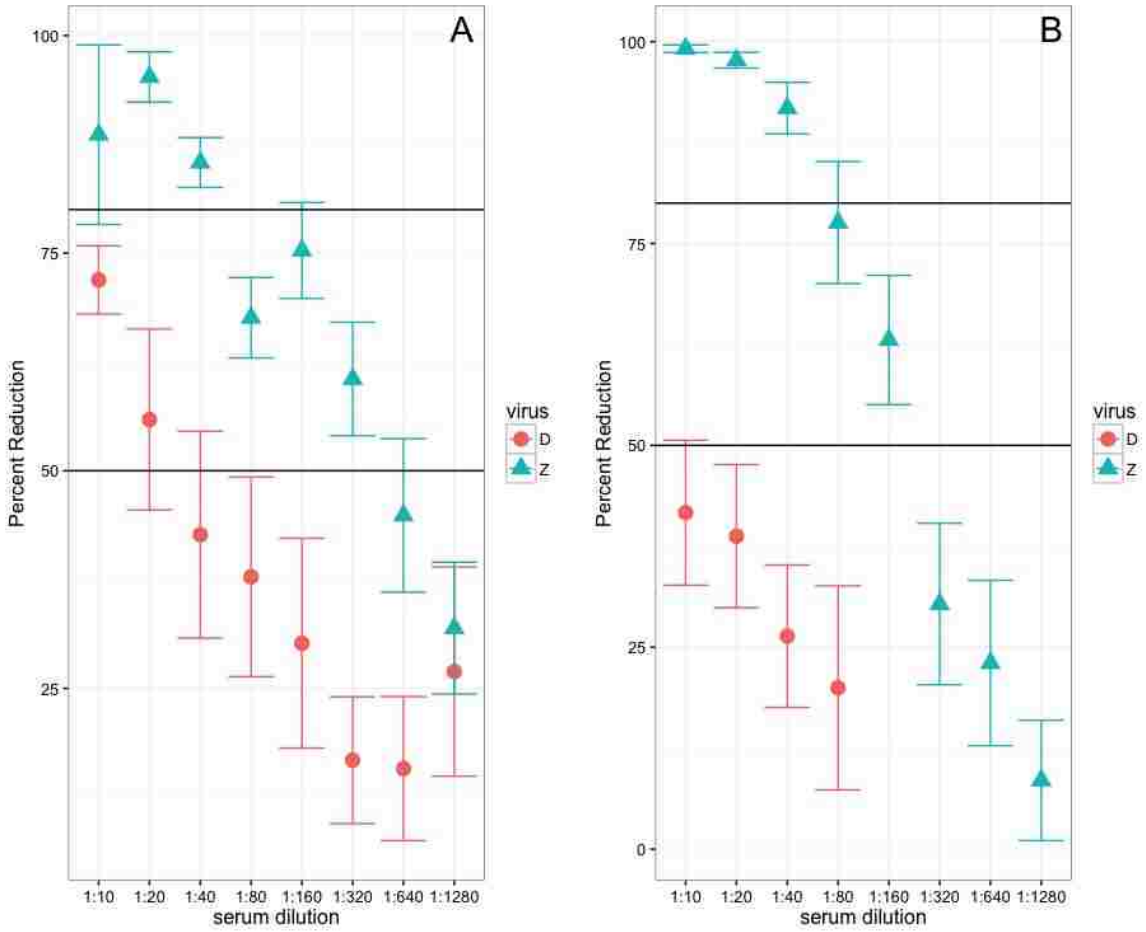

Figure 3.1: For each time point, the mean percent neutralization +/- 95% confidence interval for each serum dilution and virus challenge. A) time point 1 B) time point 2. (ZIKV green, DENV2 red).

