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Understanding the Severe Consequences of Zika Virus Infection

Emerging Infectious Diseases Dissertation
William Valiant

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Graduate Program,
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UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

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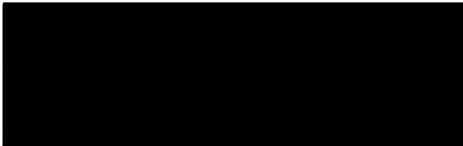
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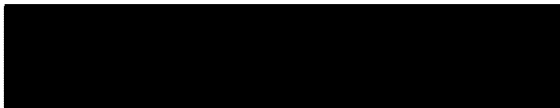


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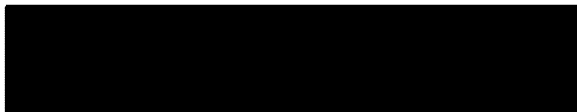


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Abstract

Zika virus (ZIKV) is a Flavivirus that emerged into the Americas in 2015, leading to a public health emergency. ZIKV infection has been associated with microcephaly and other birth defects when mothers are infected during the first trimester of pregnancy. As an emerging virus, the consequences of ZIKV infection are not fully understood. We sought to investigate the potential interactions between ZIKV and Dengue virus (DENV) as well as to understand the pathogenesis of ZIKV within a target cell of the retina.

We hypothesized that ZIKV-induced antibodies have the capability to enhance DENV infection, due to the structural similarity of the viruses. We found that rhesus macaques that have previously been infected with ZIKV experience enhanced DENV infections. Additionally, ZIKV infection results in an IgG1 antibody response that is highly cross-reactive with DENV, but is not able to neutralize DENV. These cross-reactive antibodies are able to enhance DENV-infection *in vitro*. The presence of DENV-neutralizing antibodies within ZIKV-convalescent serum results in a shift of the enhancing titer of the serum, indicating that the presence of DENV-neutralizing antibodies can protect from ZIKV-induced enhancement. These results from experimental infection of rhesus macaques were mirrored in humans naturally infected with ZIKV.

Müller cells, a glial cell of the eye, have been identified as the target of ZIKV infection within the retina. Müller cells are responsible for maintenance of homeostasis and also play a role in regeneration of retinal damage. We found that ZIKV infection of Müller cells results in the production of Interferon lambda (IFN λ) and the production of Interferon Stimulated Genes. If the IFN λ produced during infection is neutralized, then the level of ZIKV replication in the cells is decreased but Müller cells treated with IFN λ prior to infection are resistant to ZIKV.

146 These results highlight the potential severe outcomes of ZIKV infection that were not
147 understood as ZIKV emerged. ZIKV infection may have consequences for later DENV infection
148 and further epidemiologic studies are required to determine if areas affected by the ZIKV
149 outbreak will be at risk for outbreaks of severe dengue. Accompanying the adaptive immune
150 response, there is a strong innate immune response to ZIKV infection, but that response may lead
151 to pathogenic outcomes by promoting ZIKV replication. Further understanding of how the
152 immune response reacts to ZIKV may lead to potential therapeutics to prevent congenital zika
153 syndrome.

155	Chapter 1:
156	The Interactions of Zika Virus and Dengue Virus
157	William G. Valiant

Zika virus and Dengue virus

Zika virus (ZIKV) was first isolated from a sentinel monkey in the Zika forest in Uganda in the 1950s^{1,2}. At the time of discovery, it was known that ZIKV was transmitted by mosquitoes and that inter-cerebral infection of a neonatal mouse was lethal. Initial serologic studies identified that ZIKV was different from other related viruses including Dengue virus (DENV)². For about 60 years, ZIKV was not considered a public health concern until it entered South America and caused a major pandemic, resulting in the World Health Organization declaring a global public health emergency³. Zika virus exists as a single serotype, although it is divided into two lineages, the African and the Asian lineage⁴. The African lineage includes the historic isolates from Africa as well as currently circulating African strains, while the Asian lineage of ZIKV includes strains isolated from South East Asia as well as the epidemic strains isolated from the Americas⁵.

Unlike ZIKV, which has only recently emerged as a threat to public health, DENV has a global spread through the tropics as it initially spread from Africa beginning with the slave trade in the 1500s⁶⁻⁸. There are four serotypes of DENV, named DENV 1-4. Like ZIKV, DENV is a mosquito-borne virus with both urban and sylvatic cycles of transmission⁸. The sylvatic cycle of DENV is supported by infection of non-human primates (NHP) within tropical forests.

Flavivirus biology

Both ZIKV and DENV are flaviviruses. Many flaviviruses are arboviruses, such as Yellow Fever virus and West Nile virus, which are carried by mosquitoes, and Tick-Borne Encephalitis and Powassan virus, which are carried by ticks. Additionally, Hepatitis C virus is within the Flavivirus family⁹. Flaviviruses have a positive sense RNA genome that is approximately 11kb in length (Figure 1)¹⁰. The genome consists of a single open reading frame

that encodes for a polyprotein that is cleaved into the functional proteins of the virus¹⁰. The polyprotein is translated in the order of 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'¹⁰⁻¹². The structural proteins include C, the capsid protein, prM, the precursor membrane protein, which is processed into M, the membrane protein, and E, the envelope protein. The remaining proteins are non-structural. NS1 is a secreted protein involved in immune evasion¹³, NS3 and NS5 are involved in the replication of the RNA genome¹⁴, NS2A, NS4A and NS4B are involved in RNA accumulation, and NS2B acts as a cofactor for NS3¹².

ZIKV and DENV epidemiology

ZIKV was considered a rare disease until it began to cross the Pacific. The first recognized modern outbreak of ZIKV infection was on Yap island in 2007, followed by a number of additional outbreaks on other Pacific islands, such as French Polynesia¹⁵⁻¹⁷. ZIKV was first recognized in the Americas in 2015, however there is evidence that ZIKV was imported to Brazil as early as 2014 at the world canoe championships in Rio de Janeiro¹⁸. After entering Brazil, ZIKV spread within the Americas and the Caribbean, leading to a total of 86 countries reporting endemic ZIKV transmission¹⁹. In a study of DENV and Chikungunya virus (CHIKV) transmission in Haiti, three children were retrospectively found to be infected with ZIKV during 2014²⁰. There was endemic transmission of ZIKV within Florida in 2016 at the peak of the ZIKV outbreak and there has been continuous transmission within Puerto Rico since the outbreak²¹. Since the peak of the outbreak, the number of ZIKV cases has greatly decreased. In 2018 there were no cases of endemic transmission of ZIKV within the continental United States, and approximately 150 cases of endemic ZIKV infection within US territories. Overall, the epidemiology of ZIKV was similar to that of CHIKV: the mosquito-borne virus emerged in a

naïve population causing an initial explosion of cases but resulting in lasting endemic transmission at a low rate.

DENV infections occur at a steady pace in many endemic areas and infections have a serious public health impact. The WHO estimates that there are 390 million people infected with one of the four serotypes of DENV each year, with the infections spread across approximately 100 countries^{8,22}. Of these infections, the WHO estimates that 500,000 people require hospitalization each year due to severe dengue and of those hospitalized approximately 2.5% will die²². The four serotypes of DENV are each spread globally across the tropics, with co-circulation of all serotypes reported in some countries⁸. Some urban areas in Brazil are considered to be hyperendemic for DENV, with each of the four serotypes circulating at a high level^{23,24}.

Since ZIKV and DENV share the same vector, many countries have experienced endemic transmission of both ZIKV and DENV (Figure 2)^{25–27}. In countries like Brazil, DENV has been endemic for many years, and when ZIKV emerged into the country, people who were DENV-convalescent were infected. Additionally, some people experienced co-infection with ZIKV and DENV during the outbreak²⁸. As the ZIKV outbreak has waned, DENV continues to circulate within South America, so individuals who are ZIKV-convalescent may be infected with DENV in the future. Further studies are needed to understand the potential consequences of sequential infection, or co-infection with ZIKV and DENV.

The vector of ZIKV and DENV

The primary mode of infection for both ZIKV and DENV is by infected mosquito bite. Mosquitoes are able to maintain both a sylvatic and urban transmission of the virus and understanding their biology gives insights to the potential dynamics of viral transmission. The

primary vectors of DENV are *Aedes aegypti* and *Aedes albopictus*^{29,30}. There is also a DENV reservoir within NHP, which is maintained by the sylvatic cycle transmission⁸. The sylvatic cycle of DENV is maintained by distinct mosquitoes from the urban cycle. There is evidence that *Ae. taylori*, *Ae. luteocephalus*, *Ae. furcifer*, and *Ae. niveus* are responsible for the infection of nonhuman primates in the sylvatic cycle of DENV³⁰. *Ae. aegypti* is considered a domestic mosquito, while *Ae. albopictus* is considered peridomestic, and these mosquitoes will feed on an infected human then infect other people in the urban cycle of DENV infection. *Ae. aegypti* and *Ae. albopictus* are both able to breed within urban areas and are also often found inside. Both species are considered day biters, and are especially active during dawn and dusk³¹. Like DENV, the primary vectors of ZIKV are *Ae. aegypti* and *Ae. albopictus*^{16,32,33}. There are also additional species of mosquitoes associated with the sylvatic transmission of ZIKV. ZIKV was first isolated from *Ae. africanus*². Additionally, *Ae. furcifer*, *Ae. luteocephalus*, and *Ae. taylori*, have been identified as potential vectors for the sylvatic cycle of ZIKV^{16,34}. Collection of mosquitoes within ZIKV endemic areas has shown that many different species of mosquitoes are PCR positive for ZIKV including mosquitoes within the genera *Culex* and *Anopheles*^{35,36}. Although *Culex spp.* of mosquitoes are the primary vector of WNV, *Culex* or *Anopheles* mosquitoes are not capable of the transmission of ZIKV^{32,33,36}.

The biology of the vectors has implications on control of ZIKV and DENV infection. Reduction of mosquito populations has been used to control Flavivirus infections and the use of repellents and insecticides is effective at preventing mosquito-borne infections^{37,38}. Elimination of breeding sites and killing of adult female mosquitoes by dusting with pesticides within homes has also been found to control outbreaks³⁹. As climate change progresses, the predicted range of

the *Aedes* mosquitos is expected to increase. Modeling suggests that as countries north of the tropics continue to warm, there will be increased rates of DENV infection^{40,41}.

Vector Independent Transmission of ZIKV

Although ZIKV is primarily transmitted by *Aedes* mosquitoes, there is evidence of other routes of transmission. As with most Flaviviruses, there is risk of ZIKV transmission by contaminated blood products and because ZIKV can cause an asymptomatic infection, screening of blood products is necessary to fully prevent disease transmission⁴². ZIKV RNA has been isolated in vaginal secretions as well as semen both early in infection and following clearance of viremia^{43–45}. There have been recoded cases of sexual transmission of ZIKV, where a traveler returns to a non-endemic area and infects their partner following unprotected intercourse^{44,46}. Both male to female and female to male transmission of ZIKV have been recorded^{44,46,47}. There is also a recorded case of non-sexual person to person transmission of ZIKV, where the virus was likely transmitted by close contact to an infected individual by contact with their saliva or tears⁴⁸.

Dengue Fever and Severe Dengue

Infection with DENV can result in a range of illness, ranging from an asymptomatic infection to a severe infection associated with hemorrhagic fever^{22,49}. About 75% of DENV infections result in no clinical illness⁵⁰. Of those who experience symptoms, DENV infection typically results in a sudden onset fever accompanied by rash, joint and muscle pain, headache and nausea. Typical dengue fever is not a fatal illness and is self-limiting with symptoms normally lasting 3-5 days^{22,49}. DENV infection can also result in a more severe form of infection called severe dengue (which was formerly known as dengue shock syndrome and dengue hemorrhagic fever)²². Severe dengue tends to affect children and begins with a sudden-onset

fever that is similar to typical dengue fever. In severe dengue, after the fever subsides, there are signs of circulatory failure or hemorrhage. Severe dengue is fairly rare compared to dengue fever, with less than 5% of dengue cases progressing to severe dengue⁵⁰. In a WHO report, of 1.6 million cases of dengue fever, 49,000 of those cases were considered to be severe dengue⁵¹. Vascular leak syndrome during severe dengue can lead to shock and death. The vascular leakage is caused by cytokines produced during viremia, and the secreted NS1 is thought to also play a role^{52,53}.

There are no antivirals available to treat DENV infection. During severe dengue, viremia has been cleared as the shock is caused by cytokines released during the viremia, not a direct effect of viral replication. During dengue fever or severe dengue symptoms are managed with supportive treatment of fluids and plasma transfusion. Despite the lack of a specific treatment of severe dengue, the case fatality rate drops from more than 20% to less than 1% with treatment at an experienced hospital^{22,54}.

Zika disease

ZIKV infection results in a disease similar to dengue fever. About 18% of people who are infected with ZIKV experience clinical symptoms, based on a study of the ZIKV outbreak on Yap island¹⁵. Typical symptoms of ZIKV infection are rash, fever, and joint and muscle pain¹⁶. Typically ZIKV infections are mild and self-limiting, although individuals with serious complicating conditions have died from ZIKV infection⁴⁸. ZIKV infection is also associated with a number of serious complications. Adults who are infected with ZIKV have developed Guillain-Barré syndrome following clearance of viremia^{17,55,56}. An analysis of 11 outbreak locations found that approximately 2 of every 10,000 ZIKV infections resulted in Guillain-Barré syndrome⁵⁶.

ZIKV has also been identified as a teratogen, which is the main driver of the public health response to the virus. Children born to mothers infected with ZIKV while they are pregnant have increased risk of developing a neurological or ocular abnormality called congenital zika syndrome. There are reports of miscarriage, as well as death shortly after birth, of fetuses or children that were positive for ZIKV by qPCR, however there is not a firm epidemiological link between ZIKV and fetal death⁵⁷⁻⁶⁰. The most common ZIKV associated birth defect is microcephaly^{59,61,62}. Of the confirmed cases of ZIKV infection in the U.S. Zika Pregnancy and Infant Registry, 6% of all births had microcephaly⁶². The risk of microcephaly caused by ZIKV infection during pregnancy is estimated to be 0.8% to 17%, depending on the case definition of ZIKV infection^{63,64}. The epidemiologic findings of microcephaly have been supported by neurological abnormalities in mice, ferrets and primates in experimental infections⁶⁵⁻⁶⁸. The ultimate effect of microcephaly on the children is not yet understood, and further study into children with congenital Zika syndrome, as they age, is required.

Animal models

The main animal models of both ZIKV and DENV are NHP and immunodeficient mice. Both ZIKV and DENV have a sylvatic cycle with NHP as a natural reservoir, so the viruses are naturally able to replicate within NHP. Rhesus macaques, cynomolgus macaques, pigtail macaques, and marmosets have been used as models of ZIKV and DENV infection⁶⁹⁻⁷⁴. NHP, when infected with ZIVK or DENV, will experience viremia but not the typical symptoms of zika or dengue fever. For example, when rhesus macaques are infected with ZIKV, they experience peak viremia approximately 2 to 3 days post infection, with between 10^6 and 10^8 copies ZIKV RNA/mL plasma and rhesus macaques infected with DENV will experience peak viremia 5 to 7 days post infection, with between 10^5 to 10^7 copies DENV RNA/mL plasma^{70,73}.

Despite high levels of viremia, there are no clinical signs of infection: macaques do not experience fever, lethargy, or skin rash⁷⁵. When DENV-convalescent NHP or NHP given passive transfer of DENV IgG are infected with a heterologous serotype of DENV the animals will experience enhanced DENV viremia but not the life threatening symptoms of dengue hemorrhagic fever^{76–78}. In macaques infected with ZIKV, RNA can be detected in the lymph nodes, spleen, brain, within the vaginal tract and is secreted in the urine, saliva, and semen^{70,75}. Additionally, if pregnant primates are infected with ZIKV, there is prolonged viremia and transfer of the virus to fetal tissues⁷⁹.

In addition to NHP models of infection, many mouse models of ZIKV and DENV infection have been developed⁷³. Wild type mice do not support DENV or ZIKV viremia, although immune-competent mouse models can be used to study some aspects of infection^{73,80–82}. Mice with genetic defects in innate immune responses will support DENV and ZIKV viremia, and will display clinical signs following infection^{80–82}. Mice that are deficient for IFNAR1/2, or STAT2, or deficient for IRF3, IRF5 and IRF7 together will support ZIKV or DENV replication and will have fever, weight loss and eventual death following infection^{73,80–82}. Additionally mice treated with IFNAR1/2 neutralizing antibody support viremia similar to knockout mice⁶⁵. Humanized SCID mice have also been used to study DENV infection, and will die following viral infection⁸³. ZIKV infected immune-compromised mice that are pregnant will transmit the virus to their fetuses, resulting in microcephaly, fetal absorption, or fetal demise^{65,84}.

Both NHP and mouse models of ZIKV infection have drawbacks, and neither fully replicates the effects of ZIKV and DENV infection in humans. NHPs experience an asymptomatic infection but do not experience disease symptoms that 30% of people experience. Wild-type mice do not support infection while the majority of Type I IFN deficient mice die

following infection, an outcome that is rare in DENV infection and less common in ZIKV infection. Mice do allow for the screening of potential antiviral compounds or vaccines since death is a measurable outcome, while NHP allow the investigation of the natural immune response to infection.

The structural similarities between ZIKV and DENV

ZIKV and DENV are phylogenetically closely related and cluster together when phylogeny is based on the E-protein or the polyprotein as a whole (Figure 3)⁸⁵⁻⁸⁷. ZIKV and DENV have similar nucleotide sequences, with up to 55% amino-acid sequence identity between ZIKV and DENV-2, with some sequences of the envelope, such as the fusion loop, sharing high levels of identity (Figure 4)^{85,88,89}. Along with similar genomes, ZIKV and DENV have similar protein structures (Figure 5). Crystal structures of the viral envelope of ZIKV are similar in structure to DENV: the root mean square deviation between equivalent C-alpha atoms of the E and M proteins between crystal structures of ZIKV and DENV-2 was 1.8 angstroms, with a maximum detected distance of up to 8 angstroms^{90,91}. Similarly, the crystal structures of ZIKV and DENV-3 non-structural proteins possess root mean square deviations between equivalent C-alpha atoms of 1.1 angstroms⁹².

The T cell responses to ZIKV and DENV

DENV infection induces a cellular immune response of both CD4+ and CD8+ T cells which is implicated in viral clearance^{49,93}. The CD4+ and CD8+ T cell responses target a variety of epitopes on both structural and nonstructural genes, although non-structural genes have the highest T cell immunogenicity⁹⁴⁻⁹⁶. In mice lacking Type I Interferon responses, CD8+ T cells have been shown to reduce disease severity due to primary DENV infection⁹⁷. Another study found that depleting CD8+ T cells, but not CD4+ T cells, resulted in increased death following

lethal DENV challenge⁹⁸. Due to the antigenic similarity of the four serotypes of DENV, many of the T cells induced by infection with one serotype have been found to be cross-reactive with other serotypes^{99,100}. During secondary infection, pre-existing cross-reactive T cells are not protective, although they are activated and amplified by the infecting heterologous virus^{69,101}. Interestingly, during secondary infection, pre-existing cross-reactive T cells preferentially expand compared to homotypic naïve T cells due to original antigenic sin¹⁰¹. During a secondary infection that leads to severe dengue, there is production of T cell associated cytokines that leads to the pathogenic consequences of vascular leak syndrome, although the full role of T cells in the enhancement of DENV disease is not fully understood^{8,49,102}.

ZIKV infection also induces a CD4+ and CD8+ T cell response against each ZIKV protein^{70,103}. The structural proteins of ZIKV seem to be the primary targets of the cellular immune response¹⁰⁴. CD4+ T cells to ZIKV structural proteins are detected in individuals and NHPs previously infected with ZIKV^{70,105}. CD8+ T cells in mice are able to kill ZIKV infected cells within the brain, although this cellular death leads to increased damage and pathology¹⁰⁶. Despite the production of ZIKV specific T cells, the humoral response to ZIKV is sufficient for protection from reinfection¹⁰⁷. Due to the similar protein sequence of ZIKV and DENV, ZIKV-induced T cells are able to cross react with DENV^{104,108}. Mice given an adoptive transfer of ZIKV induced CD8+ T cells prior to DENV infection were protected from disease¹⁰⁸.

Overall, both ZIKV and DENV induce potent T cell responses, and these responses are able to protect against reinfection. These T cell responses are also cross-reactive, and able to protect against heterologous virus challenge if amplified through passive transfer, but a naturally produced T cell response is not able to protect from infection with a heterologous virus and secondary T cell responses may be involved in the pathology of severe dengue.

The cross-reactivity of ZIKV and DENV induced antibodies

Antibodies produced during a Flavivirus infection are often able to bind to a number of different Flaviviruses and antibodies induced by ZIKV and DENV are no exception. Serum collected from a number of patients previously infected with DENV was able to bind to lysates from cells infected with ZIKV and plasma from DENV infected individuals bound to whole ZIKV^{109,110}. Additionally, serum from both animal models experimentally challenged with ZIKV as well as humans experiencing clinical infections with ZIKV will bind to DENV in ELISAs^{111,112}.

In addition to cross reactive polyclonal serum, monoclonal antibodies (mAbs) isolated following either ZIKV or DENV infection are often cross reactive between the two viruses. Stettler et al¹¹³ identified NS1 and E protein binding mAbs from both ZIKV and DENV infected individuals with varying levels of cross reactivity. Other mAbs, such as 4G2, a mAb isolated from a mouse infected with DENV, has been shown to be able to bind to ZIKV¹¹⁴.

The cross-neutralizing capacity of ZIKV and DENV induced antibodies

There is a wide consensus that infection with ZIKV results in the production of potentially neutralizing antibodies. This is seen in experimental infection of mice or NHPs as well as in people clinically infected with ZIKV^{111,112,115,116}. Where there is some lack of consensus is whether ZIKV infection can produce antibodies that can neutralize DENV, or vice-versa. Published PRNT values from the ZIKV outbreak on Yap island showed that serum from 10 individuals who experienced ZIKV infection as their first flavivirus infection neutralized only ZIKV and not any serotype of DENV¹¹². Another study followed a single, DENV-naïve, patient in Florida who experienced ZIKV infection. In this study, no neutralization was detected of any serotype of DENV up to 148 days post ZIKV symptoms¹¹⁵.

There is some disagreement in the published literature if DENV infection can result in the neutralization of ZIKV. When ZIKV was initially described, serum from DENV-convalescent individuals in Hawaii was not able to neutralize ZIKV¹. A representative sample of a set of DENV-reactive human plasma from blood donors was tested for its ability to neutralize ZIKV¹¹⁷. Plasma samples that were considered low DENV-binding were unable to neutralize ZIKV while plasma samples that were considered high DENV-binding displayed some ZIKV-neutralizing capability at a 1:20 dilution of plasma. In a study testing the plasma from 16 different individuals previously infected with DENV, 3 were able to neutralize ZIKV, depending on the strain tested¹⁰⁹. Priyamvada et al. found that serum from individuals experiencing an acute DENV infection, or that were DENV convalescent, was able to neutralize ZIKV¹¹⁰.

Further studies have investigated the cross-neutralizing capabilities of monoclonal antibodies (mAb) induced by either ZIKV or DENV infection. Studies where many DENV-induced mAbs are tested have similar results: some but not all DENV-induced neutralizing mAbs have the capability of neutralizing ZIKV^{110,118,119}. Generally, all the cross-neutralizing mAbs bind to the E protein. Studies have isolated mAbs that bind to E domain I, E domain II and also the envelope dimer epitope^{85,110,118}. There were no regions of the DENV E that had mAbs that would consistently neutralize ZIKV; in each study, only a subset of DENV-neutralizing antibodies were also able to neutralize ZIKV. Interestingly, three different DENV-induced mAbs that bind to the fusion loop epitope that are capable of neutralizing all four serotypes of DENV were not able to neutralize ZIKV⁸⁵. A number of these DENV-induced mAbs that are able to neutralize ZIKV have been shown to protect mice from lethal ZIKV challenge^{118,119}. ZIKV-induced mAbs have also been shown to have the ability to neutralize DENV. Of a set of ZIKV-induced mAbs that bind to E domain I, E domain II and E domain III, a portion of mAbs that

bound to each domain of E were able to neutralize each of the serotypes of DENV¹¹³. Another study found that mAbs generated by vaccination with ZIKV E domain III were able to neutralize DENV-1 as well as ZIKV¹²⁰.

Overall, there is some conflicting evidence, but natural infection results in a poorly cross-neutralizing polyclonal antibody response following ZIKV or DENV infection. Within this response there are antibodies that are induced by epitopes on either ZIKV or DENV that are immunogenic and will produce a cross-reactive response. Due to the lack of polyclonal cross-neutralization these cross-neutralizing epitopes are likely only a small part of the overall humoral response, however, harnessing these cross-neutralizing responses could result in potent vaccine targets.