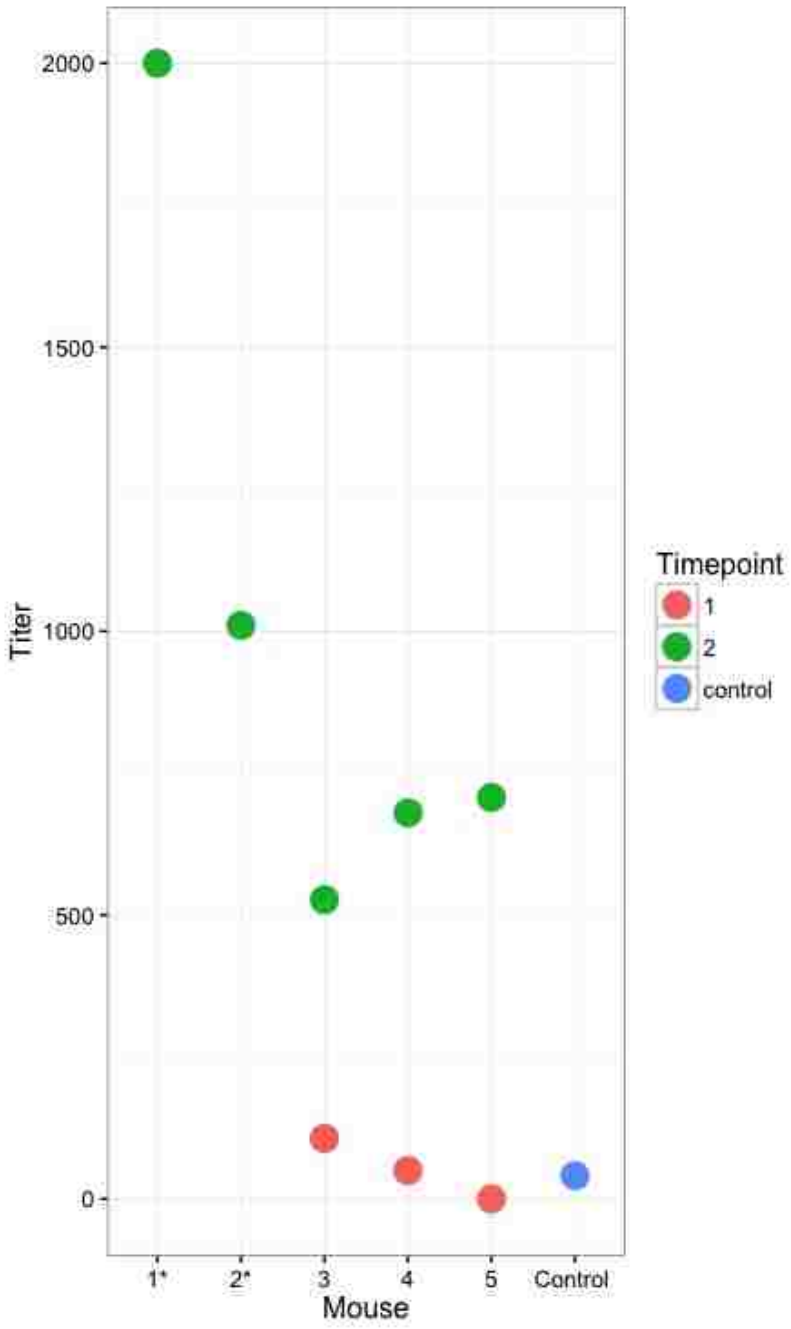

Figure 3.2: DENV2 titers as determined by plaque assay following incubation of THP-1 cells with polyclonal serum from ZIKV exposed mice after 1 initial exposure (time point 1) and following homologous boosting (time point 2) demonstrate enhancement at time point 2 only. Control is the average of 3 replicates of virus-only experiments on THP-1 cells. *Mouse was not included in calculated of average ADE titers, nor included in the statistical analyses.

3.4 DISCUSSION

Due to their common vectors and geographical distributions, ZIKV and DENV continue to co-circulate in many areas of the world. In these regions, ZIKV will account for the primary flavivirus infection for a portion of the population, meaning that these individuals will mount an antibody response to ZIKV in the absence of other flavivirus history. Understanding how this primary ZIKV infection will affect susceptibility to secondary DENV exposure is critical. Further, due to the intense circulation of ZIKV in these areas it is likely that some individuals will be naturally boosted; that is, exposed more than once to ZIKV, resulting in altered antibody profiles. A recent report demonstrates the occurrence of repeated infection with homologous DENV serotypes[175]. A similar occurrence of repeated ZIKV exposure is likely, but the effect of such a re-exposure on the antibody population and how it may affect heterologous enhancement is unknown.

Our results demonstrate that antibody population raised to a primary ZIKV exposure cross-neutralizes DENV2 at the PRNT50 level, but that cross-neutralization could be reduced with subsequent homologous exposures to ZIKV. This has implications for diagnostics and vaccine development, as the cut-off value for most vaccine studies is PRNT50, and we have shown that at that level, differentiation of ZIKV or DENV2 infections would be difficult. Further, our data suggest that while homologous boosting with ZIKV decreases the overall cross-neutralizing capacity of the antibody response to DENV2, it also may result in risk of enhancement upon subsequent DENV2 infections. This suggests a role for natural boosting in ADE risk in DENV and ZIKV endemic regions.

The characteristics of ZIKV-induced antibodies that are responsible for enhancement are not yet fully understood. Stettler et al.(2016) showed that monoclonal ZIKV antibodies specific for domains I and II of the E protein (EDI/II), highly cross-reactive to all four serotypes of DENV but weakly ZIKV-neutralizing, were able to neutralize and enhance DENV1 *in vitro* and *in vivo* in AG129 mice[203]. In contrast, our study indicates that a polyclonal antibody population less cross-neutralizing to DENV2 and more ZIKV – specific has increased DENV2 enhancing capability. Stettler et al. (2016) also evaluated ZIKV-immune human plasma, which was able to cross-react with DENV1-4 and to enhance DENV1 to a similar level as DENV3-immune plasma. However, the neutralization of DENV and ZIKV by this ZIKV-immune plasma was not shown.

Stettler et. al. (2016) showed that ZIKV-induced EDI/II-specific antibodies were weakly ZIKV neutralizing but quite strongly DENV1-neutralizing, capable of blocking DENV1 infection in approximately 50% of the cells even at low concentrations[203]. Interestingly, although ZIKV-induced EDI/II-specific antibodies bound strongly to both ZIKV and DENV E protein, these antibodies were shown to neutralize DENV much more strongly than ZIKV. A similar phenomenon was observed with DENV-induced monoclonal antibodies against the FL portion of the E protein that is 100% conserved between DENV and ZIKV, that are able to weakly neutralize DENV, but not ZIKV[179, 184, 185]. It is possible that because of ZIKV's increased thermal stability[39], it "breathes" less, and therefore has reduced exposure of certain epitopes on its surface compared to DENV virions. EDI/II have high percent homology between the two viruses but are perhaps more exposed on the surface of DENV, explaining the ability of ZIKV-induced EDI/II-specific antibodies to neutralize DENV but not ZIKV.

Flaviviruses require the number of antibodies that are bound to the virus to exceed a certain stoichiometric threshold in order to achieve neutralization[158]. Weakly neutralizing antibodies must bind in large numbers to the virus in order for neutralization to occur. Therefore, all but the highest concentrations of weakly neutralizing antibodies are considered sub-neutralizing, and these antibodies are able to enhance infection at a broad range of concentrations[158].

We hypothesize that the initial ZIKV-induced antibody response may have a larger fraction of cross-neutralizing antibodies, possibly targeting ZIKV EDI/II, in high concentrations, such that DENV is neutralized rather than enhanced. Homologous ZIKV boosting may lead to a shift in the epitope specificity of the larger fraction of antibodies, from more cross-neutralizing to more ZIKV-specific epitopes, decreasing the number of DENV2 cross-neutralizing antibodies present, which are able to enhance DENV2 at low concentrations. However, more detailed study into the epitope specificity of the polyclonal antibody population and the variation of its components over time is needed to confirm this hypothesis.