DENV-induced antibodies enhancing ZIKV

During epidemiologic studies in Thailand in the 1970s, an association was found between dengue disease severity and secondary infection, where an individual currently hospitalized for dengue was more likely to be experiencing a secondary DENV infection¹²¹. Further studies found that if DENV-convalescent serum was diluted and incubated with DENV, then monocytes could be infected at higher rates, leading to the hypothesized mechanism of antibody-dependent enhancement (ADE) (Figure 6). In ADE, antibodies against the first serotype of DENV bind to the heterologous infecting virus, leading the virus to be endocytosed by FC receptor expressing cells causing an enhanced rate of infection of phagocytes and an increased plasma viral load^{122,123}. This hypothesis has been supported by a number of experiments *in vivo* (monkeys receiving passively transferred antibody against one serotype of DENV will lead to enhanced viremia) as well as *in vitro* (infection of monocytes can be enhanced in the presence of DENV convalescent serum)^{76,78,123}. The mechanism of *in vitro* enhancement is well understood due to

the simplicity of the system but the mechanism of enhanced *in vivo* DENV infection has not been fully elucidated. Although ADE has not been definitely proven as the causative mechanism of enhanced DENV infection, many further studies have identified the presence of heterotypic DENV antibodies as a risk factor of severe dengue infection^{124,125}. As described above, infection with ZIKV or DENV results in a cross-reactive antibody response that does not potentially neutralize, leading to the hypothesis that DENV-induced antibodies could lead to enhancement of ZIKV infection.

A mAb binding to DENV, 4G2, is able to enhance DENV infection of monocytic cells, and when tested with ZIKV was able to significantly enhance the rate of ZIKV infection as well¹¹⁴. Additionally serum collected from DENV convalescent non-human primates^{111,126}, plasma¹⁰⁹ or serum^{110,127} collected from DENV convalescent individuals or mAbs isolated from DENV-convalescent individuals^{110,113,127} are able to enhance ZIKV infection of monocytic cell lines. Overall, there is little conflicting evidence that DENV-induced antibodies are able to enhance infection of ZIKV *in vitro*.

Currently, the exact causative mechanism of severe dengue is not known so results of an *in vitro* ADE assay cannot be used to extrapolate the risk of severe dengue. Instead other methods such as animal models or epidemiological studies are required. There have been a number of animal studies seeking to determine if ZIKV infection could be enhanced by the presence of DENV-induced antibodies. One of these studies passively transferred human plasma into Stat22^{-/-} mice then challenged with ZIKV¹¹⁷. Mice receiving antibodies that bound to DENV were more likely to die than mice receiving PBS, indicating an enhanced disease due to ZIKV. Rhesus macaques that had previously been infected with DENV were challenged with ZIKV and in two separate studies there was no detectable change in ZIKV viremia between the DENV

478 convalescent and naïve animals^{111,126}. Additionally, an epidemiologic study conducted in Brazil
479 followed outcomes of ZIKV infection in patients that had been previously exposed to DENV,
480 compared to patients who were experiencing ZIKV as their first Flavivirus infection¹²⁸. There
481 were no differences in disease outcome or level of viremia detected, although only 65 patients
482 were included in this case series.

483 Beyond investigating the ability for DENV-induced antibodies to enhance ZIKV disease
484 in adults, some studies have investigated the transmission of ZIKV to fetuses in the presence of
485 potentially enhancing antibodies. When pregnant wild type mice were infected with ZIKV, there
486 were higher rates of ZIKV transmission to the fetus in mothers that were DENV-immune¹²⁹.
487 Passive transfer of 4G2 alone also resulted in increased transmission. Additionally, the fetuses of
488 Stat22-/- mice had higher ZIKV viral loads if the mother was given a passive transfer of human
489 plasma that was seropositive for DENV¹³⁰. Supporting the role of DENV-induced antibodies in
490 transmission of ZIKV to the fetus during infection, it was found that the rate of infection of
491 trophoblasts was increased in the presence of DENV-binding antibodies¹³⁰.

492 When weighting the conflicting evidence of enhanced ZIKV infection the strength of the
493 different models must be considered. The model of *in vitro* enhancement is a simple system
494 compared to a human, as there is only a single type of cell that has been immortalized, being
495 infected with ZIKV or DENV. Mice are not typically susceptible to DENV or ZIKV infection so
496 when used in infection models, mice typically need to have a portion of their immune system
497 knocked out. Additionally, for the mice to experience enhanced ZIKV infection, they needed
498 passive transfer of human serum given to them IP immediately prior to infection. Rhesus
499 macaques are susceptible to ZIKV infection without modifications to their immune system
500 although they only support viremia and do not experience disease^{70,71,131}. Since NHPs can

survive infection with ZIKV or DENV, the post infection state is more similar to that of a human than that of an immune deficient mouse injected with antibody. Of course, humans are the most accurate type of ZIKV infection compared to any other model. When considering epidemiological data of enhanced ZIKV or DENV, it is important to consider the number of humans studied, as there is a great deal of variation within human populations and infections do not happen within a controlled environment. It is also important to consider how common a potential outcome is, especially when attempting to produce data showing an outcome does not happen. The epidemiologic study stating the lack of enhanced ZIKV following DENV infection is done in a single population with a very small scale as simply a case series. The evidence for enhanced ZIKV has been produced using either a simplistic cell culture system or an animal model that is unable to accurately represent infection, whereas the evidence stating the lack of enhancement in an animal model unable to accurately capture ZIKV disease or a small scale case series. Evidence from the more accurate models of infection does not support the existence of an enhanced ZIKV phenotype however there is not definitive evidence that this phenotype does not happen. The studies of enhanced ZIKV in pregnant mice do provide an alternate outcome to enhanced ZIKV infection, where preexisting DENV antibodies result in higher rates ZIKV transmission to fetuses. Further studies of how DENV-induced antibodies interact in natural infections will be required to fully understand the potential epidemiological consequences.

ZIKV-induced antibodies enhancing DENV

Since infection with ZIKV has been shown to induce a cross reactive antibody response, that does not neutralize DENV, a number of studies have investigated the ability of ZIKV-induced antibodies to enhance later DENV infection. The first evidence that this was possible came from testing serum from mice exposed to ZIKV for its ability to enhance DENV-2 *in vitro*,

which lead to an increase in the rate of infection of THP-1 cells¹¹⁶. Another group tested the ability of mAbs isolated from ZIKV infected individuals to enhance DENV-1 infection, and some of the tested antibodies enhanced the rate of DENV-1 infection of K562 cells¹¹³. However, human serum collected over the course of a human's infection with ZIKV was unable to enhance DENV-2 infection of K562 cells but was able to enhance the infection of ZIKV¹¹⁵.

The meaning of this conflicting evidence is difficult to parse apart, as the system of *in vitro* enhancement is very simple: antibodies bind to DENV and likewise bind to the Fc-receptor on the surface of the monocytic cell line allowing the virus to enter and subsequently infect the cell. As covered above, there is agreement in the published literature that ZIKV infection leads to DENV-binding antibodies so one would expect that serum from a ZIKV infected individual, which would contain DENV-binding antibodies, would be able to enhance DENV infection *in vitro*. This is what is seen from both mouse and macaque serum, however human serum did not consistently enhance infection despite being shown to contain DENV-binding antibodies.

There is less published evidence of the *in vivo* enhancement of DENV by ZIKV induced antibodies. A recent study investigated the risk of enhanced DENV in mice born to ZIKV-convalescent mothers¹³². When infected with DENV-2, pups born to ZIKV-convalescent mothers experienced significantly increased disease symptoms, compared to pups born to naïve mothers, and all of pups died within 6 days post infection. Additionally, passive transfer of antibodies from ZIKV-convalescent mice to pups resulted in increased viral load following DENV-2 challenge, indicating that ZIKV-induced antibodies are sufficient for the enhancement of DENV. There is currently no epidemiological evidence of the occurrence of severe dengue following ZIKV infection. Without the evidence of this outcome in humans it is difficult to exactly extrapolate risk seen in mice and likely further, large scale, epidemiological studies will be

needed to fully understand if those who are ZIKV convalescent are at greater risk of severe dengue.

The nature of enhancing antibodies

For an antibody to cause ADE, it must bind to an infectious virion. Antibodies binding to nonstructural proteins will not cause enhancement, which has led to development of DENV vaccines that target NS1 to avoid the possibility of enhancement^{52,133}. Antibodies that bind to DENV prM or domains I or II of the E protein are poorly neutralizing and highly cross reactive, leading to promotion of enhancement¹³⁴. Antibodies that bind the E domain III of DENV are potently neutralizing and often cross-neutralizing against other serotypes of DENV, but when these antibodies are diluted beyond neutralizing titers they will cause enhancement^{134–136}. There is also evidence that the interaction between enhancing antibody and FcR may have a consequence on DENV disease severity. Humans have distinct FcR subtypes, and multiple subtypes have been implicated in enhancement of DENV¹³⁷. Infection of cell lines has found that FcRI and FcRII are capable of ADE of DENV^{138–140}. Individuals experiencing enhancement of DENV had higher levels of FcRIIIA binding IgG compared to individuals experiencing secondary dengue without complication¹⁴¹. Antibodies that interact strongly with FcRs, such as afucosylated Fc glycans and the subclass of IgG1 have a higher capacity for enhancement of DENV¹⁴¹.

Neutralization and ADE

An individual with high neutralizing titers against DENV or ZIKV will be protected from infection, as neutralizing antibodies are considered a primary correlate of protection from Flavivirus infection^{142–144}. However, as these protective neutralizing antibodies are diluted, they become able to cause enhancement. This is exemplified by maternally transferred antibody.

While antibodies transferred from a DENV-convalescent mother to her child are initially neutralizing and protective, as the child ages the concentration of the antibodies lowers and the child becomes at risk for severe dengue, until the DENV-binding antibody levels decrease further^{136,137,145}. This leads to severe dengue in children less than one year of age who are experiencing a primary dengue infection, as described by Halstead et al.¹²¹. If neutralizing mAbs are diluted *in vitro*, they can enhance DENV infection on monocytes^{134,135}. Since there is no evidence of production of potentially cross-neutralizing antibodies following ZIKV or DENV infection, secondary infection can result in a risk of enhancement.

Conclusions

Overall there is a great deal of agreement that infection with either ZIKV or DENV results in an antibody response that can bind to both viruses. There is some evidence that these antibodies can cross-neutralize however most models of infection result in neutralization of only the infecting virus. DENV induced antibodies consistently enhance ZIKV *in vitro*, however only mouse models of ZIKV infection show enhancement by DENV antibodies. Conversely, only some studies have shown enhancement of DENV by ZIKV induced antibodies, and tested ZIKV-convalescent human serum has been unable to enhance DENV, but when ZIKV antibody was transferred to mice which were then challenged with DENV there was enhanced viremia and death. Further epidemiologic studies will be required to determine if there will be risks of enhanced ZIKV or DENV in human populations due to the ZIKV outbreaks that were seen in the past years.

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925 **Figure 1: The Flavivirus genome and polyprotein structure**

926 The linear order of the positive sense RNA genome is presented (A) The three genes of the 5'
927 end of the genome are structural genes, while the remaining genes are non-structural. The
928 genome is translated as a single polyprotein (B). The polyprotein is cleaved after translation into
929 functional proteins.

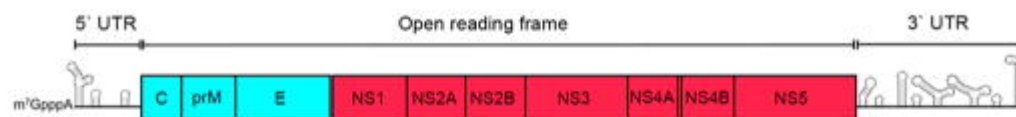
930

931 Reprinted with permission from Barrows, Nicholas J., et al. "Biochemistry and molecular
932 biology of Flaviviruses." *Chemical reviews* 118.8 (2018): 4448-4482. Copyright 2018 American
933 Chemical Society.

934

Figure 1:

A



B

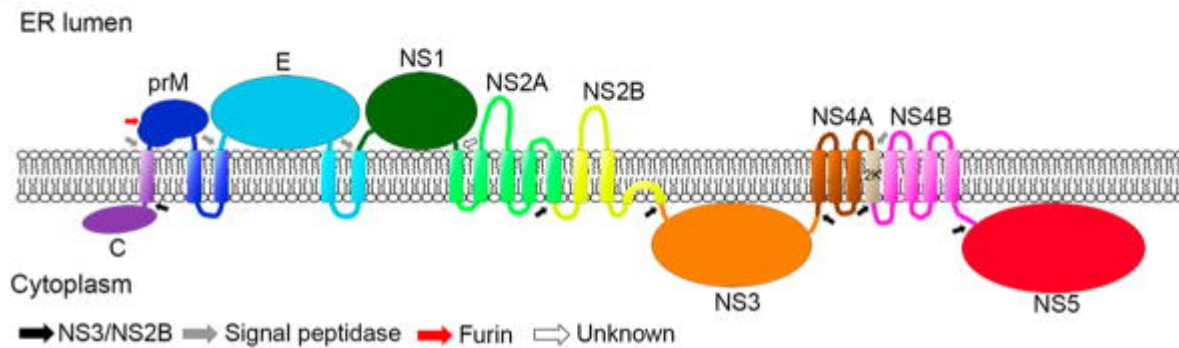


Figure 2: The global range of ZIKV and DENV

Countries where there is autochthonous (endemic) transmission of ZIKV (A) or DENV (B) are shown in red. There is transmission of both ZIKV and DENV across the tropics and many countries have reported transmission of both viruses. Northern countries are not at risk for either ZIKV or DENV since *Aedes* mosquitoes are unable to live at higher latitudes.

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Figure 2A:

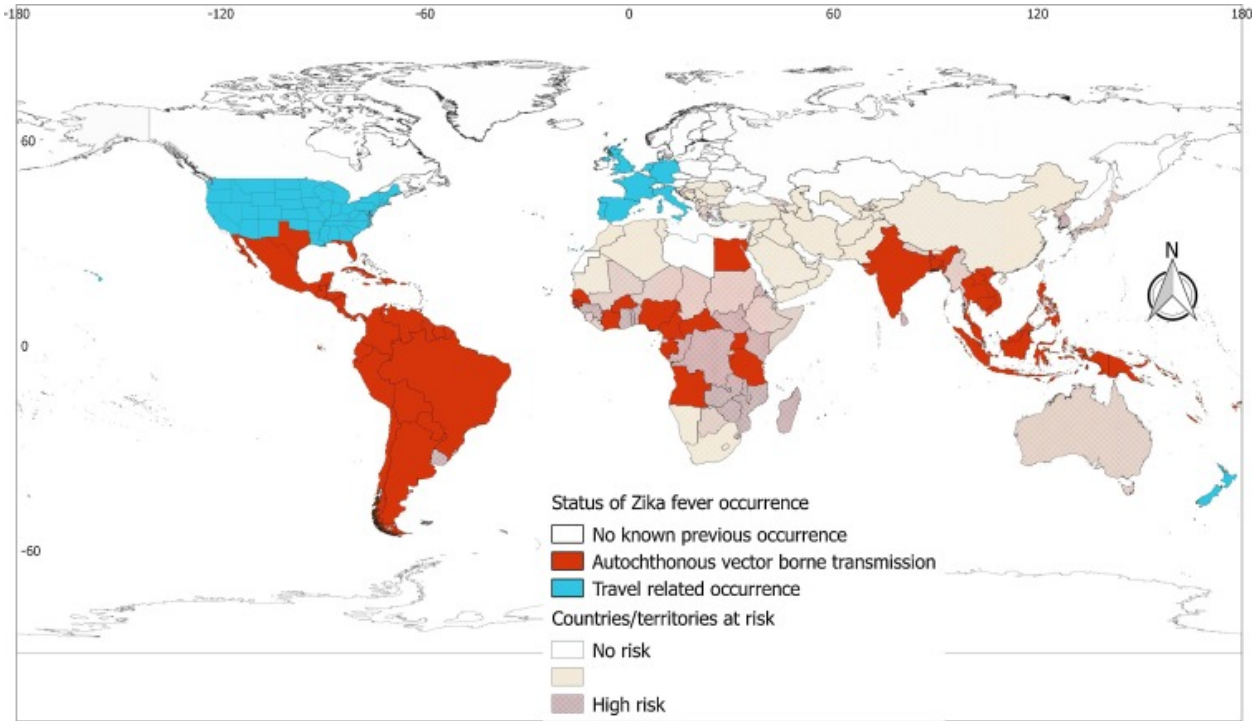


Figure 2B:

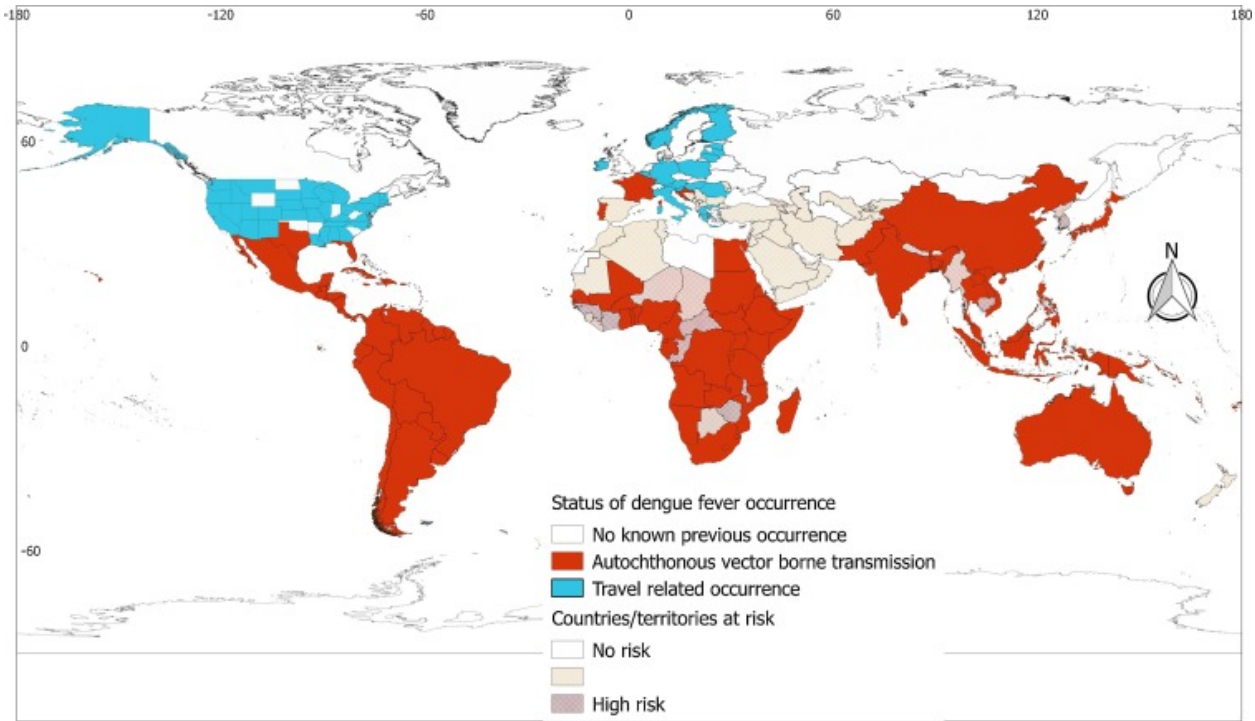
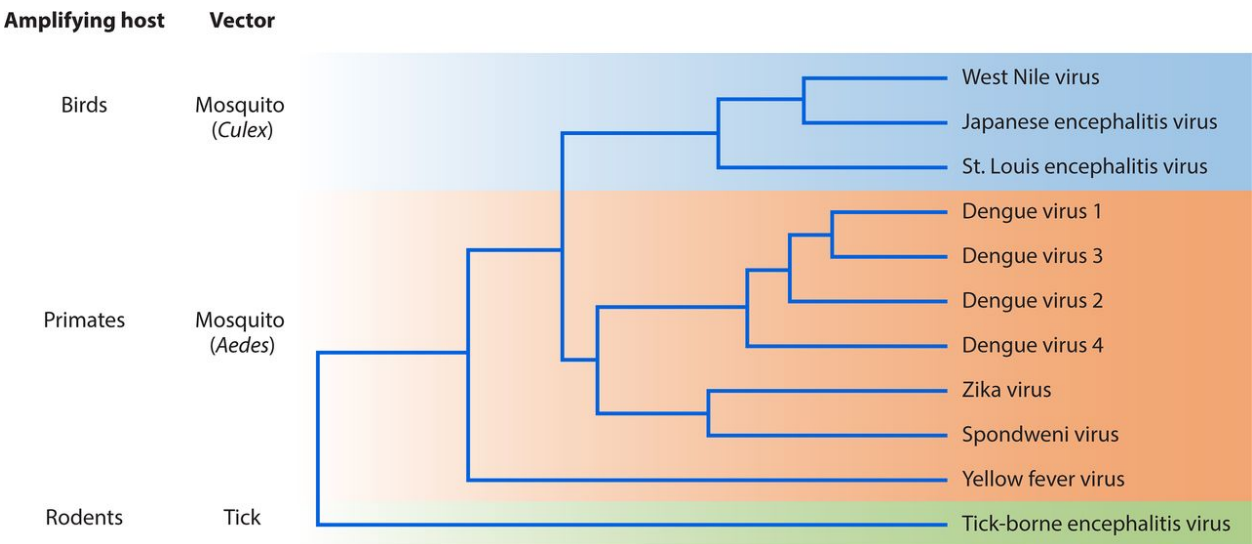


Figure 3: The phylogenetic relationship of Flaviviruses

The genetic relationship of selected Flaviviruses as determined by the amino-acid sequence of the polyprotein. ZIKV and DENV, along with other human pathogens transmitted by *Aedes* mosquitoes, are closely related.

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Figure 3:



970 **Figure 4: Conserved amino acid identity of ZIKV and DENV E protein**

971 The amino acid sequences of domain I of the E protein of 4 strains of ZIKV, and well as 1 strain
972 of each serotype of DENV are aligned and presented. There is a high degree of conserved
973 sequences within the 40 amino acids presented. The conserved asparagine glycosylation site is
974 highlighted in blue.

975

976 From Sirohi, Devika, et al. "The 3.8 Å resolution cryo-EM structure of Zika
977 virus." *Science* 352.6284 (2016): 467-470. Reprinted with permission from AAAS.

978

Figure 4:

A



133 ENLEYRIMLSVH-GSQHSGMI---GYETDEDRAKVEVTPNSPR 171
133 ENLEYRIMLSVH-GSQHSGMIVNDTGYETDENRAKVEVTPNSPR 175
133 ENLEYRIMLSVH-GSQHSGMIVNDTGHETDENRAKVEITPNSPR 175
133 ENLEYRIMLSVH-GSQHSGMIVNDTGHETDENRAKVEITPNSPR 175
133 ENLKYSVIVTVHTGDQH--QVGNESTEHE---GTTATITPQAPT 170
133 ENLEYTIVITPHSGEEH--AVGNDTGKH---GKEIKITPQSSI 170
133 ENLKYTVIITVHTGDQH--QVGNETQ-----GVTAEITPQAST 168
133 ENLEYTVAVTVHNGDTH--AVGNDTSSH---GVTATITPRSPS 170

Figure 5: Structure of the DENV and ZIKV Capsid

Dimers of E are assembled into sets of 3 to form 30 “rafts” that form the envelope of the virus.

The arrangement of E dimers on DENV (left) and ZIKV (right) virions is shown, with one of the “rafts” highlighted in white for each virion.

Reprinted with permission Heinz, Franz X., and Karin Stiasny. "The antigenic structure of Zika virus and its relation to other Flaviviruses: implications for infection and immunoprophylaxis." *Microbiol. Mol. Biol. Rev.* 81.1 (2017): e00055-16. Copyright 2017 American Society for Microbiology.