Globalization of trade and travel, continued urbanization, and climate change are just some of the factors that have led to increases in arbovirus emergence and expansion[243]. While ZIKV will likely continue to co-circulate with DENV, other closely related flaviviruses may emerge in the future, and the immunological interaction among related viruses needs to be elucidated. Additionally, the effects of natural boosting must be further studied, as it affects not only the specificity of the antibody population such as cross-neutralization capacity, but also the spectrum of susceptibility due to potential infection enhancement or protection. It is critical to understand the dynamics of these

antibody interactions in order to improve our ability to accurately diagnose, predict clinical outcomes, anticipate follow-up priorities and sequelae, as well as inform vaccine development studies.

CHAPTER 4: CONCLUSION

Mouse models are valuable tools used to study the development of disease *in vivo* in controlled conditions, especially in the light of the elevated cost and scarcity of some non-human primate models[109]. Mice have the added advantage of a short life-cycle that allows researchers to perform longitudinal studies and to evaluate disease outcomes at different life-stages in limited amounts of time. Studies in which ZIKV infection of pregnant mice resulted in fetal brain malformations were crucial to demontrating the causal link between ZIKV and congenital malformations[244], showing that ZIKV in the absence of other factors was capable of fetal neurological and placental pathogenesis. Similarly, proof of concept of antibody-dependent enhancement (ADE) was achieved *in vivo* when mice pre-treated with DENV antibodies developed increased disease severity and mortality upon heterologous DENV infection, demonstrating the role of previous immunity and Fc-receptor bearing cells in severe DENV disease[153]. In addition, mouse models are often the first step in vaccine and therapeutic development, providing a cost-efficient *in vivo* setting where protection from disease can be easily demonstrated.

In this body of work, we have characterized a type I interferon (IFN) deficient mouse model that allows robust ZIKV replication in the serum and in tissues and that develops characteristic ZIKV-induced lesions while maintaining a high survival rate. In addition, we found that the immunocompetent C57BL/6 mouse model, while not susceptible to ZIKV, is still able to develop a strong antibody response upon ZIKV exposure that allows for the exploration of antibody characteristics in an intact immune system.

In vitro studies have shown that ZIKV virus strains derived from both the African and the Asian lineages are capable of infecting primary human dendritic cells, neural progenitor cells, astrocytes, and placental cells. Which of the two lineages is more virulent varies by study and cell type: in dendritic cells, African strains can cause apoptosis and increased, rapid replication compared to the Asian strains[31]; in neural cells, the results vary[16, 121, 122]; in amniotic epithelial cells, Nicaraguan isolates produce more progeny than the prototypical African strain[129]. *In vivo*, the involvement of ZIKV in fetal brain damage and placental pathology has only been demonstrated after infection with strains from the Asian lineage. However, in older mice, infection with African strains causes similar brain lesions as infection with Asian strains, as well as a similar development of disease. Infection of 5-6-week-old A129 mice with the African isolate ZIKV MP1751 induced similar infection kinetics as infection with FSS13025 belonging to the Asian lineage, such as peak viremia at 2 dpi, loss of approximately 20% of initial weight around 6 dpi, and signs of disease that include hunched posture and ruffled fur, and eventual euthanasia[98, 101]. In addition, infection with the African isolate ZIKV MP1751 in A129 mice induced similar brain lesions as infection with the H/PF/2013 Asian isolate in the AG129 model[104], including neuronal degeneration in the hippocampus and inflammatory cell infiltration in the meninges. In 5-week-old immunocompetent C57Bl/6 mice pre-treated with anti-IFN antibody, infection with the African ZIKV strain DAK AR D 41525 also resulted in neuronal death and inflammatory cell infiltrate, most prominently in the hippocampus[105]. These results indicate that in adult mice, African isolates can cause similar brain lesions to Asian isolates. Whether

the same is true of fetal mouse brains after congenital infection with African strains remains to be seen.