Figure 5:

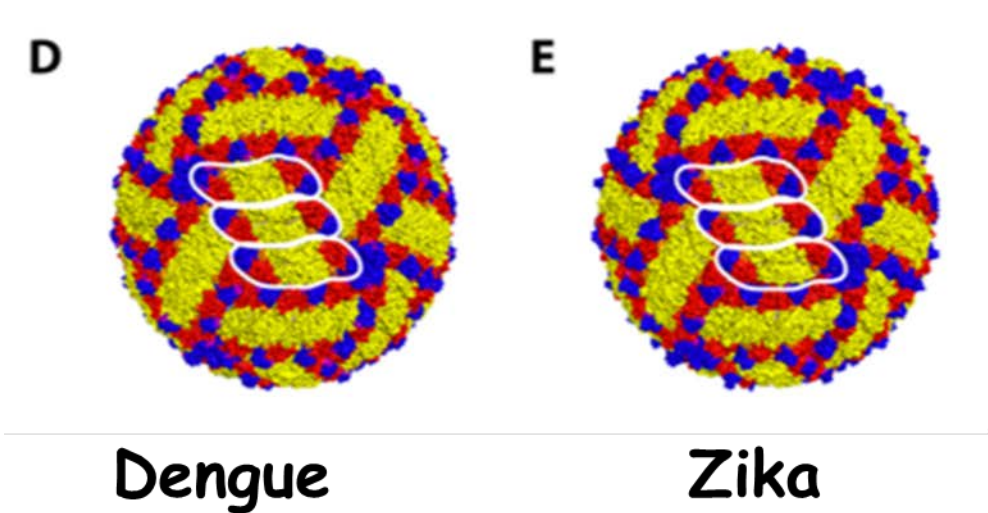
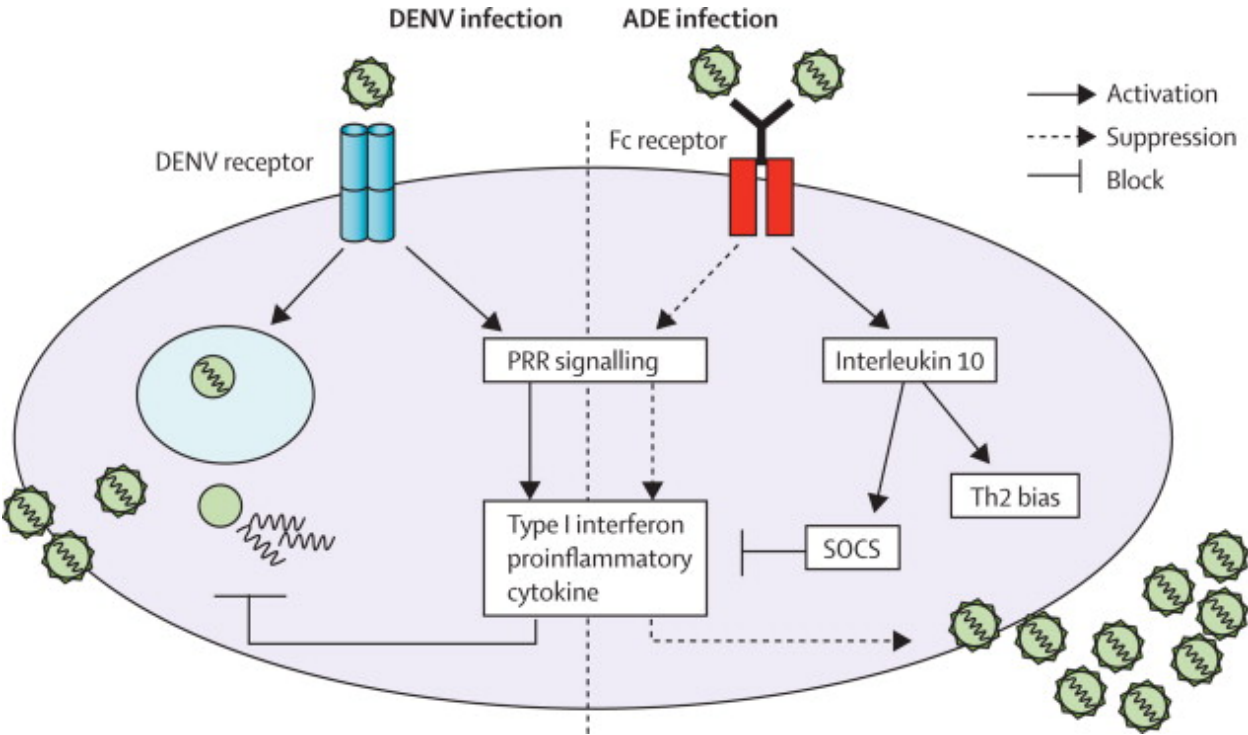


Figure 6: The mechanism of ADE

During typical DENV infection (left) the DENV virion enters the cell by binding to its receptor. Following receptor binding, the virion is endocytosed and goes on to replicate. In ADE (right) the DENV virion is bound by a preexisting cross-reactive antibody, which binds to an Fc receptor on the surface of the cell. Following endocytosis, the virion goes on to replicate within the cell with altered innate immune signaling, leading to increased viral production.

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Figure 6:



1018 **Chapter 2:**
1019 **Prior Exposure to Zika Virus Significantly Enhances Peak Dengue-2 Viremia in Rhesus**
1020 **Macaques**
1021 Jeffy George[#], William G. Valiant[#], Mary J. Mattapallil, Michelle Walker, Yan-Jang S. Huang,
1022 Dana L. Vanlandingham, John Misamore, Jack Greenhouse, Deborah E. Weiss, Daniela
1023 Verthelyi, Stephen Higgs, Hanne Andersen, Mark G. Lewis, & Joseph J. Mattapallil
1024
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1026 [#]These authors contributed equally to this work
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1029 **Published as:**
1030 “George, J., Valiant, W. G., Mattapallil, M. J., Walker, M., Huang, Y. J. S., Vanlandingham, D.
1031 L., ... & Higgs, S. (2017). Prior exposure to Zika virus significantly enhances peak dengue-2
1032 viremia in rhesus macaques. *Scientific reports*, 7(1), 10498.”
1033

Abstract

Structural and functional homologies between the Zika and Dengue viruses' envelope proteins raise the possibility that cross-reactive antibodies induced following Zika virus infection might enhance subsequent Dengue infection. Using the rhesus macaque model, we show that prior infection with Zika virus leads to a significant enhancement of Dengue-2 viremia that is accompanied by neutropenia, lymphocytosis, hyperglycemia, and higher reticulocyte counts, along with the activation of pro-inflammatory monocyte subsets and release of inflammatory mediators. Zika virus infection induced detectable Dengue cross-reactive serum IgG responses that significantly expanded after Dengue-2 virus infection. Serum from Zika virus immune animals collected prior to Dengue-2 infection showed significant capacity for *in vitro* antibody dependent enhancement of Dengue-1, 2, 3 and 4 serotypes suggesting that pre-existing immunity to Zika virus could potentially enhance infection by heterologous Dengue serotypes. Our results provide first *in vivo* evidence that prior exposure to Zika virus infection can enhance Dengue infection, which has implications for understanding pathogenesis and the development of vaccines.

Introduction

Zika virus (ZIKV) is a flavivirus transmitted by *Aedes aegypti* mosquitoes and has recently reemerged as a major public health concern worldwide¹. ZIKV infection causes mild febrile illness in most people but has been associated with microcephaly in infants, and Guillain-Barré Syndrome in adults. Interestingly, the reemergence of ZIKV infection geographically coincides with Dengue endemic areas in South America. Dengue virus (DENV), like ZIKV, is a flavivirus transmitted by the *Aedes* mosquitoes and causes mild, acute infection in most people. However, in a subset of people, secondary exposure to a heterologous serotype has been associated with significant enhancement of infection, which is thought to be mediated by antibodies induced during primary infection against one serotype cross reacting with another serotype of DENV. Antibody dependent enhancement (ADE) is accompanied by the release of pro-inflammatory mediators and vascular leakage leading to dengue hemorrhagic fever (DHF).

Studies have documented that antibodies induced during DENV infection cross react with ZIKV suggesting that antibody responses are induced against shared antigenic epitopes^{2,3}. Others have shown that Zika virus E protein shares ~50% sequence homology with DENV E protein⁴, and significant structural homology between ZIKV and DENV E proteins has been reported to induce conformation dependent antibody responses that were highly cross reactive³. The E protein is a primary target for antibody responses during DENV infection^{2,5}, and a number of ZIKV cross-reactive monoclonal antibodies were found to be specific to the DENV E protein². Recent studies demonstrated that antibodies induced against ZIKV E protein significantly enhanced DENV infection *in vitro* and lethally enhanced DENV disease in mice⁶. Likewise, Kawiecki et al⁷ demonstrated that polyclonal serum from mice infected with ZIKV significantly enhanced DENV replication *in vitro*. Though the potential for ZIKV immune serum to enhance

DENV infection has been clearly shown in these *in vitro* studies, there is little or no evidence to date showing that pre-existing immunity to ZIKV alters the course of DENV infection *in vivo*. We sought to address this question using the rhesus macaque model for ZIKV and DENV infection^{8,9}.

Results

Prior infection with Zika virus significantly enhances Dengue-2 viral loads

The kinetics of both DENV-2 and ZIKV viremia (Fig. 1a-b) were examined using plasma samples that were collected longitudinally at different time points until sacrifice. In line with earlier studies^{8,9}, plasma ZIKV viral loads peaked at ~ 4 – 5 logs/ ml by day 3 post infection (pi) (Fig. 1a) whereas plasma DENV-2 viral loads in ZIKV naïve animals peaked at ~ 4 -5 logs/ ml by day 5 pi (Fig. 1b). Both ZIKV and DENV-2 viral loads declined to baseline levels by 14 days pi.

In contrast to ZIKV naïve animals, however, there was a significant increase in the plasma DENV-2 viral loads of ZIKV immune animals as determined by one-way ANOVA ($F(14,52) = 4.501, p = <0.0001$) and Tukey's post hoc analysis for multiple comparisons; ZIKV immune animals had peak DENV-2 viral loads that was ~1.5 log higher than ZIKV naïve DENV-2 infected animals (Fig. 1b); mean DENV-2 peak viral load was ~113,000 copies/ml of plasma for ZIKV naïve animals whereas it was 5,747,000 copies/ml of plasma for ZIKV immune animals. The extent of increase was similar to earlier studies that examined the kinetics of DENV-2 viremia in macaques that were immune to DENV 1, 3, or 4 serotypes¹⁰ raising the possibility that ZIKV likely mimics a primary DENV infection whereas DENV-2 infection in ZIKV immune animals resembled infection with a heterologous serotype. DENV-2 viremia

declined to baseline levels by day 14 – 28 pi. ZIKV immune animals experienced a significant decrease in body weight and higher body temperatures following DENV-2 infection as compared to ZIKV naïve animals infected with DENV-2 (Fig. 1c). Enhanced viremia in ZIKV immune animals was accompanied by significant neutropenia, lymphocytosis, hyperglycemia, decreased mean cell volume (MCV) along with significantly higher reticulocyte counts as compared to ZIKV naïve DENV-2 infected animals. There were no significant differences between the ZIKV naïve and immune groups of animals in other parameters examined. These findings appear to be in line with earlier studies; Onlamoon et al¹¹ showed that infection of rhesus macaques the same strain of DENV-2 as used in our study was associated with severe leucopenia, neutropenia, platelet counts along with decreased hemoglobin levels and hematocrit values¹¹. Similar observations have been reported in human DENV infection¹²⁻¹⁴. We did not observe either symptoms of rash, conjunctivitis or signs of hemorrhage in our animals.

Enhancement of DENV-2 viral loads is associated with a significant increase in pro-inflammatory cytokine responses

Next we examined if increased DENV-2 viremia in ZIKV immune animals was associated with altered levels of pro-inflammatory cytokines (Fig. 2). Previous studies have reported that enhancement of DENV infection was characterized by the release of inflammatory mediators that was associated with increased pathogenesis^{15,16}.

We observed a significant increase in plasma levels of Interferon-inducible T-cell alpha chemo-attractant (I-TAC), Monocyte chemo-attractant protein-1 (MCP-1), interleukin-8 (IL-8), Macrophage inhibitory factor (MIF), Eotaxin, Macrophage derived chemokine (MDC), Interferon gamma-induced protein-10 (IP-10), Epidermal growth factor (EGF), and Fibroblast growth factor (FGF) in ZIKV immune animals at day 5 – 14 after challenge with DENV-2 as

compared to ZIKV naïve DENV-2 infected animals. The kinetics of inflammatory cytokine responses coincided with the kinetics of enhanced viremia suggesting a role for viral replication in the induction of these cytokine responses. We did not observe significant changes in other cytokines measured (Fig. S1). Studies have reported that DENV infection was associated with significant increases IL-8, MCP-1, I-TAC and numerous other cytokines^{17,18}, whereas others have reported an increase in IL-8 and MCP-1 during primary DENV-2 infection in macaques¹⁹. A significant increase in pro-inflammatory mediators was, however, not accompanied by overt symptoms such as rash, hemorrhage etc. that are usually associated with DHF which may be related to the limitations of the rhesus macaque model as some studies have shown^{10,20-22}.

CD14⁺ and CD14⁺CD16⁺ monocytes are significantly activated in Zika immune animals after Dengue infection

Monocyte/macrophages have been shown to be the primary target cells for DENV infection^{23,24}, and a major source of pro-inflammatory cytokines²⁵. To determine if the enhancement of DENV viremia in Zika immune animals was accompanied by changes in monocyte/macrophage subsets, we examined the proportion and activation state of these cell subsets in peripheral blood samples from ZIKV immune animals that were collected longitudinally prior to and after DENV-2 infection; samples were collected at day 0, 5, 56 after ZIKV infection prior to DENV-2 infection, and at day 63 (day 7 post-DENV-2) and day 112 (day 56 post-DENV-2) after ZIKV infection and compared to ZIKV naïve DENV-2 infected animals (Fig. 3a). Monocyte/macrophage subsets were discriminated based on the expression of CD14 and CD16 on Lin⁻HLA-DR⁺CD20⁻CD11c/123⁻ myeloid cells (Fig. S2) and divided into classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), non-classical (CD14⁻CD16⁺), and macrophage (CD14⁻CD16⁻) subsets as described previously²⁶.

The proportions of CD14⁺CD16⁻ monocytes declined in both ZIKV naïve and ZIKV immune animals after DENV-2 infection that coincided with peak viremia ($F(7,29) = 3.998$; $p = 0.0036$). Decline in CD14⁺CD16⁻ monocytes were accompanied by an increase in the proportions of CD14⁺CD16⁺ monocyte subsets ($F(7,29) = 3.307$; $p = 0.0104$). These findings are in line with previous studies showing that CD14⁺CD16⁻ monocyte subsets were lower in DENV patients whereas CD14⁺CD16⁺ subsets increased as compared to healthy controls²⁷. Others have shown that CD14⁺CD16⁺ monocytes significantly expanded in rhesus macaques after DENV infection²⁸. There was no apparent difference in the proportion of either CD14⁻CD16⁺ or CD14⁻CD16⁻ subsets between the groups.

To assess if monocyte/macrophage subsets were activated during infection, we examined the expression of Toll-like-receptor-4 (TLR-4) on these subsets (Fig. 3b-c). Our results showed that the expression of TLR-4 was significantly upregulated on both CD14⁺CD16⁻ and CD14⁺CD16⁺ monocyte subsets, and on CD14⁻CD16⁻ subsets of macrophages in ZIKV immune animals at day 63 post-ZIKV infection (day 7 post-DENV-2) as compared to day 0 and 56 post-ZIKV infection (time points prior to DENV-2 infection) suggesting that DENV-2 infection was accompanied by a significant activation of inflammatory subsets of monocyte/macrophages. Previous studies have reported that TLR-4 expressing CD14⁺ cells significantly increased in DENV patients^{27,29}, and DENV directly activated human macrophages via TLR-4 leading to the induction and release of pro-inflammatory cytokines, and this effect was inhibited with TLR-4 antagonists, and anti-TLR-4 antibody³⁰. Others have shown that non-classical monocytes (CD14⁺CD16⁺) produce significant levels for pro-inflammatory cytokines³¹.

ZIKV immune animals display high levels of DENV cross-reactive binding antibody responses but show little neutralizing activity against DENV-2

Previous studies have shown that subneutralizing concentrations of DENV specific antibodies enhanced DENV infection in humans³². To determine if ZIKV infection induced DENV-2 cross-reactive neutralizing antibody (nAb) responses, we examined the potential of serum collected from ZIKV immune animals prior to infection with DENV-2 (Group B; day 56 post-ZIKV infection), and from ZIKV naïve DENV-2 infected animals (Group A; day 56 post-DENV-2 infection) to neutralize ZIKV and DENV-2 using plaque reduction neutralization antibody (PRNT) assays (Fig. 4a). Pre-ZIKV (Group B) and pre-DENV-2 (Group A) serum were used as negative controls.

As expected, we observed high PRNT₅₀ and PRNT₉₀ nAb titres against DENV-2 but not against ZIKV (PRNT_{50/90} = or <10) in the ZIKV naïve DENV-2 challenged animals (Group A). Likewise, ZIKV immune animals (Group B) displayed significantly high nAb responses against ZIKV but not against DENV-2 (PRNT_{50/90} = or <10) suggesting that ZIKV infection induced either little or no detectable DENV-2 cross-reactive nAb responses at the time points examined. These findings are in line with previous studies showing little or no DENV-2 specific nAb titres (PRNT₉₀ = or <10) in primary infection with ZIKV³³. Likewise, Collins et al³⁴ recently showed that little or no durable cross neutralizing antibodies against ZIKV were induced by DENV infection.

To determine if ZIKV infection induced high levels of cross-reactive non-nAb responses against DENV, we examined the relative levels of DENV specific IgG in serum from both ZIKV naïve DENV-2 immune (Group A; day 0 and 56 post DENV-2) and ZIKV immune animals (Group B; day 0 and 56 pi prior to DENV-2 infection and at day 7 after DENV-2 infection) using a DENV specific ELISA assay for serum IgG³⁵. Significant levels of anti-DENV IgG were readily detectable in the serum of both groups of animals (Fig. 4b) suggesting that ZIKV

infection induced significant levels of anti-DENV specific cross-reactive antibodies. The levels of DENV specific IgG was in line with what has been previously reported in DENV-2 infected rhesus macaques at the similar time points after infection¹⁹. Anti-DENV IgG titres significantly increased in ZIKV immune animals at day 7 after challenge with DENV-2 (Fig. 4b; day 63 post ZIKV infection) suggesting that DENV infection was associated with a significant expansion of DENV-2 cross-reactive anamnestic B cell responses.

Serum from ZIKV immune animals display significant *in vitro* capacity to enhance infection by heterologous DENV serotypes

Previous studies have shown that polyclonal serum from ZIKV infected mice significantly enhanced DENV-2 replication *in vitro*⁷ whereas Stettler et al⁶ reported that antibodies induced against ZIKV E protein though poorly neutralizing significantly enhanced DENV infection in mice. To assess if the serum from ZIKV immune animals was capable of mediating ADE, we tested the potential of serum collected from ZIKV immune animals prior to DENV-2 challenge (Group B; day 56 post ZIKV infection) to enhance DENV infection *in vitro* using K562 cells, an Fc receptor expressing leukemia cell line^{36,37}, along with DENV reporter virus particles (RVP)³⁸.

The DENV RVPs are structurally intact Dengue virions that are capable of only a single round of replication and encode a sub-genomic GFP replicon that is expressed following infection of target cells³⁸. The RVPs do not readily infect K562 cells in the absence of DENV specific antibodies. RVPs were obtained from Integral Molecular (Philadelphia, PA) for use in ADE assays. Serum was serially diluted 10-fold and incubated with DENV-1, 2, 3 and 4 RVPs prior to culture with K562 cells. Cells were harvested after 48 hours and the frequency of GFP expressing cells were determined by flow cytometry (Fig. 4c). Concentration of RVP's was

titrated using ZIKV positive serum and K562 cells (Fig. S3). No serum and cells only controls were included in each experiment.

Our results showed that the frequency of K562 cells expressing DENV-1, 2, 3 and 4-GFP were significantly higher in the presence of ZIKV immune serum (Fig. 4d-e) suggesting that ZIKV infection induced cross-reactive antibodies against all four DENV serotypes. The proportion of K562 cells infected with DENV-1 and 2 RVPs that expressed GFP were significantly higher than DENV-3 and 4 RVPs suggesting that ZIKV infection likely induced antibodies that preferentially cross-reacted with DENV-1 and 2 serotypes as compared to the other two serotypes (Fig. 4e). Interestingly, except for DENV-1, the fold enhancement was highest at 1:10 dilution of serum (Fig. 4d), as compared to either neat or lower serum dilutions, suggesting that sub-concentrations of antibodies equally enhanced infection of DENV-2, 3 and 4 serotypes.

Discussion

ZIKV is an emerging pathogen that is circulating in DENV endemic areas. Structural similarities between ZIKV and DENV E proteins raises the concern that cross-reactive antibody responses induced by ZIKV especially in DENV naïve populations could potentially lead to more severe pathogenic outcomes following primary infection with DENV. Our findings provide *in vivo* evidence that prior exposure to ZIKV significantly enhances primary DENV-2 viremia that was accompanied by leucopenia, neutropenia, increased reticulocyte counts, high blood glucose and thrombocytopenia, changes that are usually associated with severe DENV infection. These changes were accompanied by a significant activation of monocyte subsets and secretion of pro-inflammatory cytokines, a majority of which chemoattract monocyte/macrophages.

Numerous studies have demonstrated a key role for activated monocyte/macrophages in Dengue pathogenesis^{13,25}.

ZIKV infection induced high levels of DENV cross-reactive antibodies with little or no neutralizing activity against DENV-2. This was, however, not surprising as previous studies have reported little or no DENV-2 specific nAb titres during primary infection with ZIKV³³. Serum from ZIKV infected animals were, however, found to induce significant *in vitro* ADE of DENV-2 suggesting that the *in vivo* enhancement of DENV-2 viremia we observed may be mediated by non-neutralizing or sub-neutralizing concentrations of DENV-2 cross-reactive antibody responses. Interestingly, these antibody responses were found to enhance infection of other heterologous serotypes such as DENV- 1, 3 and 4 *in vitro* suggesting that pre-existing ZIKV specific immune responses could potentially to enhance infection with all serotypes of DENV. Additional studies are needed to determine if prior exposure to ZIKV enhances infection with DENV- 1, 3 and 4 *in vivo*. Though it is difficult to determine the exact mechanisms that may have contributed to the *in vivo* enhancement of DENV-2 viremia, our results suggest that ADE could play a role.

Though the ZIKV exposed animals experienced significant enhancement of viremia and release of pro-inflammatory mediators, why they did not display symptoms associated with DHF is not clear. There is no prior evidence of enhanced viremia with Dengue infection in rhesus macaques except for one study that reported significantly high plasma viremia and symptoms of hemorrhage in rhesus macaques that were challenged with a high dose of DENV-2 intravenously¹¹. These animals experienced changes in CBC and serum chemistry similar to what we observed in our studies even though the animals in our studies were challenged with a lower dose of DENV-2 subcutaneously. It is probable that the absence of DHF like symptoms is

1256 due to the limitation of the NHP model as has been previously reported³⁹. Though DHF was not
1257 observed in our studies, the significant differences between ZIKV naïve and immune animals
1258 after DENV-2 challenge suggests that prior exposure to ZIKV has the potential to enhance
1259 DENV associated pathogenesis.

1260 Additional and larger studies are needed to determine if the changes we observed in
1261 ZIKV immune animals can be recapitulated with other strains of DENV-2, and if similar
1262 enhancement of DENV-2 occurs when ZIKV immune animals are infected with DENV-2 after
1263 longer periods of convalescence than the 8 weeks used in our study. Likewise, further clinical
1264 and translational studies are essential to better understand the effect of prior ZIKV infection on
1265 the course of DENV pathogenesis in human subjects. In conclusion, the data reported here have
1266 implications for understanding the pathogenesis of both ZIKV and DENV infections especially
1267 in DENV naïve populations, and will aid in the development of better vaccines that can protect
1268 from these infections.

1269

Materials and Methods

Animals, infection and samples

Nine rhesus macaques of Indian origin acquired by Bioqual Inc. (Rockville, MD) were used in this study. All animals were seronegative for ZIKV and DENV. Animals were housed at Bioqual and cared for in accordance with local, state and federal policies in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility. All animal experiments were reviewed and approved by Institutional Animal Care and Use Committee at Bioqual Inc., and samples were acquired through a tissue sharing protocol. The animals were divided into two groups; Group A (n = 4) was infected with only DENV-2 subcutaneously and sacrificed at day 56 post infection, and Group B (n = 5) were first infected with ZIKV subcutaneously first for a period of 56 days and then infected with DENV-2 subcutaneously at day 56 post-ZIKV infection and sacrificed at day 56 post-DENV-2 infection (day 112 post-ZIKV infection). Animals were infected subcutaneously with 10^6 TCID₅₀/ ml of Zika virus (Puerto Rico Strain; Genbank KU501215) and 10^5 TCID₅₀/ ml of DENV-2 virus (strain 16681). The challenge titer was based on previous studies^{9,19}.

Peripheral blood samples were collected longitudinally from animals in Group A and B at day 0, 1, 2, 3, 4, 5, 7, 14, 28 and 56. Additional samples were obtained from animals in Group B at day 57, 58, 59, 60, 61, 63, 70, 84, and 112 following DENV-2 infection. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation and cryopreserved along with plasma and serum at each time point. Cumulative Blood Counts (CBC) and analysis of serum chemistry was performed at IDEXX Laboratories, Inc. (Rockville, MD).

Absolute quantification of plasma viral loads by qRT-PCR

Plasma viral loads were determined using real-time quantitative RT-PCR assay. RNA was extracted from the plasma using the QIAamp MinElute Virus spin kit (Qiagen) and reverse transcribed using a mixture of random hexamers and anchored oligo-dT primers. Synthesized cDNA was PCR amplified using Zika⁴⁰ (forward: GGAAAAAAGAGGCTATGGAAATAATAAAG, reverse: CTCCTTCCTAGCATTGATTATTCTCA, probe: AGTTCAAGAAAGATCTGGCTG) and DENV-2 (forward: CAGGGTGTGGATTCAAGAAAACCCATGG, reverse: TGCTTGTTAACCCAATCAATGAGCC, probe: ACTCCAGTG/ZEN/GAATCATGGGAGGAAATCCCA) specific primers and probes. The PCR reaction was set up in triplicate using Taq-polymerase (Bioline USA, Inc., (Taunton, MA) and assayed in the 7500 Taqman instrument (Applied Biosystems) using the following conditions: 48°C for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 1 minute at 60°C. The number of Zika and Dengue-2 viral copies was determined using standards generated from RNA that was extracted from the Zika and Dengue-2 viral stocks. Viral RNA was quantified and diluted at a concentration of 10⁷ copies and reverse transcribed as described above. Reverse transcribed cDNA was serially diluted to generate a standard curve ranging from 1 copy to 10⁶ copies / reaction. The limit of detection was 50 copies / ml.

Antibody dependent enhancement assay with DENV reporter viral particles

ADE assays were performed using serum from ZIKV exposed animals along with DENV-1, 2, 3, and 4 Reporter Virus Particles (RVP). RVPs³⁸ are replication incompetent viral particles that express GFP after a single round of replication and were obtained from Integral Molecular Inc., (Philadelphia, PA). The serum was heat inactivated at 56°C for 30 minutes, serially diluted 10 fold, and mixed with 10uL of RVP at a ratio of 1:1. After incubation for 1

1315 hour at 37C, 5% CO₂, the serum RVP mix was added to ~80,000 K562 cells in 30ul of RPMI-10
1316 and incubated for 1 hour at 37⁰C, 5% CO₂. After 1 hour, the cells were washed twice with
1317 RMPI-10 to remove unbound virus, and resuspended in RPMI-10 and cultured in a 96 well tissue
1318 culture plate for 48 hours. After culture, cells were washed in 1 x PBS and fixed in 0.5%
1319 Paraformaldehyde and analyzed using an LSR II flow cytometer. Collected data was analyzed
1320 using Flowjo 9.6 software.

1321 **Cytokine levels in plasma**

1322 Cytokine levels were determined using plasma that was collected longitudinally from
1323 each animal. Plasma cytokine levels were examined using the Cytokine Monkey Magnetic 29-
1324 Plex Panel for Luminex™ Platform (Thermofisher Scientific, Waltham, MA). This kit allows
1325 simultaneous quantification of 29 cytokines namely, FGF-basic, IL-1b, G-CSF, IL-10, IL-6, IL-
1326 12, RANTES, Eotaxin, IL-17, MIP-1α, GM-CSF, MIP-1β, MCP-1, IL-15, EGF, IL-5, HGF,
1327 VEGF, IFNγ, MDC, I-TAC, MIF, IL-1RA, TNFα, IL-2, IP-10, MIG, IL-4 and IL-8. Briefly,
1328 plasma samples were diluted 1:2 in assay diluent and used as per manufacturer's instructions.
1329 The assay was repeated for each sample and the average concentration was determined for each
1330 cytokine. Plates were analyzed using Luminex xMAP technology on a Bio-plex 200 system
1331 (Biorad). Collected data was analyzed, and concentrations were determined using Bioplex
1332 manager software 6.1. The operator was blinded to the identity of the samples prior to each assay
1333 and data was unblinded after analysis.

1334

1335 **Antibodies and flow cytometry**

1336 Peripheral blood mononuclear cells (PBMC) were labeled with a panel of anti-CD3-
1337 Pacific blue (PB), CD8-PB, VIVID live dead stain, CD20-BV650, CD14-FITC, CD11c-PE,

1338 CD123-PE, HLA-DR-ECD, CD16-Cy-7-APC and TLR-4-APC. Monocyte subsets were
1339 discriminated based on the differential expression of CD14 and CD16 on CD3/CD8/VIVID-
1340 HLA-DR+CD20-CD11c/123- myeloid cells. All the antibodies were titrated using rhesus
1341 macaque PBMC. Labeled cells were washed and fixed in 0.5% PFA and analyzed on an LSR-II
1342 flow cytometer. One million total events were collected for analysis. Collected data was
1343 analyzed using Flowjo 9.6 software. The operator was blinded to the identity of the samples and
1344 data was unblinded after analysis.

1345 **Plaque reduction neutralization test**

1346 PRNT assays were performed using serum that was collected longitudinally. Briefly, 24-
1347 well plates were set to 100% confluence with Vero cells. Serum was heat inactivated for 30
1348 minutes at 56°C in a water bath. After initially diluting the serum at 1:5 in DMEM with 1% FBS,
1349 and it was diluted serially at a ratio of 1:10. ZIKV or DENV-2 viral stocks were quick thawed at
1350 37°C and diluted in DMEM containing 1% FBS to a titer of 4×10^2 plaque forming units
1351 (pfu)/ml. An equal volume of virus was added to each dilution of serum, mixed, and incubated
1352 for 1 hour at 37°C. After aspirating the media 75uL of virus and serum mix containing ~30
1353 pfu/well of virus was added to a 24 well plate. Each dilution was assayed in duplicate. After
1354 incubating the plates at 37°C in 5% CO₂ for 1 hour, 1 ml of 2 x EMEM containing 10% FBS
1355 mixed with an equal volume of 2% methylcellulose, warmed to 37C, was added to each well.
1356 The plates were incubated for a period of 5 days for ZIKV and 6 days for DENV-2 at 37C, 5%
1357 CO₂. At the end of incubation, the plates were fixed by adding 10% formalin to each well, and
1358 stained with hematoxylin and eosin and the number of plaques were counted. Percentage
1359 neutralization was determined using the formula: (number of plaques in dilution of interest) /

(number of plaques in virus only control well * 100). The operator was blinded to the identity of the samples prior to each assay.

DENV specific IgG ELISA

Serological assays for DENV specific IgG were performed as per manufactures instructions (IBL-America, Minneapolis, MN) using serum that was collected longitudinally. Briefly, serum samples were diluted 1:21 in sample diluent and dispensed into the microwell strips. Negative control, positive control and calibrator were used as provided with the kit. Assay diluent alone instead of serum was used as reagent blanks. All the samples and controls were setup in duplicates. After incubating for 20 minutes, the microwell strips were washed 3x with wash buffer, and 100 ul of the enzyme conjugate was added to each well. The microwell strips were incubated for 20 minutes, washed 3x with wash buffer and 100ul of TMB substrate was added to each well. The reaction was stopped after 10 minutes using the stop buffer and the O.D was determined at 450 nm using an ELISA plate reader. The operator was blinded to the identity of the samples prior to each assay and data was unblinded after analysis.

Data analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Prism Software, Inc. San Diego, CA). Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error. All data generated during this study are included in the manuscript and available on request.

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Figure 1: Pre-exposure to ZIKV enhances DENV-2 viremia *in vivo*. Kinetics of (a) ZIKV viral loads in plasma of ZIKV naïve (Group A; n = 4) and ZIKV infected (Group B; n = 5) rhesus macaques prior to DENV-2 infection. (b) DENV-2 viral loads in plasma from ZIKV naïve (Group A; n = 4) and ZIKV immune (Group B; n = 5) rhesus macaques after DENV-2 infection. Animals in Group A were not infected with ZIKV prior to infection with DENV-2, whereas the animals in Group B were infected with DENV-2 at day 56 post-ZIKV infection. (c) Analysis of body weight, body temperature, neutrophil and lymphocyte counts, blood glucose, mean cell volume (MCV), RBC, hematocrit, platelet and reticulocyte counts in blood samples that were collected longitudinally from animals in Group A (n = 4) and B (n = 5). Data are shown as % change relative to each animal's day 0 of DENV-2 challenge values. Line represents day 0. Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error and * indicates $p < 0.05$.

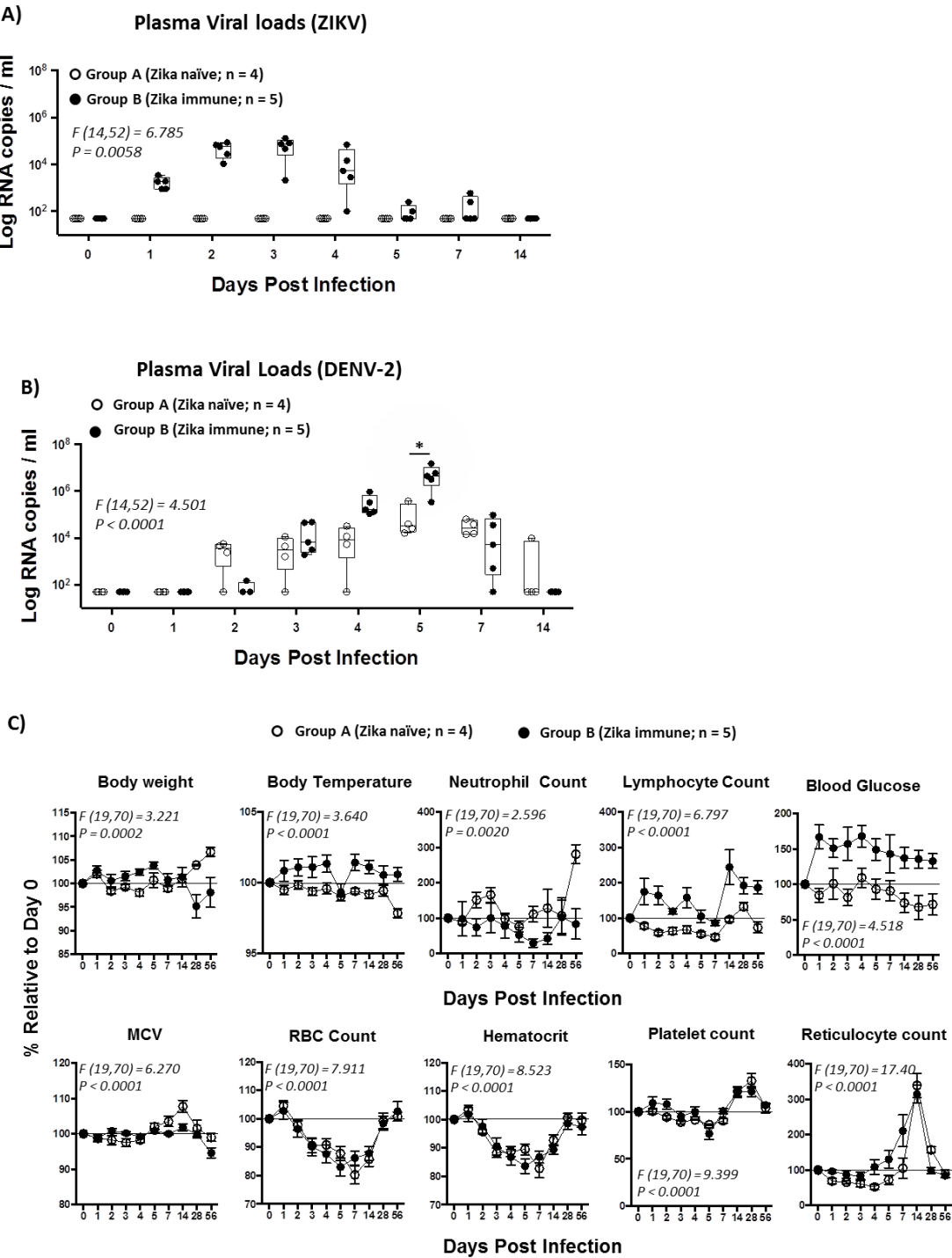


Figure 2: Pro-inflammatory cytokine levels are significantly upregulated in ZIKV immune animals after DENV-2 challenge. Kinetics of Interferon-inducible T-cell alpha chemoattractant (I-TAC), Monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8), Macrophage inhibitory factor (MIF), Eotaxin, Macrophage derived chemokine (MDC), Interferon gamma-induced protein-10 (IP-10), Epidermal growth factor (EGF), and Fibroblast growth factor (FGF) in plasma that were collected longitudinally from ZIKV naïve (Group A; n = 4) and ZIKV infected (Group B; n = 5) rhesus macaques after to DENV-2 challenge. Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error and * indicates $p < 0.05$.

Figure 2:

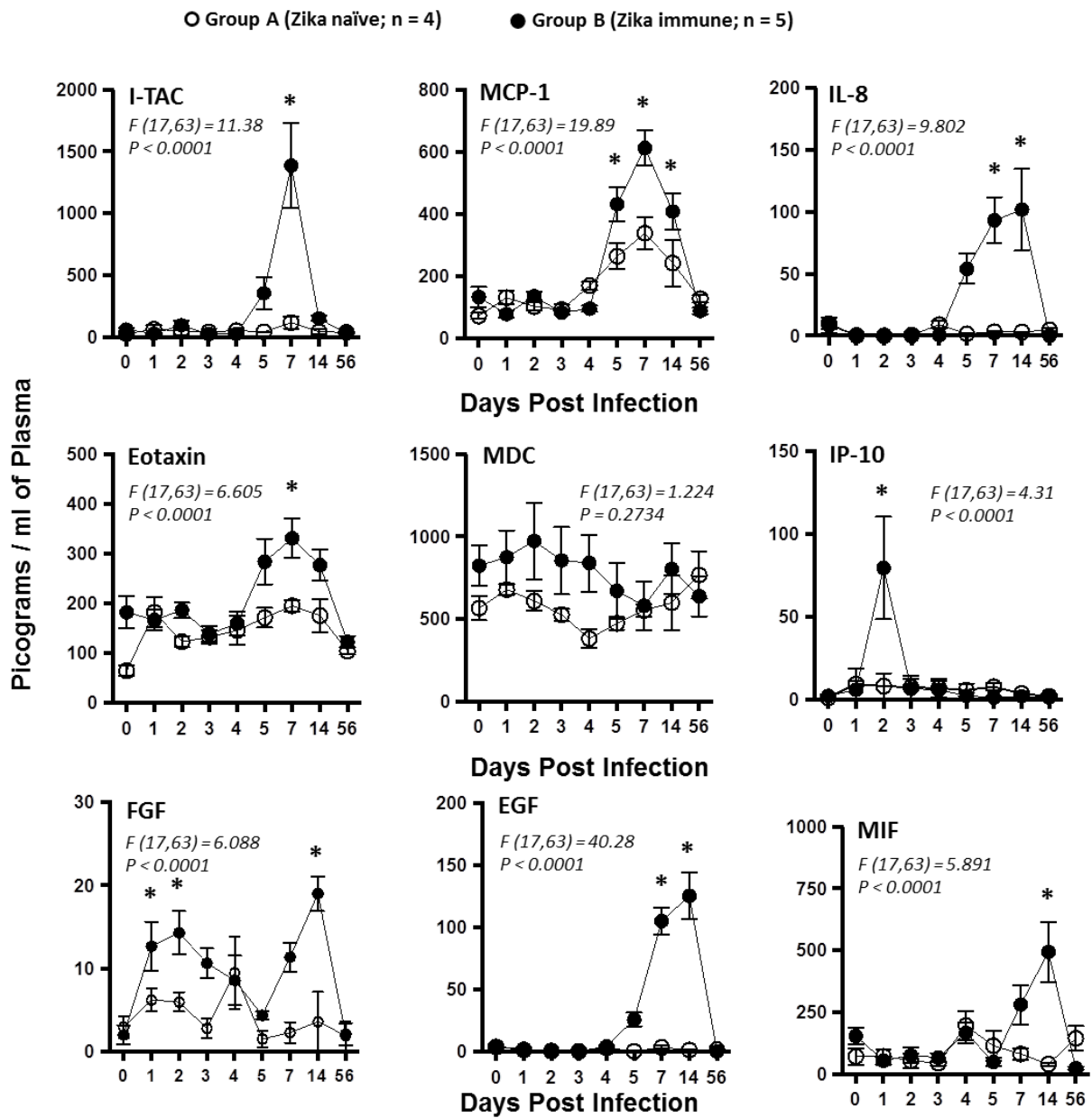


Figure 3: Pro-inflammatory monocyte subsets are significantly activated in ZIKV immune animals after DENV-2 infection. (a) Proportions of CD14⁺CD16⁻, CD14⁺CD16⁺, CD14⁻CD16⁺ and CD14⁻CD16⁻ subsets in peripheral blood from ZIKV naïve animals (Group A; n = 4) at day 0, 5 and 56 after DENV-2 challenge, and ZIKV immune animals (Group B; n = 5) at day 0, 5, 56, 63 and 112 post ZIKV infection. Day 0, 5 and 56 correspond to time points after ZIKV infection but prior to DENV-2 infection whereas day 63 and 112 correspond to Day 7 and Day 56 after DENV-2 infection. (b) Representative dot plots showing the discrimination of CD14 and CD16 subsets of monocytes/ macrophages, and histograms showing the level of TLR-4 expression on subsets of monocyte/macrophages in peripheral blood. (c) Mean fluorescence intensity (MFI) of TLR-4 expression of CD14⁺CD16⁻, CD14⁺CD16⁺, CD14⁻CD16⁺ and CD14⁻CD16⁻ subsets in peripheral blood from ZIKV naïve animals (Group A; n = 4) at day 0, 5 and 56 after DENV-2 challenge, and ZIKV immune animals (Group B; n = 5) at day 0, 5, 56, 63 and 112 post ZIKV infection. Day 0, 5 and 56 correspond to time points prior to DENV-2 infection whereas day 63 and 112 correspond to Day 7 and Day 56 after DENV-2 infection. Peripheral blood mononuclear cells that were collected longitudinally from each animal were used for analysis. Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error and * indicates $p < 0.05$.

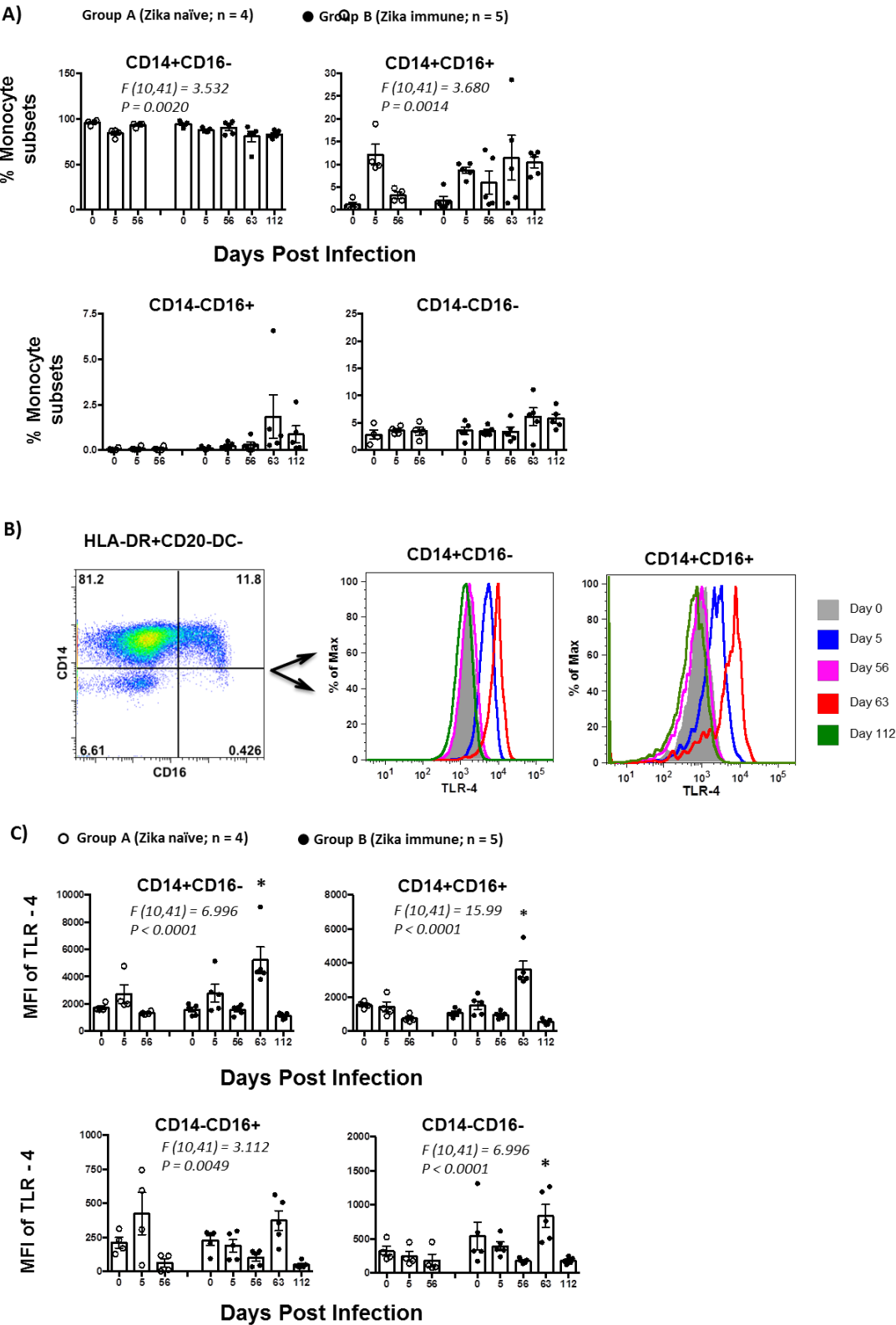
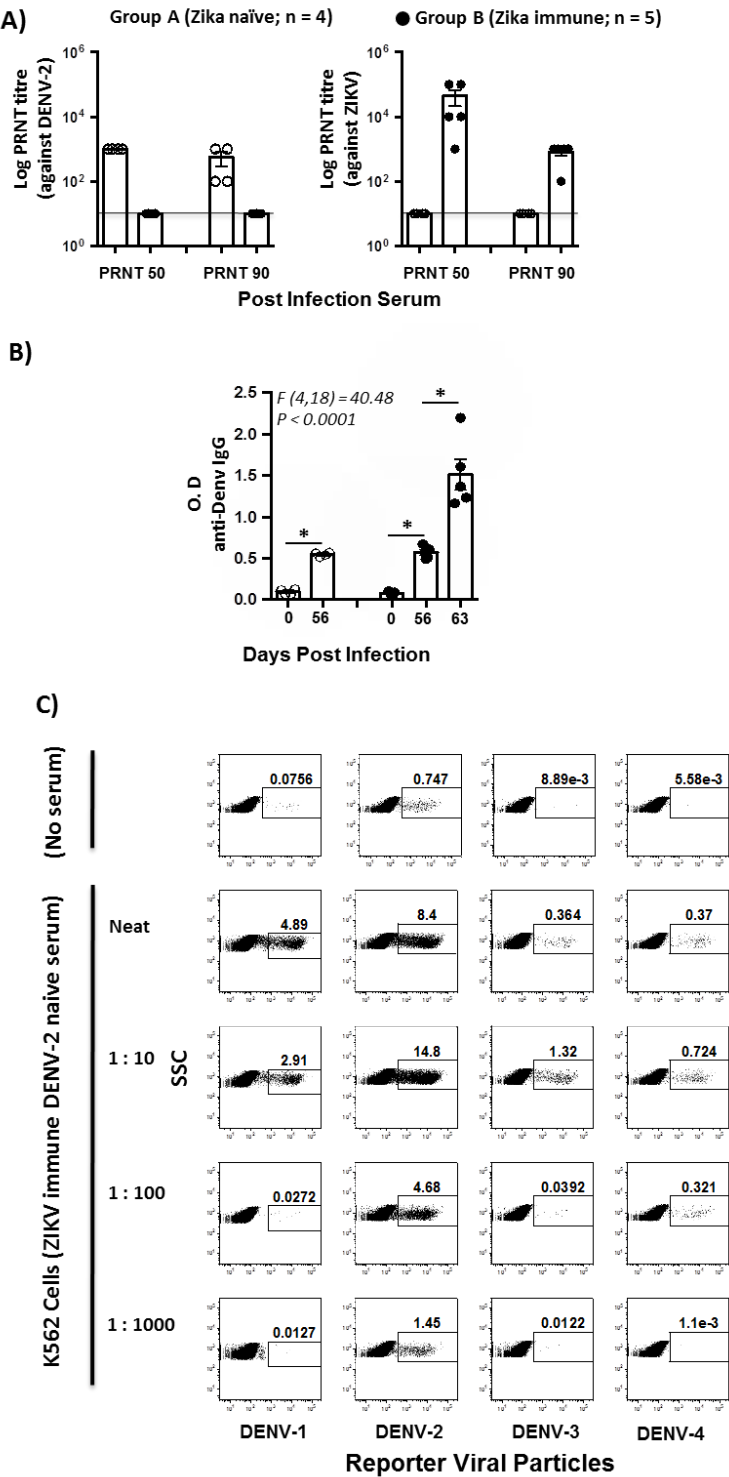


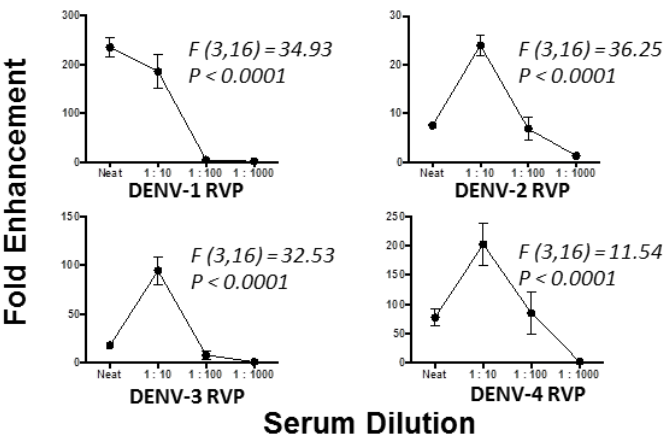
Figure 4: Serum from ZIKV immune animals collected prior to DENV-2 challenge significantly enhances DENV infection *in vitro* (a) Plaque reduction neutralization (PRNT) 50 and 90 titres against DENV-2 and ZIKV using serum from ZIKV naïve DENV-2 challenged animals (Group A; n = 4) and ZIKV immune animals (Group B; n = 5) prior to DENV-2 challenge. Line represents the limit of detection at 1:10 dilution. (b) Serum IgG levels in ZIKV naïve animals (Group A; n = 4) at day 0 and day 56 after DENV-2 challenge, and ZIKV immune animals (Group B; n = 5) at day 0, 5, 56 and 63 post ZIKV infection with day 0, 5 and 56 corresponding to time points prior to DENV-2 infection whereas day 63 corresponding to Day 7 after DENV-2 infection. Group B animals were infected with DENV-2 at day 56 after ZIKV infection. Serum samples collected longitudinally were used for analysis. (c) Representative FACS plots showing infection of K562 cells with DENV-1, 2, 3 and 4 reporter viral particles (RVP) using day 56 serum from ZIKV immune animals (Group B; n = 5) collected prior to DENV-2 challenge. K562 cells were incubated with either neat or serially diluted serum at 1:10, 1:100, and 1:1000 dilution in the presence of RVP's and GFP expression was examined by flow cytometry. (d) Fold antibody dependent enhancement of DENV-1, 2, 3 and 4 RVP infection of K562 cells using serum from ZIKV immune animals (Group B; n = 5) that was collected at day 56 after ZIKV infection but prior to DENV-2 challenge and (e) Frequency of RVP+ K562 cells infected with DENV-1, 2, 3 and 4 RVP at 1: 10 dilution of serum from ZIKV immune animals (Group B; n = 5) that was collected at day 56 after ZIKV infection but prior to DENV-2 challenge. Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error and * indicates $p < 0.05$.