In addition to affecting the brain and placenta, both African and Asian strains of ZIKV have been shown to infect and cause lesions in the gonads of male mice that result in a reduced size of the testicles, testicular atrophy, depletion of spermatogonia, and infertility[136]. Ifnar1 -/- mice infected with an Asian strain evidenced ZIKV infection of the epithelial cells of the epididymis[136], and our experiments in Chapter 2 confirm the ability of the MR766 African strain to infect the same cells. In addition, in our study we visualized a large quantity of these cells inside the seminal fluid. Epididymal epithelial cells were shown to degenerate and necrotize in the Ifnar1 -/- after infection; we hypothesize that some of these infected cells can lose their attachment to the epididymal wall and provide an additional source of ZIKV transmission in the semen.

ZIKV RNA can be detected in testicular cells and mature sperm in the lumen of the epididymis up to 21 days post-infection in immunocompetent C57Bl/6 mice pre-treated with anti-Ifnar1 antibody, corresponding to the prolonged detection of ZIKV RNA in the in the semen of infected human males[136]. In humans, lesions in the gonads have not been reported, and the pathogenesis of ZIKV in these tissues is unknown. However, case reports of involvement of both lineages of ZIKV in the male reproductive organs exist. ZIKV has been detected in spermatozoa in the semen of an infected patient[66], and together with RNA detection in the semen for prolonged periods of time[34], these findings suggest that ZIKV replicates in the gonads and can be found in sperm cells in the semen. It is also possible that both genotypes of ZIKV can cause lesions in human masculine gonads, as evidenced by a reports of hematospermia in a patient during the

French Polynesian outbreak[65], and also of hematospermia accompanied by prostatitis symptoms in an imported case from Senegal[245]. If epithelial cells of the epididymis become infected in humans as they do in mice, infected epithelial cells that have sloughed off into the ejaculate could provide an explanation for the reported case of ZIKV transmission by a vasectomized male[69]. In vasectomized patients the ductus deferens is blocked so that the ejaculate does not contain sperm cells, however, infected epithelial cells could still provide a source of ZIKV infection. Further studies in a human system of the cellular components of ZIKV sexual transmission, as well as the lesions induced by ZIKV infection in the gonads, are needed. In mice, the lesions caused by ZIKV lead to infertility[136]; whether the same occurs in humans has yet to be determined.

Conjunctivitis is a common symptom in ZIKV infection, but there are increasing reports of more severe ZIKV-associated ocular lesions, both in adults and in congenital cases. Adults have been found to develop uveitis and chorioretinal lesions, as well as to shed virus from conjunctival exudat[21, 22, 84]. Neonates from ZIKV-infected mothers present a wide array of lesions involving the retina, optic nerve, lens, and iris[115-118]. Until the present time, all of the reported human cases with severe ocular lesions have been either imported from Latin America or have occurred in Brazil, and are therefore are associated with the Asian strains currently circulating in the Americas. Mouse experiments *in vivo* have also demonstrated ZIKV involvement in the eye[100]. Both the Brazil Paraiba 2015 and the H/PF/2013 strains of ZIKV were able to cause lesions in the eyes of Ifnar1 -/- mice similar to those reported in human cases, such as infectious shedding of virus in tear fluid and infection of the cornea, iris, lens, retina, choroid

complex, and optic nerve, accompanied by lesions in several ocular tissues. Infection with these ZIKV strains also caused C57Bl/6 immunocompetent 8-day-old mice to develop lesions in optic areas.