1590 **Figure 4:**
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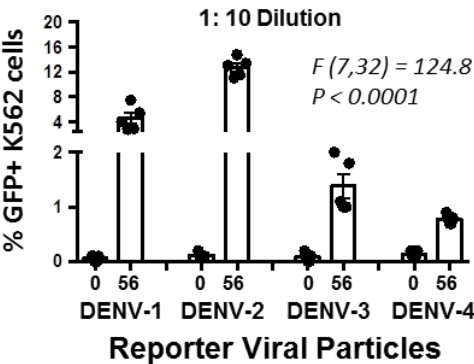


1593 **Figure 4 (continued):**

D) ZIKV Immune DENV-2 naïve Serum (Group B; n = 5; day 56 pi)



E) ZIKV Immune DENV-2 naïve serum (Group B; n = 5; day 56 pi)

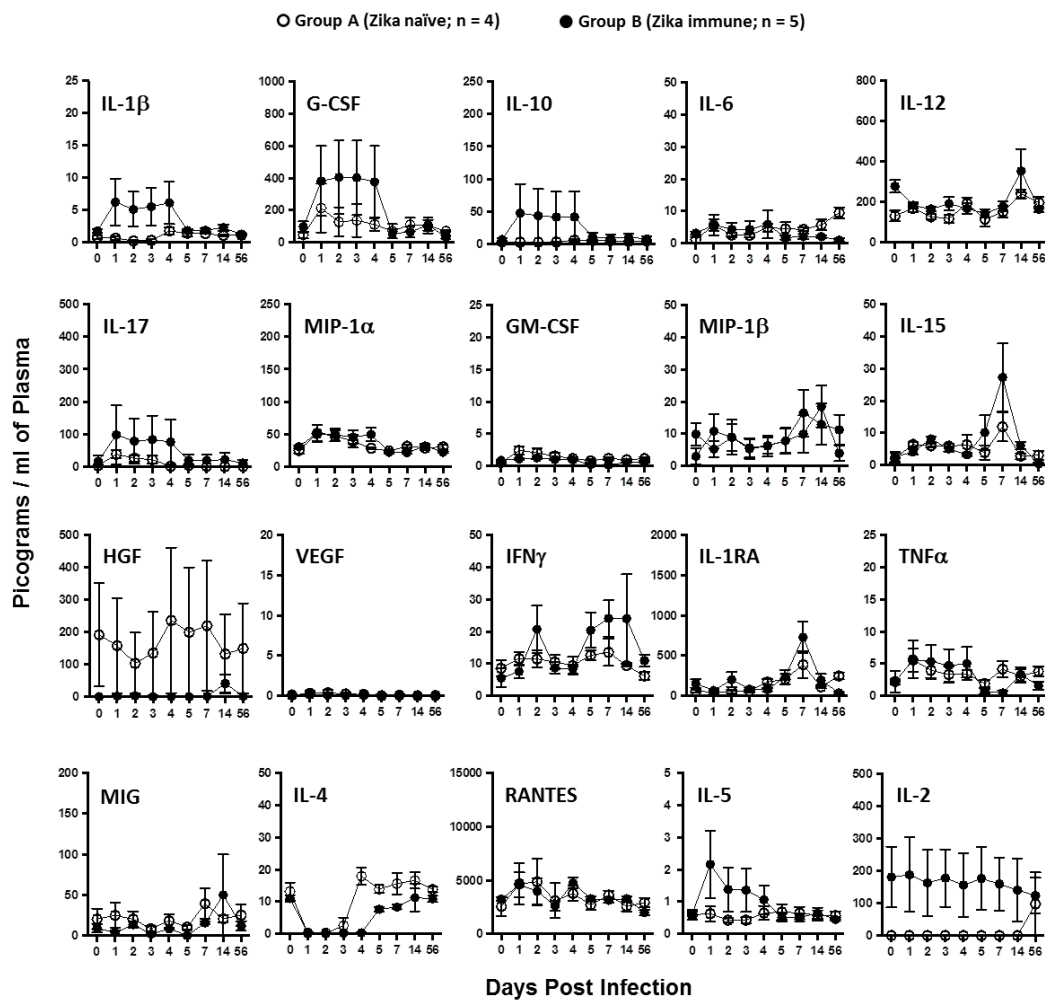


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Fig. S1: Plasma cytokine Levels. Kinetics of IL-1 β , G-CSF, IL-10, IL-6, IL-12, IL-17, MIP-1 α , GM-CSF, MIP-1 β , IL-15, HGF, VEGF, IFN γ , IL-1RA, TNF α , MIG, IL-4, RANTES, IL-5, and IL-2 in plasma that were collected longitudinally from ZIKV naïve (Group A; n = 4) and ZIKV immune (Group B; n = 5) rhesus macaques after DENV-2 challenge. No significant differences were observed between the groups. Differences between groups were determined using One-way ANOVA and differences between time points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error and * indicates $p < 0.05$.

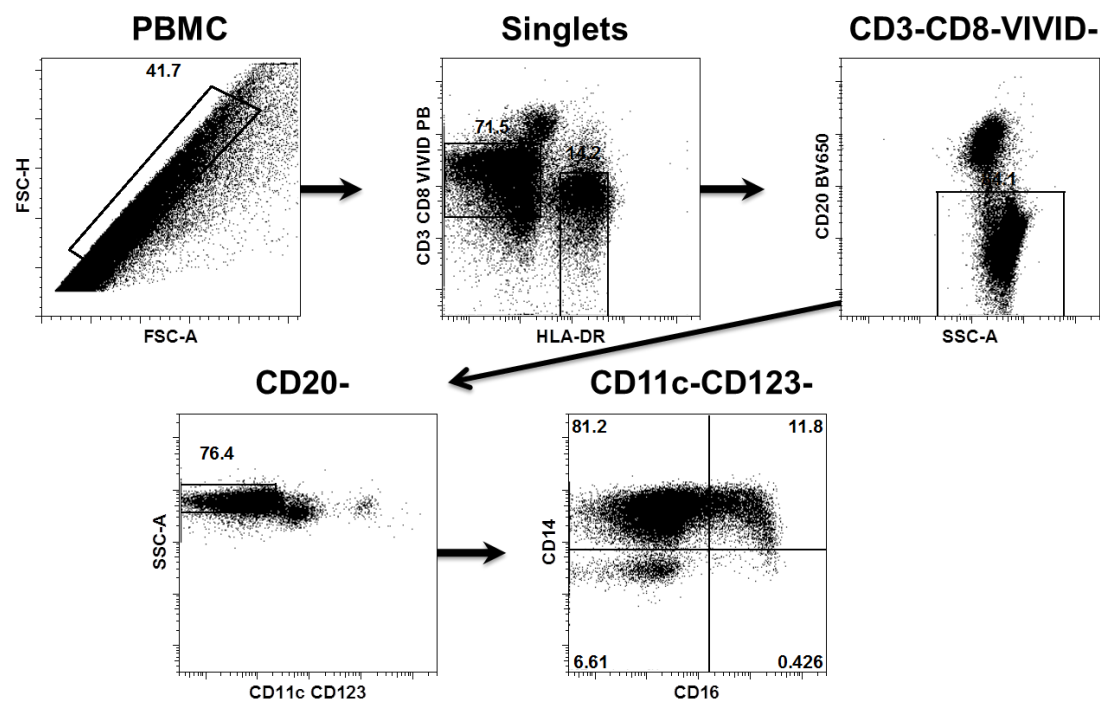
1605 **Figure S1:**



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1607 **Fig. S2: Gating strategy used to discriminate monocyte/macrophage subsets in peripheral**
1608 **blood.** Peripheral blood mononuclear cells (PBMC) were stained with a panel of markers to
1609 discriminate CD3⁻CD8⁻VIVID⁻HLA-DR⁺CD20⁻CD11c/CD123⁻CD14 and CD16 subsets.
1610

1611 **Figure S2:**



1612

Fig. S2

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1614 **Fig. S3: Titration of DENV - 1, 2, 3 and 4 Reporter viral particles (RVP) using K562 cells.**

1615 The concentration of DENV RVP to be used in the antibody dependent enhancement of infection
1616 is K562 cells was determined using ZIKV positive serum. Heat inactivated serum was incubated
1617 with different concentrations of RVP and then cultured with K562 cells for 48 hours. The
1618 frequency of GFP expressing cells was determined by flow cytometry.

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1620 **Figure S3:**

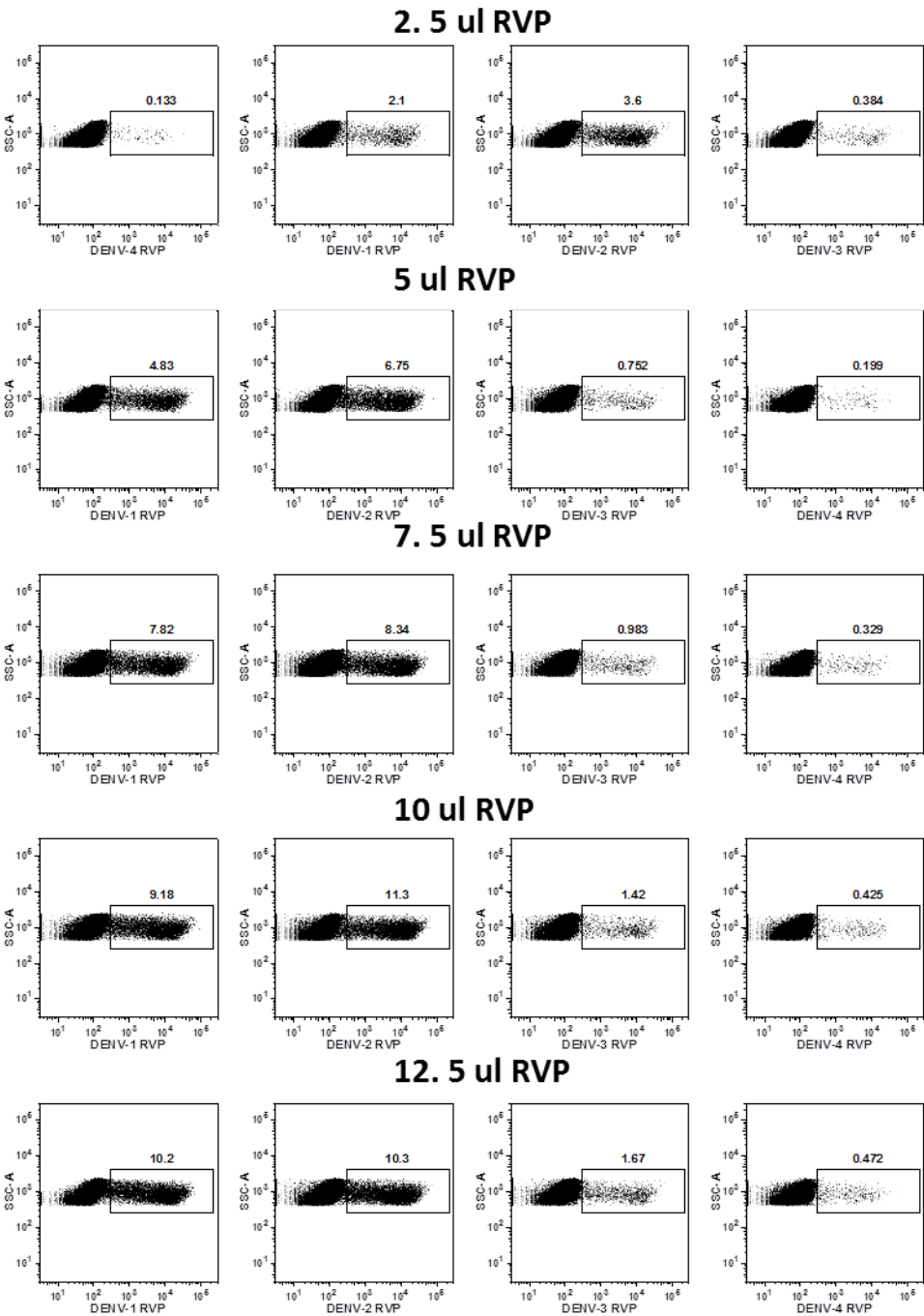


Fig. S3

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1623 **Chapter 3:**

1624 **Zika Convalescent Macaques Display Delayed Induction of Anamnestic Cross-Neutralizing**

1625 **Antibody Responses after Dengue Infection**

1626 William G. Valiant, Yan-Jang S. Huang, Dana L. Vanlandingham, Stephen Higgs, Mark G.

1627 Lewis, & Joseph J. Mattapallil

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1631 J. J. (2018). Zika convalescent macaques display delayed induction of anamnestic cross-

1632 neutralizing antibody responses after dengue infection. *Emerging microbes & infections*, 7(1), 1-

1633 11.”

Abstract

Structural similarities between Zika (ZIKV) and Dengue virus (DENV) leads to the induction of cross-reactive responses. We have previously demonstrated that ZIKV exposed macaques significantly enhance DENV viremia. Here we show this enhancement of DENV infection occurred in the presence of high levels of DENV cross-reactive IgG1 subclass of antibodies with low DENV neutralizing antibody (nAb) activity ($< 1:10$). The DENV-2 nAb levels after ZIKV infection were, however, higher than those induced in DENV-2 only infected animals suggesting that ZIKV induced cross-nAb against DENV. Surprisingly, DENV-2 infection of animals previously infected with ZIKV was not accompanied by an anamnestic increase in cross-nAb levels till about 1 week after DENV-2 infection. This delay coincided with enhanced DENV-2 viremia indicating that high levels of cross-bAb in the absence of high nAb plays a role in enhancement. Serum collected 8 weeks post-DENV-2 infection had high levels of nAb and showed delayed antibody dependent enhancement (ADE) of infection (1:100 dilution) as compared to serum collected prior to DENV-2 infection (1:10 dilution). Examination of serum from macaques that were simultaneously infected with both ZIKV and DENV-2 showed high levels of nAb and delayed ADE responses raising the possibility that the low levels of cross-nAb induced by ZIKV infection could be overcome by co-immunization against ZIKV and DENV infection. Taken together, our results provide additional insights into the nature and kinetics of cross-reactive antibody responses during ZIKV infection and identify a critical correlate of protection from enhancement of DENV infection during ZIKV convalescence.

Introduction

Zika virus (ZIKV) is a flavivirus that exhibits high levels of serological cross-reactivity with dengue virus (DENV)¹⁻⁵. We recently reported that prior exposure to ZIKV significantly enhances DENV infection in rhesus macaques *in vivo*⁶. Serum from ZIKV immune animals displayed a significant capacity to mediate *in vitro* antibody dependent enhancement (ADE) of all four serotypes of DENV suggesting a potential role for cross-reactive antibodies induced by ZIKV in ADE of DENV infection. ADE has been well documented in DENV infections⁷⁻¹² and others have reported that ZIKV induced antibodies enhance DENV-2 replication *in vitro*¹³. Previous studies have shown that serum from both acute and convalescent DENV infected subjects demonstrate significant neutralizing antibody (nAb) activity against ZIKV suggesting that prior DENV infection could potentially prevent infection with ZIKV³. On the other hand, Montoya et al¹⁴ examined antibody cross-neutralization after ZIKV and DENV infection and reported low levels of cross-reactivity in ZIKV infected subjects against DENV-1 to 4 serotypes. Likewise, other studies have reported that ZIKV induces little or no cross-nAb responses against DENV^{15,16}.

In line with the above studies, rhesus macaques previously infected with ZIKV were shown to have PRNT₅₀ titres of <1:10 against DENV at the time of DENV challenge, though these animals had significant levels of DENV cross-reactive binding antibodies (bAb)⁶. This finding raised the possibility that conformational DENV cross-reactive epitopes capable of inducing nAb responses against DENV were likely minimally immunogenic thereby contributing to low levels of neutralizing activity after DENV infection. We sought to address this question using serum that was collected longitudinally from ZIKV convalescent macaques after infection with DENV-2. We hypothesized that if ZIKV infection induced DENV specific nAb responses

then we would detect an anamnestic amplification of these responses immediately after DENV-2 challenge. We examined sera from ZIKV exposed animals at 0, 1, 4, 7 and 56 days after DENV-2 infection and compared these responses to DENV-2 only infected animals. Our results show that ZIKV infection induces DENV cross-nAb but at titres that remain below the detectable levels ($< 1:10$) during the first few days after DENV-2 infection when there was significant enhancement of viremia. Anamnestic increases in cross-nAb titres become readily detectable ($> 1:10$) by day 7 post-DENV-2 infection that coincided with a decrease in DENV-2 viremia.

Results

Kinetics of cross-reactive IgM and IgG responses induced by Zika and Dengue virus

Numerous studies have reported induction of cross-reactive antibodies by related Flaviviruses. There is, however, limited information regarding the early kinetics of responses induced by ZIKV and DENV. To address this gap, we examined the evolution of DENV-2 cross-reactive IgM and IgG responses induced by ZIKV infection prior to DENV-2 infection and compared them to DENV only infected animals (Fig. 1). Our results show that similar levels of cross-reactive IgM responses were induced against ZIKV and DENV in both groups of animals that peaked at day 14 after infection (Fig. 1a-b). Interestingly, IgM levels induced in DENV-2 only infected animals were less apparent at day 7 PI than those induced in ZIKV infected animals prior to DENV-2 infection. IgM responses declined to near pre-infection levels by 56 days post-infection (PI).

Unlike IgM responses, we observed interesting differences in the kinetics of IgG levels between the two groups of animals (Fig. 1c-d). In DENV-2 only infected animals, both DENV-2 and ZIKV binding IgG levels remained near baseline at day 7 PI and become discernable by day

14 PI (Fig. 1c). DENV-2 binding IgG levels peaked at day 28 PI whereas, ZIKV binding IgG peaked earlier at day 14 PI. Unlike DENV-2 binding IgG that remained high during the first 56 days of infection, ZIKV binding IgG levels declined significantly to near baseline levels by 56 days PI suggesting a divergence in the kinetics of cross-reactive IgG responses induced by DENV-2 infection (Fig. 1c).

In contrast to DENV-2 only infected animals, the levels of both DENV-2 and ZIKV binding IgG in ZIKV infected animals were readily detectable by day 7 PI (Fig. 1d). Binding IgG levels peaked at day 14 and stayed high during the first 56 days of infection. As expected, the levels of ZIKV binding IgG were significantly higher than DENV-2 binding IgG at day 56 PI (Fig. 1d).

Both ZIKV and DENV induce little or no cross-neutralizing antibody responses

Next we determined if the high levels of cross-reactive binding antibody (bAb) responses induced during both ZIKV and DENV-2 infection was accompanied by the induction of cross-nAb responses against both these viruses. To address this question, we first examined the ZIKV and DENV-2 nAb titres in serum that was collected at 56 days PI using plaque reduction neutralizing test (PRNT). We observed significantly high titres of ZIKV nAb in ZIKV infected animals, and high DENV-2 nAb titres in DENV-2 only infected animals (Fig. 2a-b); both groups displayed a PRNT₅₀ titre of 1,000 – 10,000.

DENV-2 only infected animals induced little or no cross-nAb responses against ZIKV with a PRNT titre of < 1:10 (Fig. 2a). Likewise, the PRNT₅₀ titres against DENV-2 in ZIKV infected animals were <1:10 (Fig. 2b) suggesting that infection with either ZIKV or DENV-2 alone induces little or no cross-nAb activity *in vivo*. These findings were not surprising, as previous studies have reported that serum from ZIKV infected human subjects failed to cross-

neutralize DENV¹⁷. Likewise, Dejnirattasai et al¹⁸ showed that plasma from DENV infected donors variably neutralized different strains of ZIKV.

To confirm if nAb activity against DENV-2 was completely absent in macaques infected with ZIKV and not neutralizing at lower dilutions, we examined nAb titres against DENV-2 using 2-fold dilution of serum that was collected at 56 days after ZIKV infection (Fig. 3a); we observed low levels of neutralization against DENV-2 in ZIKV infected animals prior to DENV-2 infection (day 0 of DENV-2 challenge; ~40% neutralization) as compared to DENV-2 only infected animals (day 0 of DENV-2 challenge; ~10% neutralization) suggesting that prior infection with ZIKV induced DENV cross-nAb responses albeit at significantly low levels. These results appear to be in line with studies suggesting that ZIKV lies outside the DENV serocomplex¹⁴.

Anamnestic neutralizing antibody responses remain low during the first few days after Dengue infection in ZIKV immune animals

Previous studies have shown the titres of DENV specific nAb correlate with reduced probability of severe DENV infection¹⁹. To determine if low nAb played a role in the enhancement of DENV-2 infection in ZIKV exposed animals, we examined the early kinetics of DENV-2 nAb responses in serum that was collected from ZIKV exposed animals at days 1, 4, 7, 14 and 56 after DENV-2 infection and compared them to day 0 of DENV-2 infection (day 56 post-ZIKV infection). Neutralizing antibody titres during the first 4 days after DENV-2 infection did not differ from that of day 0 levels suggesting that there was no anamnestic increase in the nAb titres immediately after infection (Fig. 3a-f). Neutralizing antibody titres increased by day 7 post-DENV-2 infection to detectable levels with titres <1:100. Detectable nAb titres at day 7 post-DENV-2 infection coincided with a decline in plasma viremia (Table 1). By day 56 post-

DENV-2 infection, nAb responses against DENV-2 were readily detectable. These findings indicate that DENV-2 infection failed to boost cross-nAb induced by prior ZIKV infection during the early stages of infection.

As enhancement of DENV infection has been associated with binding cross-reactive antibodies in the absence of detectable nAb activity, we assessed the levels of envelope binding IgM and IgG at 1, 4 and 7 days after DENV-2 infection using whole virus based ELISA assays to determine if bAb levels unlike nAb were boosted immediately after infection (Fig. 4a-b). Similar to nAb titres, we observed little or no anamnestic increase in either DENV-2 binding IgM or IgG levels during the first 4 days after infection with DENV-2. However, by day 7 post-DENV-2 infection the levels of cross-reactive IgG had increased significantly suggesting that there was an anamnestic antibody response that became apparent only 1 week after infection. These findings suggest that the enhancement of DENV-2 viremia likely occurred due to the high prevalence of cross-reactive binding IgG that were induced by ZIKV infection prior to DENV-2 infection and remained high at the time of DENV-2 infection. It is difficult to directly discern from our studies if the higher binding IgG responses we observed 7 days after DENV-2 infection was an anamnestic or a naïve response to DENV-2 challenge. However, based on the kinetics of IgG responses in DENV-2 only infected animals where IgG levels were near baseline levels at day 7 post-DENV-2 infection (Fig. 1c), it is possible to infer that the enhanced IgG response at day 7 post-DENV-2 in ZIKV exposed animals were most likely consisted of an anamnestic IgG response.

ZIKV infection induces predominantly IgG1 subclass of ZIKV specific and DENV cross-reactive IgG

We have previously shown that DENV-2 enhancement of viremia in ZIKV immune animals was accompanied by release of various pro-inflammatory mediators associated with severe DENV infections⁶, and others¹² have implicated IgG1 subclass of antibodies in the induction of DHF/DSS. To determine if ZIKV infection induced different subclasses of IgG, we examined the ZIKV and DENV-2 envelope binding IgG1, 2, and 3 from serum from ZIKV immune animals that was collected at day 14 and 56 PI prior to DENV-2 infection using whole virus based ELISA and compared them to DENV-2 only infected animals (Fig. 5a-d). We could not obtain reliable reagents to IgG4 subclass hence restricted our analysis to the 3 subclasses. Positive controls were set up simultaneously for each subclass using purified rhesus macaque IgG1, 2 and 3 (Supplementary Fig. 1).

Our results showed that the predominant subclass of IgG induced against ZIKV and DENV-2 in DENV only infected animals were IgG1 (Fig. 5a-b). Likewise, both DENV-2 and ZIKV cross-reactive IgG subclass induced in ZIKV exposed animals were IgG1 (Fig. 5c-d). There was no major change in the subclass of IgG in both groups of animals between day 14 and 56 PI. DENV-2 infection in ZIKV exposed animals did not alter the subclass of IgG with the primary subclass of IgG induced against both ZIKV and DENV-2 being IgG1 (Fig. 5e-f).

The potential for *in vitro* ADE decreases with higher neutralizing antibody titres

Our earlier studies⁶ have shown that serum from ZIKV exposed animals prior to DENV-2 infection displayed a significant potential for ADE that peaked at a serum dilution of 1:10. To assess if the *in vitro* ADE of DENV-2 infection was altered in the presence higher DENV-2 nAb titres, we compared the potential for *in vitro* ADE using sera from the ZIKV exposed animals that was collected prior to DENV-2 infection (Fig. 6a) and at 56 days after DENV-2 infection (Fig. 6b). Our results showed that enhancement peaked at a dilution of 1: 100 at day 56 post-

DENV-2 infection as compared to a 1:10 dilution in serum that was collected at day 56 after ZIKV infection but prior to DENV-2 challenge suggesting that the threshold for enhancement is significantly higher in the presence of elevated nAb titers against DENV-2.

Simultaneous infection with ZIKV and DENV-2 induces high neutralizing antibody titres against both viruses

Given the correlation between high nAb responses and low *in vitro* ADE, we hypothesized that higher nAb levels if induced simultaneously against both ZIKV and DENV-2 could likely reduce the potential for enhancement of infection. We tested this hypothesis by co-infecting rhesus macaques with ZIKV and DENV-2 simultaneously and assessing nAb titres (Fig. 6c), and the potential for *in vitro* ADE of DENV-2 infection (Fig. 6d). Our results showed that co-infection induced high levels of both ZIKV and DENV-2 nAb titres that were similar to the nAb titres induced against DENV-2 in DENV-2 only infected animals or against ZIKV in ZIKV exposed animals prior to DENV-2 infection (Fig. 2a-b). Sera from the co-infected animals that were collected at 56 days post-infection displayed *in vitro* ADE of DENV-2 infection at a dilution of 1:100 (Fig. 6d) as compared to a 1:10 dilution for sera that was collected prior to DENV-2 infection (Fig. 6a) suggesting that higher nAb titres delays the induction of ADE responses *in vitro*.

Discussion

Antibody cross-reactivity among related flaviviruses have been extensively documented. Less clear is the effectiveness of cross-nAb responses in protection from infection. Though enhancement of infection with heterologous serotypes of DENV has been well studied, there is limited information regarding the potential for ZIKV induced responses to protect from DENV

infection. We have previously reported enhancement of DENV viremia in rhesus macaques that had been previously exposed to ZIKV. Here we show that this enhancement occurred in the presence of high titres of pre-existing cross-reactive bAb with little or no DENV cross-nAb activity. Previous studies¹⁶ have shown that ZIKV infection induced little or no cross-reactive nAb against DENV. Likewise Dick et al¹⁵ using intracerebral neutralization test in mice showed that ZIKV immune serum failed to protect mice from DENV challenge.

What was surprising, however, was the lack of an early anamnestic antibody response in ZIKV immune animals immediately after DENV infection, a time period that coincided with enhancement of viremia. ZIKV immune animals displayed a significant enhancement of DENV-2 viremia as compared to ZIKV naïve animals that peaked at 5 days post-DENV-2 infection and, declined by day 7 post-infection (Table 1). These findings were somewhat surprising given the high levels of DENV-2 viremia in ZIKV immune animals during the first week of infection; plasma DENV-2 viral loads were ~ 4 logs / ml of plasma at day 3, ~ 5.4 logs / ml of plasma at day 4, and ~ 6.5 logs / ml of plasma at day 5. The lack of significant anamnestic boosting of ZIKV induced DENV cross-nAb responses likely points to the time it takes for a secondary immune response to emerge. On the other hand, enhancement was likely dictated by the high levels of pre-existing cross-reactive binding antibodies that are present at the time of DENV-2 infection.

We examined enhancement in ZIKV infected animals during early convalescence (56 days post-ZIKV infection). Would cross-reactive titres remain at such high levels over a longer period of convalescence, and if those levels can enhance *in vivo* infection is not known and still remains to be determined. We have previously shown that serum that was collected from macaques 16 weeks after ZIKV infection enhanced infection *in vitro* with all serotypes of

DENV²⁹. Additional studies are needed to examine the effect of sera from long-term convalescent subjects to determine if the potential for enhancement exists over long periods of time. It was, however, exciting to note that the decline in enhanced DENV viremia in ZIKV immune animals coincided with the emergence of detectable nAb responses against DENV raising the possibility that high cross-neutralizing antibody titres if induced could potentially prevent the enhancement of DENV infection even in the presence of high levels of binding cross-reactive antibody responses. In line with this argument, the *in vitro* capacity of serum to induce ADE was significantly higher very early after DENV-2 infection as compared to serum that was collected at 8 weeks post-DENV-2 infection when nAb titres against DENV-2 was significantly high; peak enhancement was observed at a dilution of 1:10 using sera that was collected prior to DENV-2 infection as compared to 1:100 dilution of serum that was collected 56 days after DENV-2 infection. It is interesting to note the nAb titres though below detectable levels in ZIKV immune animals prior to DENV-2 infection (<1:10 at day 0), were higher than those induced in ZIKV naïve DENV-2 infected animals during the first 1 week after infection (Fig. 3a-d); ~10% neutralization in ZIKV naïve DENV-2 infected animals vs ~40% neutralization in ZIKV infected animals DENV-2 infected animals at day 0 of DENV-2 infection suggesting that there are shared conformational nAb inducing epitopes between ZIKV and DENV that could potentially be harnessed for protection. Robbiani et al²⁰ reported that antibody responses induced against DENV-1 envelope domain III prior to ZIKV infection was associated with higher ZIKV specific nAb responses titers after ZIKV infection. These antibodies appear to cross-neutralize DENV-1 and ZIKV but not other Flaviviruses. Valiant et al²¹ recently reported that serum from a human subject with high levels of nAb against both ZIKV and DENV displayed *in vitro* ADE of DENV infection at a dilution of 1: 10,000 as compared to a 1:10

dilution of serum from a subject who had high levels of nAb only against ZIKV but not against DENV. Could these responses translate to protection from enhancement *in vivo* remains to be determined.

Vaccine based strategies using a combination of ZIKV and DENV vaccines could potentially induce such high levels of nAb responses against both ZIKV and DENV. Though we did not directly address this hypothesis using challenge studies, we did observe significantly high levels of nAb titres against both ZIKV and DENV-2 in rhesus macaques that were co-infected with ZIKV and DENV-2 at the same time suggesting that vaccinating against both viruses simultaneously could likely protect from enhancement. Additional studies are needed to address this hypothesis.

It was interesting to note that the primary subclass of IgG induced after ZIKV infection against both whole ZIKV and DENV viruses was IgG1. The subclass did not change after DENV-2 infection in ZIKV immune animals. The exact reasons for preferential induction of IgG1 is not clear and may be related to the specific microenvironment induced within the B cell follicles. The induction of IgG1, however, may have implications for the enhancement of DENV infection as numerous studies have reported. Wang et al¹² demonstrated that patients with DHF/DSS had cross-reactive non-neutralizing antibodies that had a high affinity for FcγRIIIA and were primarily of IgG1 subclass. Thein et al²² showed that serum from subjects with acute dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) contained higher levels of anti-DENV IgG1 subclass of IgG. Likewise, Posadas-Mondragon et al²³ reported that IgG1 subclass of DENV specific IgG was the predominant subclass during both primary and secondary DENV infections that significantly increased in the patients with secondary DENV

infection and DHF. Others²⁴ have shown that high levels of DENV specific IgA, IgG1 and IgG4 antibodies in serum were markers of high risk for DSS and DHF.

In conclusion, our findings provide additional new insights into the early kinetics of cross-bAb and nAb responses induced against DENV following ZIKV infection and raise the prospect that higher levels of nAb if induced against both viruses simultaneously could potentially overcome the effect of enhancing bAb and protect from adverse events such as enhancement immediately following DENV infection during ZIKV convalescence.

Materials and Methods

Samples

Archived serum samples that were collected from rhesus macaques that were used in this study. All animal experiments had reviewed and approved by Institutional Animal Care and Use Committee at Bioqual Inc., and samples were acquired through a tissue sharing protocol. ZIKV naïve animals had been infected with 10^5 TCID₅₀ of DENV-2 (strain 16681) subcutaneously and serum samples were collected longitudinally at day 0, 7, 14, 28 and 56 post-DENV-2 infection. Likewise, serum was collected longitudinally at day 0, 7, 14, 28 and 56 from animals who had been infected subcutaneously with 10^6 TCID₅₀ of ZIKV (Puerto Rico Strain; Genbank KU501215) for a period of 8 weeks ($n = 5$). Additionally, serum samples were obtained after challenge with 10^5 TCID₅₀ DENV-2 (strain 16681) subcutaneously at day 0 (day 56 post-ZIKV infection), 1 (day 57), 4 (day 61), 7 (day 63), 14 (day 70) and 56 (day 112) post-DENV-2 infection ($n = 5$). Plasma viral loads were determined as described previously⁶. To assess the effect of co-infection, we collected serum at day 56 post-infection from rhesus macaques that were simultaneously infected with ZIKV and DENV-2 as described above ($n = 5$). Samples from each animal were assayed separately for each of the time points examined, and in all of the assays reported in the manuscript.

Plaque Reduction Neutralization Test (PRNT)

Fully confluent monolayers of Vero cells were infected with ZIKV or DENV-2 in DMEM containing 1% FBS. When severe cytopathic effect was observed the supernatant was collected, centrifuged at 500g for 10 minutes, and stored at -80°C . The titer of the stocks was determined by plaque assay using Vero cells.

PRNT assays were set up in 24-well plates that were set to 100% confluence with Vero cells. Serum was heat inactivated for 30 minutes at 56°C. Serum was initially diluted at 1:5 in DMEM with 1% FBS followed by a 10x serial dilution. ZIKV or DENV-2 were diluted in DMEM containing 1% FBS to a titer of approximately 4×10^2 plaque forming units (PFU)/ml. An equal volume of virus was added to each dilution of serum, mixed, and incubated for 1 hour at 37°C. After aspirating the media from the 24-well plate with Vero cells, and 75uL of the virus and serum mix was added to each well. Each dilution was assayed in duplicate. After incubating the plates at 37°C in 5% CO₂ for 1 hour, 1 ml of 2 x EMEM containing 10% FBS that was mixed with an equal volume of 2% methylcellulose, warmed to 37°C, was added to each well. The plates were incubated for 5 days at 37°C, 5% CO₂. Plates were fixed with 10% formalin, stained with hematoxylin and eosin, and the number of plaques in each well was counted. Percentage neutralization was determined using the formula: (number of plaques in dilution of interest) / (number of plaques in virus only control well) * 100. The operator was blinded to the identity of the samples prior to each assay.

Neutralization assay using RVP

Neutralization assays using DENV reporter viral particles (RVP) were performed as described previously^{6,25}. Briefly, serum was heat inactivated for 30 minutes at 56°C and diluted 2-fold in RPMI-10. The diluted serum was mixed in equal volume with 5uL of DENV-2 RVP (Integral Molecular, Philadelphia, PA) and incubated at 37°C in 5% CO₂ for one hour. After incubation, 40,000 Raji DC-SIGN-R cells²⁶ (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Raji/DC-SIGN Cells from Drs. Li Wu and Vineet N. KewalRamani) were suspended in 15µl of RPMI-10 were added to the serum/RVP mixture and incubated at 37°C, 5% CO₂ for one hour. Following incubation 100uL of RPMI was added and the cells were held at

1938 37°C, 5% CO₂ for three days in a 96 well U-bottom tissue culture plate. After culture cells were
1939 fixed in 0.5% Paraformaldehyde, and analyzed using an LSR II flow cytometer. Each sample
1940 was assayed in duplicate. Collected data was analyzed using Flowjo 9.6 software.

1941 **ELISA for serum IgM and IgG**

1942 96-well plates were coated overnight at 4°C a pan-flavivirus antibody (clone 4G2),
1943 followed by blocking with 1% BSA in 1xPBS overnight at 4°C. The 4G2 antibody has been
1944 extensively used in capture-based ELISA to look at both ZIKV and DENV binding antibody
1945 levels in serum^{3,18,27,28}. After washing with PBS-T (PBS with 0.5% Tween-20) cell culture stocks
1946 of ZIKV or DENV-2 diluted 1:10 in 1xPBS (~1-2 x 10⁴ pfu/ml) were added to each well and the
1947 plate was incubated at 60 minutes at 37°C. Serum samples were diluted 1:100 in PBS-T and
1948 dispensed into respective wells. PBS-T alone was used as reagent blanks. After incubating for 60
1949 minutes at 37°C, the plates were washed 3x with PBS-T, and anti-IgG-HRP (Biorad) diluted
1950 1:50,000 in PBS-T or anti-IgM-HRP (Rockland), diluted 1:10,000 was added to each well. The
1951 plates were incubated for 30 minutes at room temperature, washed 3x with wash buffer and
1952 Tetramethylbenzidine (TMB) substrate was added to each well. The reaction was stopped after
1953 15 minutes using 2M Sulfuric acid, and the O.D was determined at 450 nm using an ELISA plate
1954 reader. Samples were assayed in duplicate and the operator was blinded to the identity of the
1955 samples prior to each assay and data was unblinded after analysis.

1956

1957 **IgG Subclass ELISA**

1958 Subclass of serum IgG was determined as described previously²⁹. Cell culture stocks of
1959 ZIKV or DENV-2 were diluted 2:5 in PBS and bound to half area 96-well plate overnight at 4°C,
1960 followed by blocking with 1% BSA in PBS overnight at 4°C. Serum samples were diluted 1:10

in PBS-T and incubated on the virus coated plates for 60 minutes at 37°C. Mouse anti-rhesus macaque anti-IgG1, IgG2 and IgG3 were obtained from the NIH Nonhuman Primate Reagent Resource supported by AI126683 and OD010976, and added to each well at a dilution of 1:200 in PBS-T. The plates were incubated for 60 minutes at 37°C. After washing with PBS-T goat anti-mouse HRP was added at a dilution of 1:10,000 in PBS-T for 30 minutes at room temperature. The plate was washed and TMB solution was added to each well. The reaction was stopped using 2M Sulfuric acid after 15 minutes and the O.D. was determined at 450 nm using an ELISA plate reader. Purified rhesus macaque IgG1, 2 and 3 that was obtained from the NIH Nonhuman Primate Reagent Resource were used as positive controls (Supplementary Fig. 1). Samples were assayed in duplicate and the operator was blinded to the identity of the samples prior to each assay and data was unblinded after analysis

Antibody dependent enhancement assay

The capacity of serum to enhance DENV infection *in vitro* was performed using RVP as described previously^{6,25}. Serum was heat inactivated at 56°C for 30 minutes, serially diluted 10 fold, and mixed with 5µL of RVP at a ratio of 1:1 volume. After incubation for 1 hour at 37°C, 5% CO₂, the serum/RVP mix was added to 40,000 K562 cells³⁰ suspended in 15µl of RPMI-10 and incubated for 1 hour at 37°C, 5% CO₂. After 1 hour the cells were washed twice with RMPI-10 to remove unbound virus, resuspended in RPMI-10 and cultured in a 96 well tissue culture plate for 48 hours. After culture cells were fixed in 0.5% Paraformaldehyde, and analyzed using an LSR II flow cytometer. Collected data was analyzed using Flowjo 9.6 software. Samples were assayed in duplicate and the operator was blinded to the identity of the samples prior to each assay and data was unblinded after analysis.

Data analysis

1984 Statistical analysis was performed using GraphPad Prism Version 5.0 software
1985 (GraphPad Prism Software, Inc. San Diego, CA). Error bars represent standard error and $p <$
1986 0.05 was considered significant.

1987

1988

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1993 Uniformed Services University of the Health Sciences or any other agency of the U.S.
1994 Government.

1995 **Author contributions:** WGV performed all the experiments and analyzed the data; JJM
1996 designed and supervised the study; WGV, YSH, DLV, SH, MGL and JJM wrote the paper.

1997 **Competing interests:** The authors declare no financial conflict of interest.

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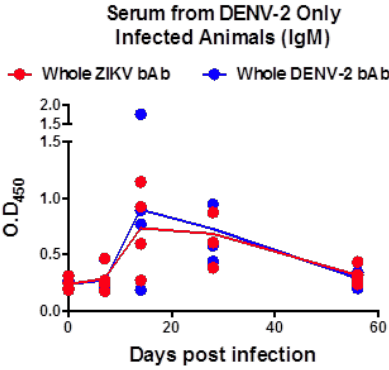
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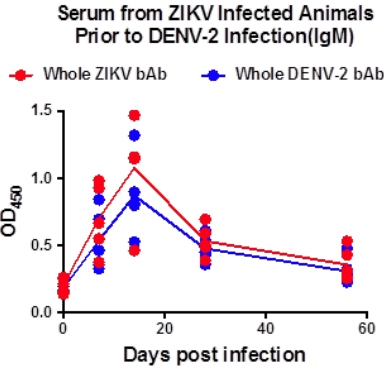
Figure 1: Kinetics of cross-reactive antibody responses. Relative binding antibody (bAb) levels against whole ZIKV and DENV were examined using ELISA. The kinetics of binding IgM in serum that was collected longitudinally from (a) DENV-2 only (ZIKV naïve) infected animals (n = 4), (b) ZIKV infected animals prior to DENV-2 infection (n = 5). The kinetics of binding IgG in serum that was collected longitudinally from (c) DENV-2 only (ZIKV naïve) infected animals (n = 4), (d) ZIKV infected animals prior to DENV-2 infection (n = 5). Statistical significance was determined using multiple unpaired t-tests and corrected for multiple comparisons using the Holm-Sidak method. A $p < 0.05$ was considered significant and * indicates significant difference between groups.

2104 **Figure 1:**

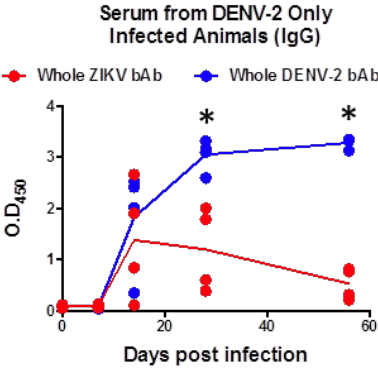
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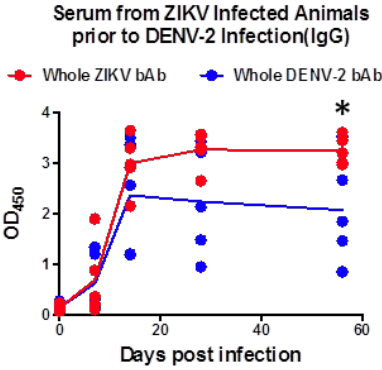
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c)



d)

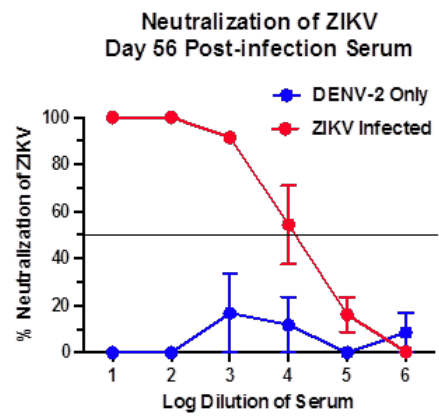


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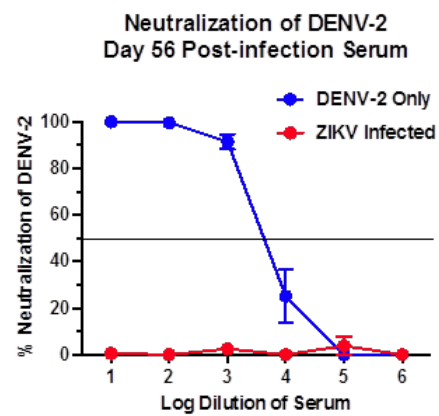
2106 **Figure 2: ZIKV infected animals display little or no cross-neutralizing antibody responses**
2107 **against DENV-2.** Percentage neutralization of (a) DENV-2 and (b) ZIKV by serum that was
2108 collected longitudinally from DENV-2 only (ZIKV naïve) (n = 4) and ZIKV infected (ZIKV
2109 immune) animals prior to DENV-2 infection (n = 5). Line represents PRNT₅₀ titres.
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2111 **Figure 2:**

a)



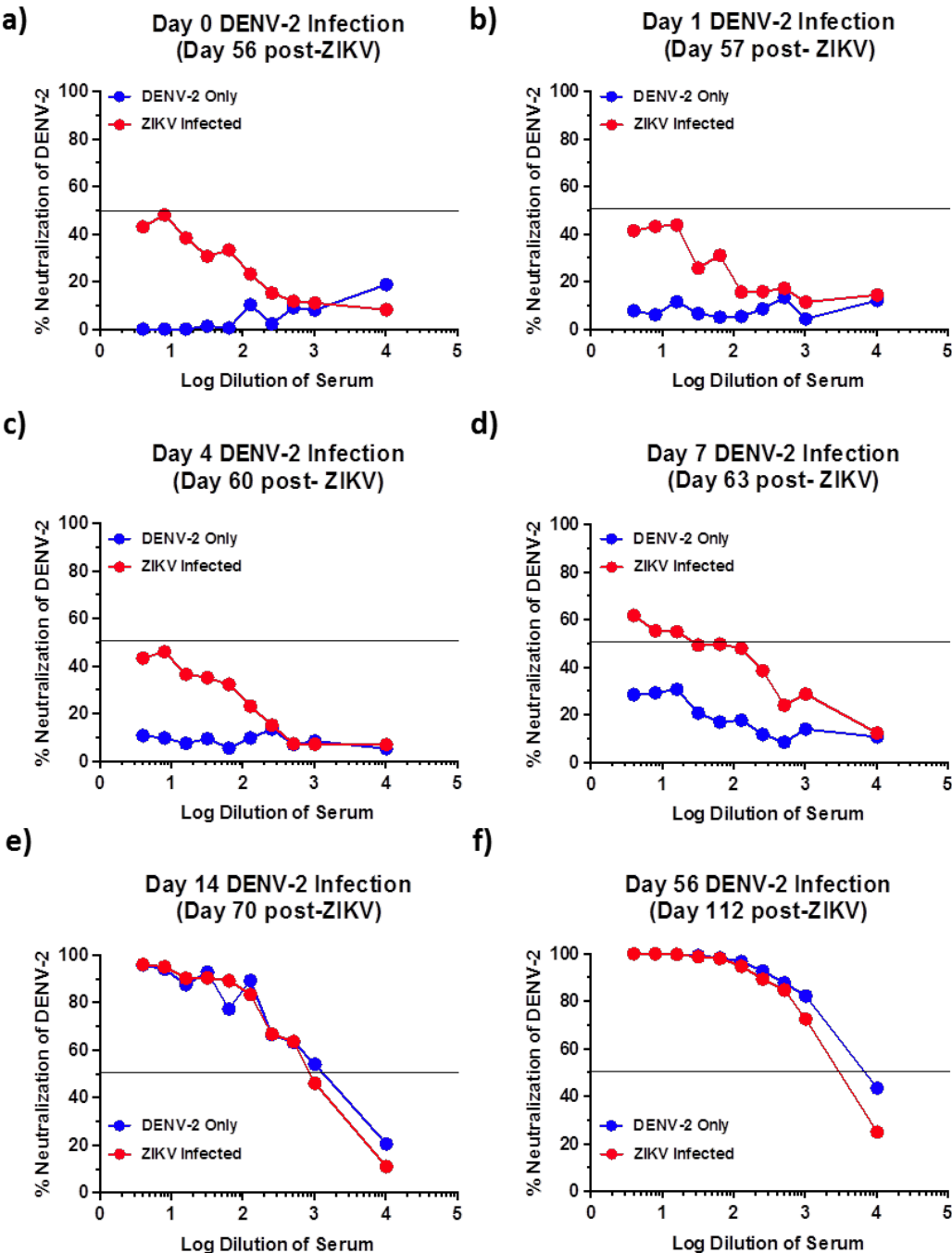
b)



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2114 **Figure 3: ZIKV immune animals display delayed anamnestic cross-neutralizing antibody**
2115 **responses after DENV-2 infection.** Kinetics of neutralizing antibody responses against DENV-
2116 2 using serum that was collected longitudinally from DENV-2 only (ZIKV naïve) and ZIKV
2117 infected (ZIKV immune) animals at a) day 0, b) day 1, c) day 4, d) day 7, e) day 14, and f) day
2118 56 after DENV-2 infection (n = 5). Line represents 50% neutralization.
2119

2120 **Figure 3:**



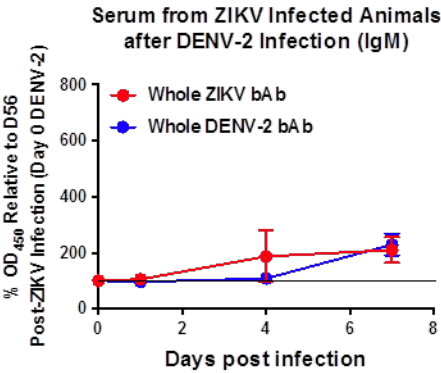
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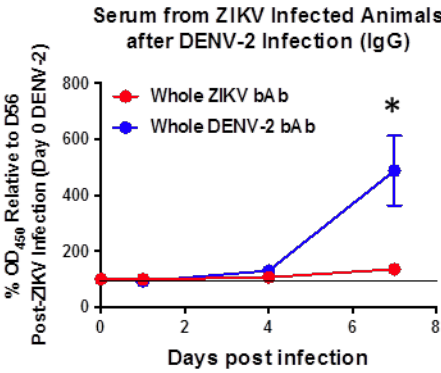
Figure 4: ZIKV immune animals display delayed binding antibody responses after DENV-2 infection. Kinetics of binding antibody (bAb) responses against DENV-2 in serum showing relative levels of a) IgM and (b) IgG against DENV-2 using serum that was collected longitudinally from ZIKV infected (ZIKV immune) animals 1, 4 and 7 days after DENV-2 infection (n = 5). Percentage OD₄₅₀ values relative to each animals day 56 post-ZIKV infection (day 0 DENV-2) values are shown. Line represents day 0 vales. Statistical significance was determined using multiple unpaired t-tests and corrected for multiple comparisons using the Holm-Sidak method. A $p < 0.05$ was considered significant and * indicates significant difference between groups.