Ocular lesions induced by ZIKV have been associated, to date, with the Asian lineage of ZIKV, more specifically the strains circulating in the Americas. However, in Chapter 2, we present evidence that the African MR766 strain is also able of causing lesions in the eyes in a susceptible ZIKV mouse model. The necrosis of ganglionar cells in the retina, the inflammatory infiltrate in the vitreous humor, and the shedding of ZIKV RNA in conjunctival exudate correspond not only to almost identical lesions observed in the Irf1 -/- model infected with Asian strains, but also to lesions observed in humans during the American outbreak [118, 132]. This would indicate that the African strains are likely capable of ocular involvement.

In the light of these findings, we suggest that African strains of ZIKV may be capable of lesions that have up to now only been reported in association with Asian strains, and that the pathogenic capability of both genotypes are similar. *In vitro* studies indicate that African strains infect human primary neural progenitor cells and placenta- derived cells, indicating that strains of the African lineage could potentially cause neurological and developmental issues in congenital infections. *In vivo* models show that African strains can also infect the brain cells of adult mice, indicating that neurological symptoms such as those reported in adults need not be caused only by Asian strains. Further experiments *in vivo* with African strains are needed to explore their ability of causing congenital anomalies. Increased surveillance in Africa in ZIKV-endemic areas would also help establish whether the African strains of ZIKV are as dangerous to pregnant

women as the Asian strains have been shown to be, and in that case appropriate preventative measures should be applied. Such surveillance would provide strong evidence that the current increase of ZIKV-associated congenital brain malformations observed in the Americas is not mainly the consequence of a particular genotype of ZIKV, but of the heightened case-count and reporting of ZIKV disease in the region.

In addition, the above findings of ZIKV infection in the brain, placenta, eye, and male gonads suggest that both the African and Asian lineages of ZIKV have tropism for immune-privileged sites. Immune privilege is a combination of anatomical, physiological, and immunoregulatory characteristics that together protect certain critical tissues from immune-mediated inflammation[246]. These tissues include the brain, eye, pregnant uterus, and testes. It is possible that due to the reduced inflammatory response in these organs, ZIKV is not cleared and is able to persist and replicate; on the other hand, it is possible that ZIKV directly targets these organs to establish reservoirs from which to disseminate. Indeed, in *in vivo* models and some human cases, ZIKV RNA has been detected from semen, conjunctival exudate, and brain after having been cleared from the serum, suggesting that it can establish infection in certain organs and continue to replicate and disseminate form there. However, ZIKV is also detected for prolonged periods of time from urine and saliva which are not produced in immune-privileged organs, suggesting that ZIKV also has the ability of establishing infection in other organs that are not protected from the immune system. A similar mechanism has been observed in the filovirus Ebola, that, like ZIKV, presents persistent RNA shedding from immune-privileged and other organs, such as the eye, semen, and lungs[247-250].

Further studies are needed to elucidate the mechanism of viral persistence in these immune-privileged sites.

The ability of WT C57BL/6 mice to develop ZIKV infection under certain circumstances but not others points at factors that may play a role in entry into immunologically protected sites. WT C57BL/6 mice did not acquire ZIKV infection in the male gonads after intraperitoneal injection, but presented severe lesions in both the testis and epididymis after intratesticullar infection[136]. In addition, congenitally ZIKV-infected WT pups did not develop ocular lesions, while postnatally infected 8-day-old pups did, suggesting that ZIKV does not easily cross the placenta in late gestation in immunocompetent mice[100]. Therefore, crossing the blood-testicular-barrier and the placenta may be limiting factors for the establishment of ZIKV infection. Further study is needed to elucidate the mechanisms of this entry. The IRF 3/7 DKO model characterized in Chapter 2 presents ZIKV infection and lesions in most of these immune-privileged sites, as well as a low mortality rate, making it a good candidate to study questions regarding ZIKV persistence and pathogenesis in immune-privileged organs.