2133 **Figure 4:**

a)



b)



2134
2135

Figure 5: IgG1 is the primary subclass of IgG in serum from ZIKV naïve and immune animals prior to and after infection with DENV-2. Relative levels of (a) DENV-2 and (b) ZIKV binding IgG1, IgG2, and IgG3 in serum that was collected at day 14 and day 56 post-infection from DENV-2 only (ZIKV naïve) (n = 4) infected animals. Relative levels of (a) DENV-2 and (b) ZIKV binding IgG1, IgG2, and IgG3 in serum that was collected at day 14 and day 56 post-infection from ZIKV infected (ZIKV immune) animals prior to DENV-2 infection (n = 5). Relative levels of (a) DENV-2 and (b) ZIKV binding IgG1, IgG2, and IgG3 in serum that was collected at day 1, 4, 7 and 56 post-infection from ZIKV infected (ZIKV immune) animals after DENV-2 infection (n = 5). Statistical significance was determined using One-way ANOVA and differences between time-points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error.

Figure 5:

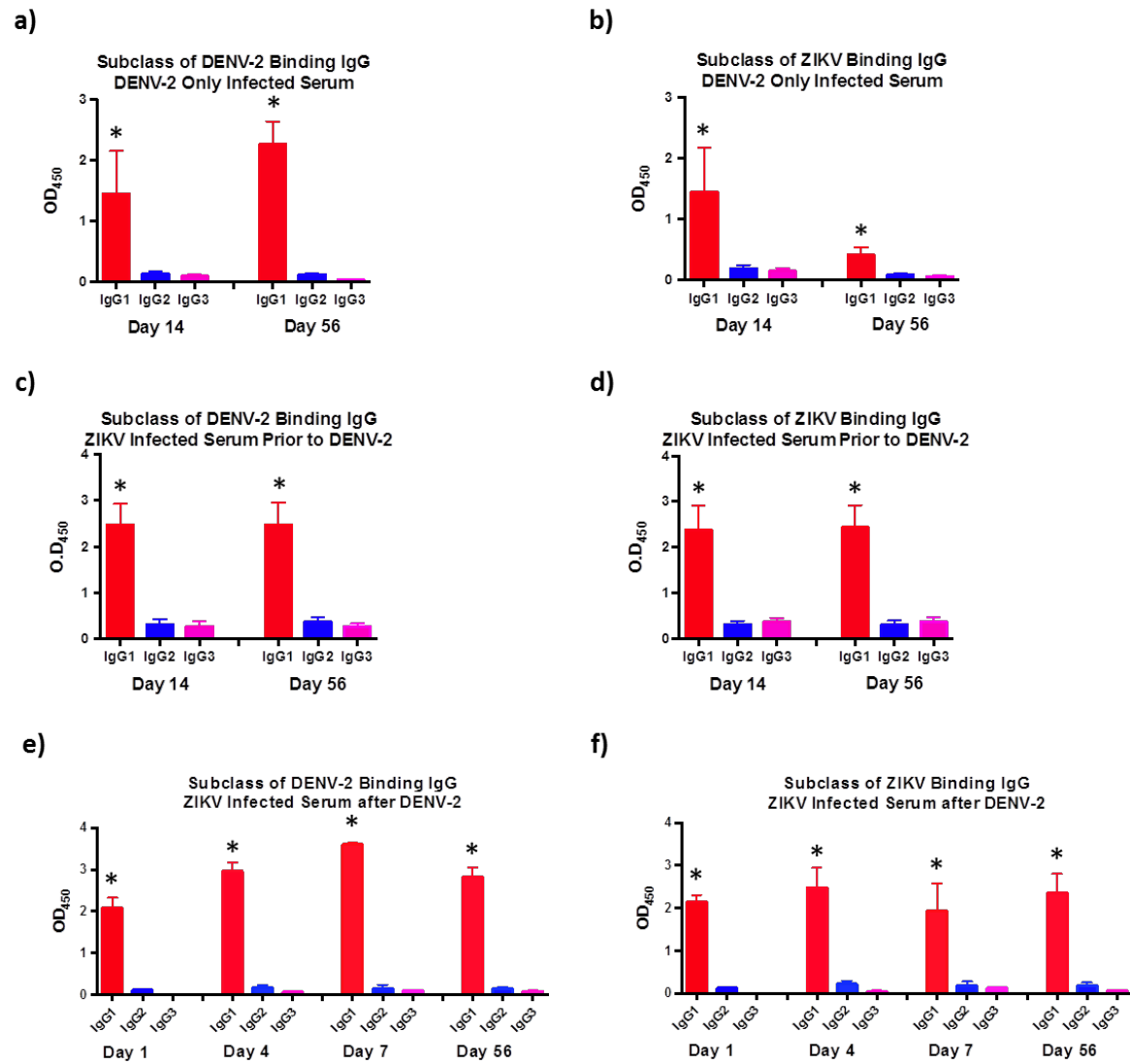
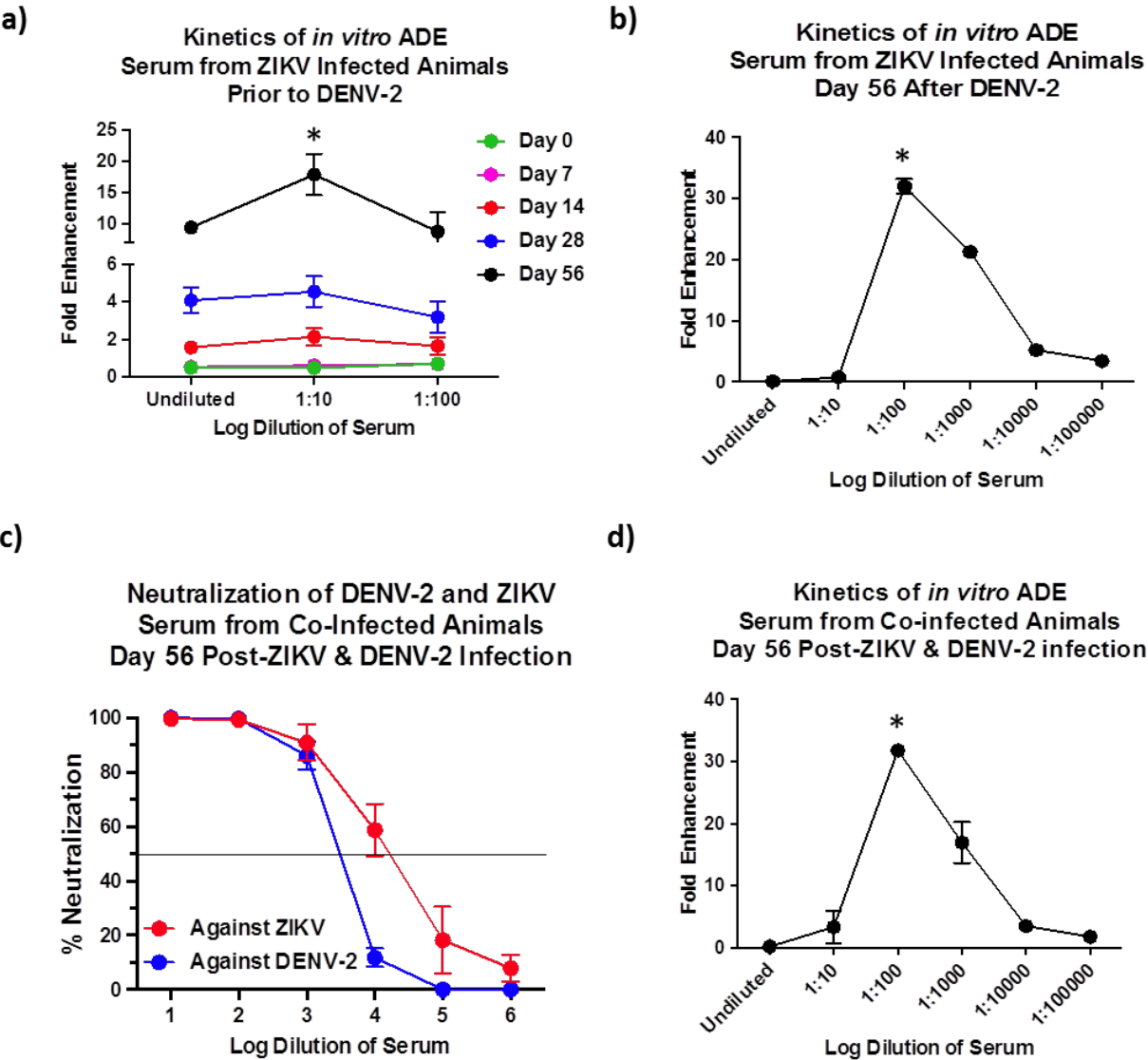


Figure 6: Serum from ZIKV immune animals infected with DENV-2 demonstrate antibody dependent enhancement at a dilution of 1:100. Fold enhancement of DENV-2 infection in K562 cells using serum that was collected a) prior to DENV-2 infection from ZIKV infected (ZIKV immune) animals (n = 5) and b) at day 56 after DENV-2 infection. Statistical significance was determined using One-way ANOVA and differences between time-points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. (c) Percentage neutralization of DENV-2 using serum that was collected at day 56 post-infection from animals that was simultaneously infected with ZIKV and DENV-2 (n = 5). (d) Fold enhancement of DENV-2 infection in K562 cells using serum that was collected at 56 days from rhesus macaques that were simultaneously infected with ZIKV and DENV-2 (n = 5). Statistical significance was determined using One-way ANOVA and differences between time-points were determined by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error.

2166 **Figure 6:**



2170 **Table 1: Kinetics of DENV-2 viremia**

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Days Post Infection	<i>Log Plasma viral loads $\pm$ SD*</i>	
	DENV-2 only infected animals	ZIKV exposed animals
1	1.7 $\pm$ 0.0	1.7 $\pm$ 0.0
2	3.1 $\pm$ 1.0	1.8 $\pm$ 0.2
3	3.1 $\pm$ 1.0	4.0 $\pm$ 0.7
4	3.5 $\pm$ 1.2	5.4 $\pm$ 0.4
5	4.7 $\pm$ 0.6	6.5 $\pm$ 0.6
7	4.4 $\pm$ 0.3	3.5 $\pm$ 1.3
14	2.3 $\pm$ 1.1	1.7 $\pm$ 0.0
28	1.7 $\pm$ 0.0	1.7 $\pm$ 0.0
56	1.7 $\pm$ 0.0	1.7 $\pm$ 0.0

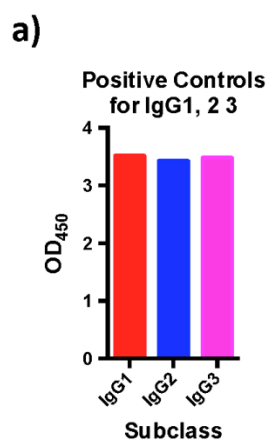
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2173 *SD = Standard deviation

2174

2175 **Supplementary Figure 1. Positive controls for subclass of IgG1, IgG2, and IgG3.** Relative
2176 levels of IgG1, IgG2, and IgG3 were determined using purified rhesus macaque IgG1, IgG2, and
2177 IgG3 coated plates.
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2179 **Supplementary Figure 1**
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2182

2183 **Chapter 4:**
2184 **Simultaneous Coinfection of Macaques with Zika and Dengue Viruses Does not Enhance**
2185 **Acute Plasma Viremia but Leads to Activation of Monocyte Subsets and Biphasic Release**
2186 **of Pro-inflammatory Cytokines**

2187 William G. Valiant, Mary J. Mattapallil, Stephen Higgs, Yan-Jang S. Huang, Dana L.
2188 Vanlandingham, Mark G. Lewis, & Joseph J. Mattapallil

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2194 Viruses Does not Enhance Acute Plasma Viremia but Leads to Activation of Monocyte Subsets
2195 and Biphasic Release of Pro-inflammatory Cytokines. *Scientific reports*, 9(1), 7877.”

2196

2197 **Abstract**

2198 The consequences of simultaneous infection with Zika (ZIKV) and Dengue (DENV)
2199 viruses are poorly understood. Here we show that rhesus macaques experimentally coinfectd
2200 simultaneously with ZIKV and DENV-2 demonstrated ZIKV or DENV replication without an
2201 enhancement of either infection. Coinfection was accompanied by an increase in the proportions
2202 of CD14+CD16+ pro-inflammatory subsets of monocytes and release of pro-inflammatory
2203 cytokines in the plasma. Numerous cytokines such as I-TAC, Eotaxin, RANTES, MCP-1, IFN γ
2204 and MIG demonstrated a biphasic peak that coincided with the differences in kinetics of ZIKV
2205 and DENV replication suggesting that viral replication likely differentially modulated the release
2206 of these cytokines. Red blood cell indices significantly declined during acute infection
2207 suggesting transient anemia, and was accompanied by elevated levels of muscle, liver and renal
2208 injury markers. These findings have implications for understanding the pathogenesis of
2209 coinfection in ZIKV and DENV endemic regions. This is the 1st report of an experimental
2210 coinfection using the rhesus macaque model for ZIKV and DENV infections.

2211 **Introduction**

2212 Zika virus (ZIKV) and dengue virus (DENV) are flaviviruses that are transmitted by
2213 *Aedes aegypti* mosquitoes and co-circulate in the same endemic regions. Numerous reports have
2214 documented coinfection of the same individual with both ZIKV and DENV¹⁻⁶. Monoinfection
2215 with either ZIKV or DENV usually causes mild febrile illness in most individuals though ZIKV
2216 infection in some pregnant women has been associated with congenital brain abnormalities in the
2217 newborn, and Guillain-Barré Syndrome in adults⁷⁻⁹, whereas secondary DENV infection has
2218 been shown to cause dengue hemorrhagic fever. Interestingly, secondary exposure to DENV
2219 after prior exposure to ZIKV has been associated with significant enhancement of infection that
2220 was associated with cross- reactive antibodies that were induced during primary infection with
2221 ZIKV¹⁰⁻¹³. As has been reported during antibody dependent enhancement (ADE) of DENV
2222 following infection with a heterologous serotype, the enhancement of DENV infection after
2223 ZIKV exposure was accompanied by the release of pro-inflammatory mediators and activation of
2224 monocyte/ macrophages¹⁰⁻¹³.

2225 Although the potential for ADE has been well documented following secondary exposure
2226 to a heterologous serotype of DENV, little is known about the pathogenic outcome of
2227 simultaneous infection with ZIKV and DENV. *Aedes aegypti* mosquitoes were shown to be
2228 infected with both ZIKV and DENV and capable of transmitting these viruses simultaneously
2229 suggesting that there is a potential for mosquitoes to transmit both viruses at the same time to the
2230 human host¹⁴. Chaves et al¹⁵ demonstrated that *A. aegypti* mosquitoes were highly permissive to
2231 coinfection with ZIKV and DENV and readily transmitted both viruses to BALB/c mice. In line
2232 with this, approximately 27% of arbovirus infected human subjects examined in Nicaragua were
2233 found to be viremic for ZIKV, DENV and CHIKV¹⁶. Likewise, detectable levels of ZIKV and

2234 DENV genomes were reported in the serum of two travelers who returned from French Polynesia
2235 and New Caledonia¹⁷.

2236 Other studies have reported coinfection with ZIKV and DENV in human patients.
2237 Carrillo-Hernandez et al¹⁸ reported that 6.7% of the 82 subjects they examined in Columbia for
2238 infection with ZIKV, DENV and Chikungunya (CHIKV) were co-infected with ZIKV and
2239 DENV. Azeredo et al¹⁹ reported that out of the 106 confirmed cases of ZIKV, DENV and
2240 CHIKV, 38% and 26.8% were infected with either DENV or ZIKV alone, whereas 13.4% of
2241 subjects were positive for both ZIKV and DENV. Estofolete CF et al²⁰ examined 1254
2242 suspected cases of arbovirus infections in Sao Jose Rio Preto between January and November of
2243 2016, and found that 12 of the subjects were co-infected with ZIKV and DENV. A majority of
2244 coinfecting individuals reported symptoms of myalgia, headache, fever, exanthema, arthralgia,
2245 and a minority of them reported conjunctival hyperemia, abdominal pain, and vomiting, whereas
2246 2/ 12 subjects reported alarm signs of DENV although none of them showed signs of severe
2247 dengue. We have previously shown that coinfection with ZIKV and DENV was associated
2248 induction of high levels of neutralizing antibodies against both viruses leading to a delayed
2249 induction of ADE^{12,21}. Other studies²² have shown that coinfection with ZIKV and DENV
2250 decreased the potential of CD4⁺ T cells to secrete cytokine such as IFN γ and TNF α ²².

2251 There is little information about the acute consequences of simultaneous coinfection with
2252 ZIKV and DENV. We sought to address this gap in our knowledge using the rhesus macaque
2253 model^{10,21,23-29} where we infected macaques with both ZIKV and DENV-2 simultaneously and
2254 assessed the effect on the kinetics of plasma viremia, plasma cytokine levels, and
2255 monocyte/macrophage activation. Our results show that both ZIKV and DENV replicated at
2256 levels similar to what have been reported in monoinfected animals without any enhancement of

viremia^{10,21}. Acute viremia was associated with activation of monocyte/ macrophage subsets and release of numerous pro-inflammatory mediators that have implications for pathogenesis in the coinfecting host.

Results

Coinfection with ZIKV and DENV-2 does not alter plasma viral kinetics

We have previously shown that prior exposure to ZIKV significantly enhanced DENV-2 viremia in rhesus macaques that was associated with high levels of DENV cross-reactive non-cross-neutralizing antibodies induced by ZIKV^{10,21}. We sought to determine if simultaneous infection with ZIKV and DENV-2 in the absence of cross-reactive antibody responses would lead to enhancement of infection. Rhesus macaques (n = 5) were infected simultaneously with ZIKV (10⁶ TCID₅₀) and DENV-2 (10⁵ TCID₅₀) at the same site subcutaneously. We examined ZIKV and DENV-2 viral loads in plasma samples that were collected longitudinally over a period of 8 weeks (Fig. 1). Our results showed that ZIKV viral loads peaked at 5 logs/ ml of plasma at day 3 post-infection (PI) whereas DENV-2 viral loads peaked at 4 logs/ ml of plasma as reported previously¹⁰ suggesting that coinfection did not have a substantial effect on the replication kinetics of both viruses. Surprisingly, ZIKV viremia was readily detectable at day 1 PI as compared to day 2 PI for DENV-2 yet early ZIKV replication was not found to enhance DENV-2 viremia. Low levels of ZIKV was detectable in 2/ 5 animals at day 7 PI whereas 4/ 5 animals had < 3 logs of DENV-2/ ml of plasma at day 7 PI. By day 14 PI both ZIKV and DENV-2 plasma viral loads were below the levels of detection.

Acute viral replication is associated with alterations in serum markers of tissue damage during coinfection with ZIKV and DENV-2

To determine if simultaneous infection with ZIKV and DENV was accompanied by tissue damage, we examined the levels of Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), Alkaline phosphatase, albumin and Creatine Phosphokinase (CPK), in serum samples that were collected longitudinally after infection and compared them to pre-infection values (Fig. 2a-f).

SGPT, also called Alanine aminotransferase (ALT) and SGOT, also called Aspartate aminotransferase (AST) are liver enzymes that play a role in hepatocyte integrity, whereas Alkaline phosphatase is a canicular enzyme that is essential for bile production³⁰. Serum albumin is a marker for liver function mass as albumin is synthesized in the liver³⁰. An increase in the serum levels of SGPT and SGOT is indicative of acute and chronic liver injury³¹. Our results showed that coinfection with ZIKV and DENV-2 significantly enhanced serum levels of both SGPT ($F(9,40) = 12.39, p < 0.0001$) and SGOT ($F(9,40) = 15.57, p < 0.0001$) during the 1st week of infection, with both enzymes reaching peak levels at day 4 PI, suggesting that coinfection is accompanied by increased levels of serum markers associated with acute liver injury. There was no significant difference in the level of Alkaline phosphatase whereas serum albumin levels ($F(9,40) = 3.003, p = 0.0079$) declined significantly following infection though the level of decline was variable. Previous studies have reported that ZIKV was isolated from the subjects who presented with jaundice during an outbreak in Africa^{32,33}. Wu et al³⁴ at reported that a patient infected with ZIKV experienced signs of liver injury, a decrease in albumin levels, and an increase in lactic dehydrogenase, alpha-hydroxybutyric dehydrogenase and creatine kinase in the serum. Others have reported similar changes in rhesus macaques infected with ZIKV³⁵, whereas increases in both SGPT and SGOT levels during acute stages of DENV infection have been well documented. There is, however, little or no information regarding changes in liver

enzymes during the early acute phase of coinfection with ZIKV and DENV. SGPT and SGOT levels declined to baseline or below baselines by day 7 – 14 PI.

CPK is a tissue specific enzyme that is significantly expressed only in skeletal muscle, heart and brain and studies have shown that serum levels of CPK are elevated during muscle injury³⁶⁻⁴². Coinfection with ZIKV and DENV-2 was associated with a significant increase in serum CPK levels during the 1st week of infection ($F(9,40) = 2.607, p = 0.0180$) suggesting that infection is likely accompanied by acute muscle damage.

Numerous studies have reported that ZIKV is shed in the urine for long periods of time, whereas others have reported that ZIKV damages the epithelial cells in the kidneys⁴³⁻⁵⁰. To determine if coinfection with ZIKV and DENV-2 was associated with abnormal kidney function, we examined the ratio of Blood Urea Nitrogen to Creatinine (B/C ratio) in serum that was collected longitudinally and compared them to pre-infection values (Fig 2f). The B/C ratio has been used extensively to examine kidney function and acute injury^{51,52}. Our results showed that B/C ratio was significantly elevated following infection with ZIKV and DENV-2 and stayed elevated though 8 weeks of infection ($F(9,40) = 3.960, p = 0.0011$). Taken together these results suggest that coinfection alters normal kidney function during the early stages of infection.