In DENV, severe disease presentations such as dengue hemorrhagic fever and dengue shock syndrome are characterized by severe thrombocytopenia, hemorrhagic manifestations, and plasma leakage[251]. The peak of symptom severity and vascular leakage coincides temporally with a decrease in viral load but an increased circulation of cytokines and chemokines such as TNF alpha, (interleukin) IL-6, IL-8, IL-10, that are produced by monocytes, macrophages and T-cells, called a cytokine storm. These soluble factors play a role in viral clearance, but are also thought to induce the

increased vascular permeability that leads to severe dengue symptoms[252, 253]. These severe symptoms have also been associated with increased viremia, which may lead to an increase of cellular activation and cytokine production that mediates the disease[253]. Antibody dependent enhancement is thought to produce an increase in viremia[150], thanks to the more efficient infection of Fc-receptor bearing cells, thereby resulting in increased disease severity.

During our characterization of ZIKV infection in the IRF 3/7 DKO mouse model, we observed that symptom onset occurred days after viremia peak, when ZIKV RNA was barely detectable in serum, but coincided with ZIKV presence in several tissues. However, ZIKV infection has been shown not to induce DC activation or cytokine production[31], and acute cases of ZIKV have not shown an increase in pro-inflammatory cytokines[140]. Therefore, ZIKV pathogenesis is unlikely to be mediated by a cytokine storm, as it is in DENV. The fact that ZIKV RNA can be continually detected from various bodily fluids for prolonged periods of time in *in vivo* models and in humans suggests that symptoms are induced by replication in certain organs where ZIKV establishes infection, rather than by an inflammatory reaction in response to increased viremia. This is supported by the fact that severe ZIKV symptoms observed in *in vivo* models and in humans, such as paralysis, uveitis, brain calcifications, or hematospermia, are more likely to be caused by lesions in ZIKV-tropic tissues, instead of being the consequence of vascular leakage.

However, sub-neutralizing antibodies may still influence disease severity in ZIKV infection. Although dendritic cells have been shown not to activate and produce cytokines upon ZIKV infection, an increased infection of these cells could allow for a

more efficient spread of ZIKV to various organs through the blood. In addition, several cells in the placenta have the potential of developing enhanced infection through their Fc-receptors. Syncytiotrophoblasts have been shown to either not allow productive infection of ZIKV or to allow it at a low level[128, 129, 131]. However, they express neonatal Fc receptors, which would potentially allow for their increased infection and therefore the dissemination to other placental cells[129]. Hofbauer cells, that also express Fc-gamma receptors[254], are speculated to be responsible for persistent infection and productive replication in the placenta. The increased infection of these cells could determine the pregnancy outcome in congenital cases of ZIKV infection, especially in late-gestation infections when the innate immune response is more likely to block infection by other means[128, 129]. Demonstration of the role of sub-neutralizing antibodies in placental infection, however, would prove difficult in a mouse model, given that mouse trophoblasts do not express neonatal Fc-receptors[106].

Enhancement of DENV infection by ZIKV antibodies, and of ZIKV infection by DENV antibodies, has been demonstrated *in vitro* in several Fc-receptor bearing cell cultures[106, 179, 180, 184, 185, 203]. Antibody characteristics, such as concentration and ability to neutralize and bind to an infecting virus, determine their ability to enhance the infection caused by said virus[158]. These characteristics vary over time, and can be modified by infections with related viruses. Therefore, it may be that risk of severe ZIKV disease is influenced by previous DENV infections. It has been speculated that the severe symptoms occurring in the outbreak in the Americas may be consequence of the background DENV immunity in the population. The Asian genotype of ZIKV circulates in both Southeast Asian and the Americas; however, in Southeast Asia, DENV has been

hyperendemic for a longer time than in the Americas, and people are exposed to several different serotypes of DENV by an earlier age. It is possible that the broadly protective response resulting from numerous DENV exposures is protecting the Southeast Asian population from severe ZIKV disease outcomes[200]. Although serum from monotypic and heterotypic DENV-immune pregnant women did not show any difference in ZIKV enhancement[187], further studies are need to confirm whether previous DENV immunity affects the outcome of ZIKV infection.

In vivo, DENV antibodies failed to induce increased disease severity or lethality of ZIKV infection in wild type 120v/ev mice[203]. It is possible that infection was not enhanced due to the low susceptibility of this mouse model to ZIKV infection: low levels of ZIKV RNA can be detected in serum and later in tissues, but these mice do not display signs of disease or tissue lesions evidenced by histology[101]. In a more susceptible, non-lethal model presenting signs of disease and lesions, it is possible that there would be more evidence of enhancement. Measuring the severity of lesions and presence of virus in the tissues might be a more accurate measure of enhanced infection in the case of ZIKV, in addition to signs of disease and mortality. The IRF3/7 DKO mouse model characterized in Chapter 2 would be a good candidate for these experiments, due to the low mortality and the development of tissue lesions and signs of disease in mice.

It may also be possible that the ZIKV immunity now prevalent in the population in the Americas will lead to a recrudescence of symptoms upon subsequent DENV infection. *In vivo*, ZIKV antibodies have been shown to enhance the morbidity and mortality of DENV1 infection in type I and II receptor knock-out mice AG129[203]. In addition, in areas of intense transmission, re-exposure is bound to occur. Further research is

needed to establish the characteristics of ZIKV-induced antibodies responsible for enhancement. Stettler et al. (2016) showed that DENV enhancement can be mediated by ZIKV-induced monoclonal antibodies that target the E protein domains I and II (EDI/II), which are highly DENV cross-reactive[203]. They also showed that human plasma exposed to a primary ZIKV infection was DENV1-4 cross-reactive and capable of DENV1 enhancement. In Chapter 3, we show that repeated exposure to ZIKV in C57BL/6 mice increases the ZIKV-neutralizing specificity and DENV2-enhancing ability of the polyclonal antibody population. Our results suggest that a less DENV-2 cross-neutralizing population has an increased capability of DENV enhancement, which seems to diverge from the results obtained in the Stettler study. This effect of increased enhancement may be due to a change in the fraction of the polyclonal antibody population specific to certain epitopes. The number of EDI/II-specific antibodies, which are able to neutralize DENV[203], may have decreased to sub-neutralizing concentrations, allowing enhancement. A more detailed understanding of the components of a ZIKV-induced polyclonal antibody population is needed to predict the risk of enhancement.

Further investigation is needed to elucidate the effects of ”natural boosting” on the antibody population, and whether the timing and the number of the boosts change these effects. The outcome of a secondary flaviviral infection can depend on the timing of the infection, the specificity of antibody population and the number of previous infections. Detection of re-exposure to homologous virus in human cohorts is difficult due to the limitations of serological diagnosis. Therefore, mouse models, in which all the parameters can be controlled, such as the number and timing of re-exposures, are

invaluable to explore this phenomenon. We have shown that C57Bl/6 mice are a valid model to study the changes in the polyclonal antibody response induced by ZIKV infection, with the added advantage that these changes occur in the setting of an intact immune system. Understanding how immunity to ZIKV affects subsequent DEVN infection and vice-versa will help inform vaccine development and vaccination protocols. In addition, it will improve the predictive capability of outbreak models, as both protective immunity and ADE effects have been shown to influence the predicted duration of the ZIKV outbreak[255].

To conclude, I characterized two mouse models: the immunocompetent C67BL/6 mouse model that is well-suited for studies exploring ZIKV immunity; and the IRF 3/7 DKO model, that is an adequate model to evaluate ZIKV pathogenesis, persistence, and transmission, for the development of therapeutics and for vaccine studies. Using the C57BL/6 model, I was able to show that repeated exposure to ZIKV modifies the neutralizing characteristics of the polyclonal antibody response and increases the DENV2-enhancing ability of the antibodies. In addition, infection with an African strain of ZIKV in IRF 3/7 DKO mice demonstrated that this African strain is capable of causing similar lesions in the male gonads, eye, and brain as Asian strains, indicating that the severe symptoms involving these organs, reported during the American outbreak, are unlikely to be caused by a change in the virus.

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VITA

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