Red blood cell indices are significantly lower during the early acute phase of coinfection with ZIKV and DENV-2

To determine if coinfection was associated with changes in the red blood cell indices, we examined red blood cell (RBC), reticulocyte and platelet counts, hematocrit (HCT), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), and Mean Cell Volume (MCV) following coinfection with ZIKV and DENV-2 and compared them to preinfection values (Fig. 3a-g). We observed a significant drop in RBC counts ($F(9,40) =$

2326 12.39, $p < 0.0001$) and HCT ($F(9,40) = 7.180$, $p < 0.0001$) by day 2 PI that remained
2327 significantly lower till day 14 PI and recovered to baseline levels by day 28 PI (Fig. 3a-b). In
2328 contrast to RBC counts, platelet counts ($F(9,40) = 6.214$, $p < 0.0001$) declined marginally during
2329 the 1st 5 days of infection after which it increased significantly by day 7 PI and remain above
2330 baseline levels till day 56 PI (Fig. 3c). Interestingly, reticulocyte counts ($F(9,40) = 27.31$, $p <$
2331 0.0001) showed a significant decline relative to baseline during the 1st 5 days after infection and
2332 then significantly increased by day 7 PI to peak at day 14 PI and returned to baseline levels by
2333 day 56 PI (Fig. 3d). Previous studies have reported that 2/12 patients coinfecting with ZIKV and
2334 DENV experienced a decline in platelet counts²⁰.

2335 MCH measures hemoglobin (Hb) amount/ red cell and showed a steady decline and
2336 stayed below baseline levels during the 56 days of infection though this difference was not
2337 significant due to variation between animals (Fig. 3e). On the other hand, MCHC ($F(9,40) =$
2338 17.83 , $p < 0.0001$), which is a measure of the amount of Hb relative to cell size, remained at
2339 baseline levels till day 4 PI after which it significantly declined and stayed low till day 28 PI
2340 after which it recovered to near baseline levels by day 56 PI (Fig. 3f). MCV ($F(9,40) = 9.420$, p
2341 < 0.0001) which is a measure of the average size of red cells marginally declined steadily till day
2342 4 PI relative to baseline and then significantly increased at day 5 PI and stayed high till day 14 PI
2343 after which it declined to below baseline levels by day 56 PI (Fig. 3g).

2344 Taken together, the hematological changes described above suggests that coinfection
2345 with ZIKV and DENV-2 was associated with transient anemia during the acute phase of
2346 infection. The average body weight of the 5 animals (~ 7.2 Kg) used in the study did not change
2347 over the course of 8 weeks of infection. Additionally, minimal blood volumes were collected at
2348 each of the time points suggesting that these changes were not likely due to sampling.

Coinfection with ZIKV and DENV-2 is associated with a significant increase in pro-inflammatory cytokines during the course of infection

Previous studies have shown that high levels of pro-inflammatory mediators were associated with increased pathogenesis of flavivirus infections^{53,54}. To determine if coinfection with ZIKV and DENV was accompanied by release of cytokines, we quantified plasma cytokine levels using the Cytokine Monkey Magnetic 29-Plex Panel for Luminex™ Platform (ThermoFisher Scientific, Waltham, MA) that simultaneously quantifies 29 cytokines (FGF-basic, IL-1 β , G-CSF, IL-10, IL-6, IL-12, RANTES, Eotaxin, IL-17, MIP-1 α , GM-CSF, MIP-1 β , MCP-1, IL-15, EGF, IL-5, HGF, VEGF, IFN γ , MDC, I-TAC, MIF, IL-1RA, TNF α , IL-2, IP-10, MIG, IL-4 and IL-8) and compared them to pre-infection values (Fig. 4a-j and Suppl. Fig. 1).

We observed a significant increase in plasma levels of IL-17 ($F(8,36) = 6.634, p < 0.0001$), IL-4 ($F(8,36) = 23.23, p < 0.0001$), FGF ($F(8,36) = 3.294, p = 0.0064$), Eotaxin ($F(8,36) = 2.598, p = 0.0236$), RANTES ($F(8,36) = 2.831, p = 0.0152$), TNF α ($F(8,36) = 3.218, p = 0.0073$), MIG ($F(8,36) = 2.637, p = 0.0220$), I-TAC ($F(8,36) = 3.698, p = 0.0030$), MCP-1 ($F(8,36) = 5.482, p = 0.0001$), and MIP-1 β ($F(8,36) = 6.494, p < 0.0001$). Interestingly, Eotaxin, RANTES, MIG, I-TAC and MCP-1 demonstrated a biphasic peak with a lower peak at day 1 PI followed by a second higher peak at either day 5 or 7 PI. On the other hand, IL-17 and FGF (Fig. 4a and c) were significantly elevated during the 1st 3 days following infection after which they declined to baseline levels. In contrast, IL-4 (Fig. 4b) significantly increased at day 4 PI and remained elevated until day 14 PI. The rest of 19 cytokines in the panel did not differ significantly as compared to pre-infection values (Suppl. Fig. 1). Previous studies have shown that MCP-1, I-TAC and numerous other cytokines were significantly elevated during flavivirus infections^{10,55-60}. Macaques coinfecting with ZIKV and DENV-2 did not show signs of rash or

other symptoms that are normally associated with severe disease, which may be due to the limitations of the model.

Significant expansion of pro-inflammatory CD14⁺CD16⁺ monocytes during coinfection with ZIKV and DENV-2

Monocyte/macrophages are thought to be a major target cell for both ZIKV and DENV^{61,62} and a major source of pro-inflammatory cytokines⁶³. We examined the effect of coinfection on the absolute numbers of monocytes (Fig. 5a) and the frequency of monocyte/macrophage subsets in peripheral blood that was collected at day 1, 3, 4, 5, 7, 14, and 28 PI using flow cytometry and compared them to each animals day 0 values (Fig. 5b-c). Monocyte/macrophage subsets were discriminated based on the differential expression of CD14 and CD16 on Lin (CD3/8/20)⁻ HLA-DR⁺ myeloid cells and divided into classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺), non-classical (CD14⁻CD16⁺), and double negative (CD14⁻CD16⁻) subsets based on the classification reported in earlier studies⁶⁴.

There was no difference in the absolute numbers of peripheral blood monocytes during the course of infection as compared to pre-infection values (Fig. 5a). Although the absolute numbers of monocytes did not change, coinfection was associated with changes in the frequency of monocyte subsets (Fig. 5b-c). The frequency of CD14⁺CD16⁻ monocytes marginally increased following coinfection with ZIKV and DENV that did not significant differ from that of baseline. In contrast to CD14⁺CD16⁻ monocytes, the proportions of CD14⁺CD16⁺ monocyte subsets significantly increased following coinfection and stayed elevated even at day 28 PI ($F(7,32) = 2.644, p = 0.0282$). Previous studies have shown that the proportions of CD14⁺CD16⁺ subsets were increased during either ZIKV or DENV infections as compared to healthy controls^{10,55,56}. Others have reported that CD14⁺CD16⁺ monocytes were a primary source

of pro-inflammatory cytokines⁶⁵ suggesting that coinfection with ZIKV and DENV was likely associated significant activation of pro-inflammatory subsets of monocytes and release of pro-inflammatory mediators in response to ZIKV and DENV. There was no apparent difference in the proportion of CD14⁺CD16⁺ subsets relative to baseline that was most likely due to variation between animals, whereas the proportion of CD14⁺CD16⁺ subsets significantly declined during the first week after coinfection ($F(7,32) = 2.324, p = 0.0490$).

Discussion

Both ZIKV and DENV are endemic in the same regions and transmitted by *A. aegypti* mosquitoes that are prevalent in these regions. Although both these flaviviruses are known to cause mild disease in most people, there is limited information regarding the clinical outcomes of coinfection with both ZIKV and DENV. Recent studies have documented the potential for mosquitoes to be coinfecting with ZIKV and DENV and effectively transmit these infections simultaneously raising the possibility that people in endemic areas can be coinfecting with both the viruses. Alternatively, an individual could be infected by mosquitoes carrying either DENV or ZIKV and become coinfecting with both viruses. Numerous reports have documented cases of individuals who have been infected with both ZIKV and DENV though the consequences of such coinfections are poorly understood⁶⁶⁻⁶⁹. We, and others, have previously shown the prior exposure to ZIKV induces cross-reactive antibody responses that in the absence of cross-neutralization, significantly enhances DENV viremia. Interestingly, rhesus macaques that were simultaneously coinfecting with ZIKV and DENV-2 had plasma viral loads similar to what has been reported previously for ZIKV or DENV-2 monoinfecting animals¹⁰ suggesting that simultaneous coinfection did not modulate kinetics of either ZIKV or DENV-2 replication;

2418 ZIKV viremia was readily apparent at day 1 PI, whereas DENV viremia was detectable only at
2419 day 2 PI, however, there was no significant enhancement of DENV viremia in the presence of
2420 higher ZIKV viral loads or vice versa.

2421 The lack of significant changes in the kinetics of viral replication was somewhat of a
2422 surprise given the fact that the primary target cells for both ZIKV and DENV are
2423 monocytes/macrophages. Although coinfection was not accompanied by a significant change in
2424 the absolute number of monocytes in the blood, we observed a shift in the phenotype of
2425 monocyte/macrophage subsets with a significant increase in the proportions of pro-inflammatory
2426 CD14⁺CD16⁺ monocyte subsets as early as day 1 PI, that expanded further over the course of
2427 infection. Similar changes have been reported in other viral infections^{70,71}. It is likely that ZIKV
2428 and DENV either replicate within different intracellular compartments of monocyte/macrophage
2429 subsets or that they infect and replicate in different target cells, since there is little or no evidence
2430 in the literature to show if ZIKV and DENV superinfect the same cells *in vivo*. The kinetics of
2431 viral replication, however, seems to suggest that both ZIKV and DENV likely replicate
2432 independent of the each other without significantly modulating each other's replication.
2433 Additional studies are needed to address this question in greater detail.

2434 Activation of monocyte/macrophages was accompanied by significant increases in
2435 numerous pro-inflammatory cytokines that have been implicated in the pathogenesis of DENV
2436 infections⁷². Interestingly, a number of cytokines (Eotaxin, RANTES, MIG, I-TAC and MCP-1)
2437 appeared to show a biphasic peak with a lower peak at day 1 PI that coincided with the onset of
2438 ZIKV viremia and a second higher peak at day 5 PI that coincided with peak DENV-2 viremia
2439 suggesting that coinfection with ZIKV and DENV-2 appear to differentially modulate host
2440 cytokine responses. A majority of these cytokines are known to be monocyte/macrophage

derived suggesting that activation of these subsets during coinfection likely drives the release of these pro-inflammatory cytokines. Interestingly, IL-17 and FGF was significantly upregulated only during the 1st 1-3 days PI whereas IL-4 was significantly elevated only after day 4 PI, a time point that coincided with a decline in ZIKV viremia. Previous studies have reported that DENV infection was associated with secretion of Th2 cytokines such as IL-4 and IL-10⁷³. Schaeffer et al⁷⁴ showed that infection of dermal CD14⁺ cells by DENV was significantly enhanced in the presence of IL-4, whereas Fernando et al⁷⁵ reported that both IL-10 and IL-17 were elevated during the early stages of severe DENV infection. Elevated levels of IL-10 and IP-10 were found to play a role in DENV disease severity associated with vascular leakage⁷⁶. Both IL-6 and MIP-1 were significantly elevated as early as day 1 PI and remained elevated through the course of infection suggesting that the pro-inflammatory environment persists for longer periods of time even after plasma viremia levels had declined to levels below the limits of detection.

Coinfection was accompanied by a dramatic decrease in Red blood cell indices namely, RBC counts and HCT during the 1st 14 days following infection, whereas MCH steadily declined starting at day 1 PI and remained below baseline levels even at day 56 PI. Platelet and reticulocyte counts declined below baseline levels during the 1st 5 days after coinfection after which they significantly increased. On the other hand, MCHC that is a measure of the amount of Hb relative to the size of RBC remained steady during the 1st 4 days followed by a significant decline below baseline levels. Taken together, these results suggest that coinfection was associated with anemia that became apparent as early as 2 days PI. Previous studies have reported that severe DENV infection was associated with anemia in some subjects⁶⁶⁻⁶⁹.

Although coinfection was not associated with altered viral kinetics, serum levels of muscle, liver and kidney injury markers such as CPK, SGOT, SGPT and B/C ratio significantly

increased during the early stages of infection and coincided with the kinetics of plasma viremia. Previous studies have shown that increased levels of CPK during DENV infections were associated with neuromuscular weakness and myositis⁷⁷⁻⁷⁹. Elevated levels of CPK was reported to correlate with rhabdomyolysis during DENV fever^{80,81}. Others have implicated TNF α as a myotoxic cytokine⁸². In support of this hypothesis, plasma TNF α levels were found to be significantly upregulated in animals coinfectd with ZIKV and DENV-2. Likewise numerous studies have reported an increase in markers of liver dysfunction such as SGOT and SGPT during severe DENV infections in human subjects that peaked around day 5-6^{75,77,83}, and incidence of acute kidney damage have been documented during DENV infections⁸⁴. Taken together, these results suggest that coinfection with ZIKV and DENV is associated with changes in markers of acute tissue injury.

In conclusion, our results show that macaques simultaneously coinfectd with ZIKV and DENV-2 do not significantly alter the acute kinetics of either ZIKV or DENV-2 plasma viremia but is accompanied by significant activation of pro-inflammatory monocyte/macrophage subsets and release of numerous pro-inflammatory mediators that have been shown to play a role in severe disease. Interestingly, these changes coincide with changes in Red blood cell indices, and serum levels of CPK, SGPT, SGOT and B/C ratio suggesting that coinfection was accompanied by transient anemia and acute tissue injury.

Methods

Animals, infection and samples

Healthy rhesus macaques of Indian origin (n = 5; 4 males and 1 female; average body weight of 7.2 Kg; average age 9 years) acquired by Bioqual Inc. (Rockville, MD) that were seronegative for ZIKV and DENV were used in this study. Animals were housed at Bioqual and cared for in accordance with local, state and federal policies in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility. All the animal experiments were performed as per protocols, that were reviewed and approved by Institutional Animal Care and Use Committee at Bioqual Inc. in accordance with relevant guidelines and regulations, and samples were obtained through a tissue sharing protocol. All five animals were infected subcutaneously with 1 ml of 10^6 TCID₅₀/ ml of Zika virus (Puerto Rico Strain; Genbank KU501215) and 10^5 TCID₅₀/ ml of DENV-2 virus (strain 16681) at the same time and site. The challenge titer was based on previous studies^{10,85,86}.

Peripheral blood samples were collected longitudinally at day 0, 1, 2, 3, 4, 5, 7, 14, 28 and 56. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation and cryopreserved along with plasma and serum at each time point. Cumulative Blood Counts (CBC) including platelet and reticulocyte counts, Red blood cell indices (RBC counts, HCT, MCH, MCHC, MCV etc.) and serum chemistry was performed at IDEXX Laboratories, Inc. (Rockville, MD).

Absolute quantification of plasma viral loads by qRT-PCR

Plasma viral loads were determined by real-time quantitative RT-PCR using RNA that was obtained from plasma using the QIAamp MinElute Virus spin kit (Qiagen) and reverse transcribed using a mixture of random hexamers and anchored oligo-dT primers. Synthesized

2508 cDNA was PCR amplified using Zika (forward:
 2509 GGAAAAAAGAGGCTATGGAAATAATAAAG, reverse:
 2510 CTCCTTCCTAGCATTGATTATTCTCA, probe: AGTTCAAGAAAGATCTGGCTG) and
 2511 DENV-2 (forward: CAGGGTGTGGATTCAAGAAAACCCATGG, reverse:
 2512 TGCTTGTTAACCCAATCAATGAGCC, probe:
 2513 ACTCCAGTG/ZEN/GAATCATGGGAGGAAATCCCA) specific primers and probes^{10,87}. PCR
 2514 reactions were set up in triplicate using Taq-polymerase (Bioline USA, Inc., (Taunton, MA) and
 2515 assayed in the 7500 Taqman instrument (Applied Biosystems) under the following conditions: 48°C
 2516 for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 1 minute at
 2517 60°C. The number of ZIKV and DENV-2 copies were determined using ZIKV and DENV-2
 2518 standards as described previously¹⁰. The limit of detection was 50 copies / ml.

2519 **Cytokine levels in plasma**

2520 Plasma cytokine levels were determined using the Cytokine Monkey Magnetic 29-Plex
 2521 Panel for Luminex™ Platform (Thermofisher Scientific, Waltham, MA) that simultaneously
 2522 quantifies 29 cytokines namely, FGF-basic, IL-1 β , G-CSF, IL-10, IL-6, IL-12, RANTES,
 2523 Eotaxin, IL-17, MIP-1 α , GM-CSF, MIP-1 β , MCP-1, IL-15, EGF, IL-5, HGF, VEGF, IFN γ ,
 2524 MDC, I-TAC, MIF, IL-1RA, TNF α , IL-2, IP-10, MIG, IL-4 and IL-8. Plasma samples were
 2525 diluted at a ratio of 1:2 in assay diluent as per manufacturer's instructions. The assay was
 2526 repeated for each sample and the average concentration was determined for each cytokine. Plates
 2527 were analyzed using Luminex xMAP technology on a Bio-plex 200 system (Biorad). Collected
 2528 data was analyzed, and the concentrations were determined using Bioplex manager software 6.1.
 2529 The operator was blinded to the identity of the samples prior to each assay and data was
 2530 unblinded after analysis.

Antibodies and flow cytometry

Peripheral blood mononuclear cells (PBMC) were labeled with a panel of anti-CD3-Pacific blue (PB), CD8-PB, VIVID live dead stain, CD20-PB, CD14-FITC, HLA-DR-ECD and CD16-Cy-7-APC. Monocyte subsets were discriminated based on the differential expression of CD14 and CD16 on CD3/CD8/20⁻HLA-DR⁺ myeloid cells. All the antibodies were titrated using rhesus macaque PBMC. Labeled cells were washed and fixed in 0.5% PFA and analyzed on an LSR-II flow cytometer. One million total events were collected for analysis. Collected data was analyzed using Flowjo 9.6 software. The operator was blinded to the identity of the samples and data was unblinded after analysis.

Data analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Prism Software, Inc. San Diego, CA). Differences between time points were determined using One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparisons test. A $p < 0.05$ was considered significant. Error bars represent standard error. All data generated during this study are included in the manuscript and available on request.

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Author Contributions

WGV and MJM performed all the experiments and analyzed the data; JJM designed and supervised the study; WGV, MJM, SH, Y-JSH, DLV, MGL and JJM wrote the paper.

Declaration of interests

The authors declare no competing interests.

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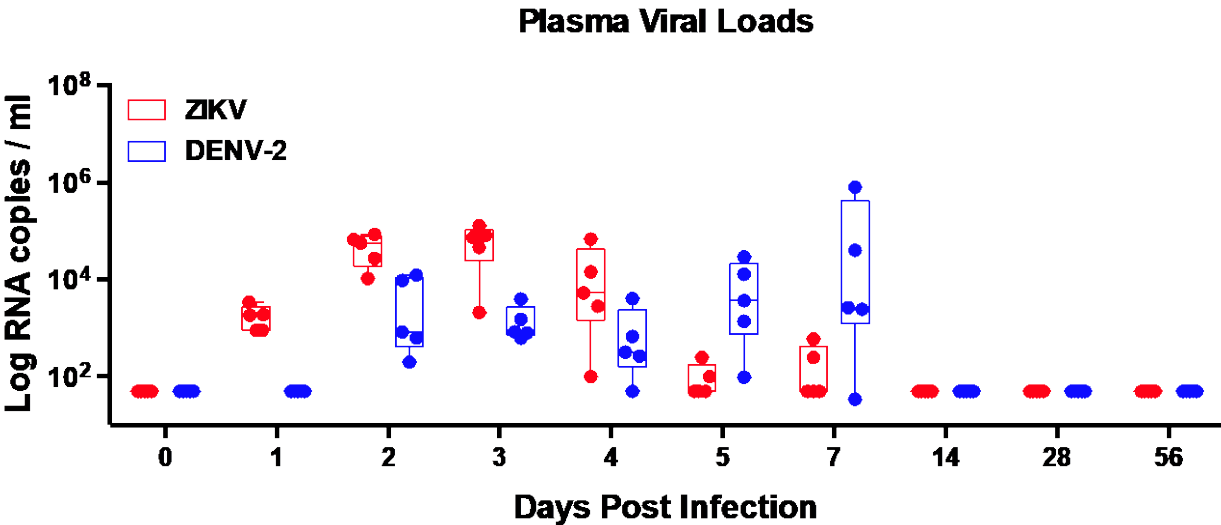
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2816

2817 **Figure 1: Simultaneous coinfection with ZIKV and DENV-2 does not alter kinetics of**
2818 **plasma viremia *in vivo*.** The kinetics of ZIKV (red) and DENV-2 (blue) viral loads in plasma of
2819 rhesus macaques (n = 5) that was simultaneously coinfectd with both viruses at the same site
2820 subcutaneously. Error bars represent standard error.
2821

2822 Figure 1:



2823

Figure 2: Serum markers of tissue injury are significantly elevated during the acute phase of coinfection with ZIKV and DENV-2. Kinetics of (a) SGPT (Serum glutamic pyruvic transaminase), (b) SGOT (Serum glutamic oxaloacetic transaminase), (c) Alkaline Phosphatase, (d) serum Albumin, (e) CPK (Creatine Phosphokinase), and (f) BUN/ creatinine ratio (B/C ratio) in serum that was collected longitudinally from rhesus macaques (n = 5) coinfecting with ZIKV and DENV-2. Line represents day 0 values. Statistical differences were determined using One-way ANOVA and a $p < 0.05$ was considered significant. Error bars represent standard error.

2832 **Figure 2:**

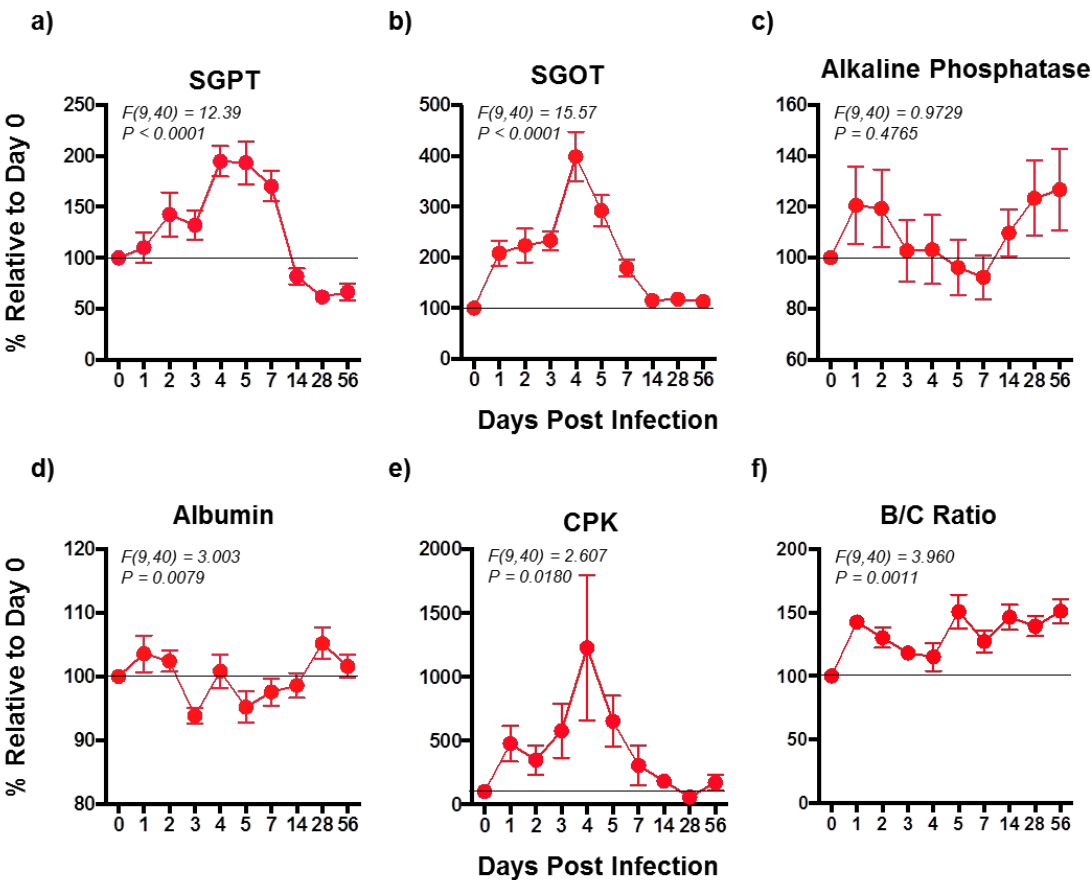
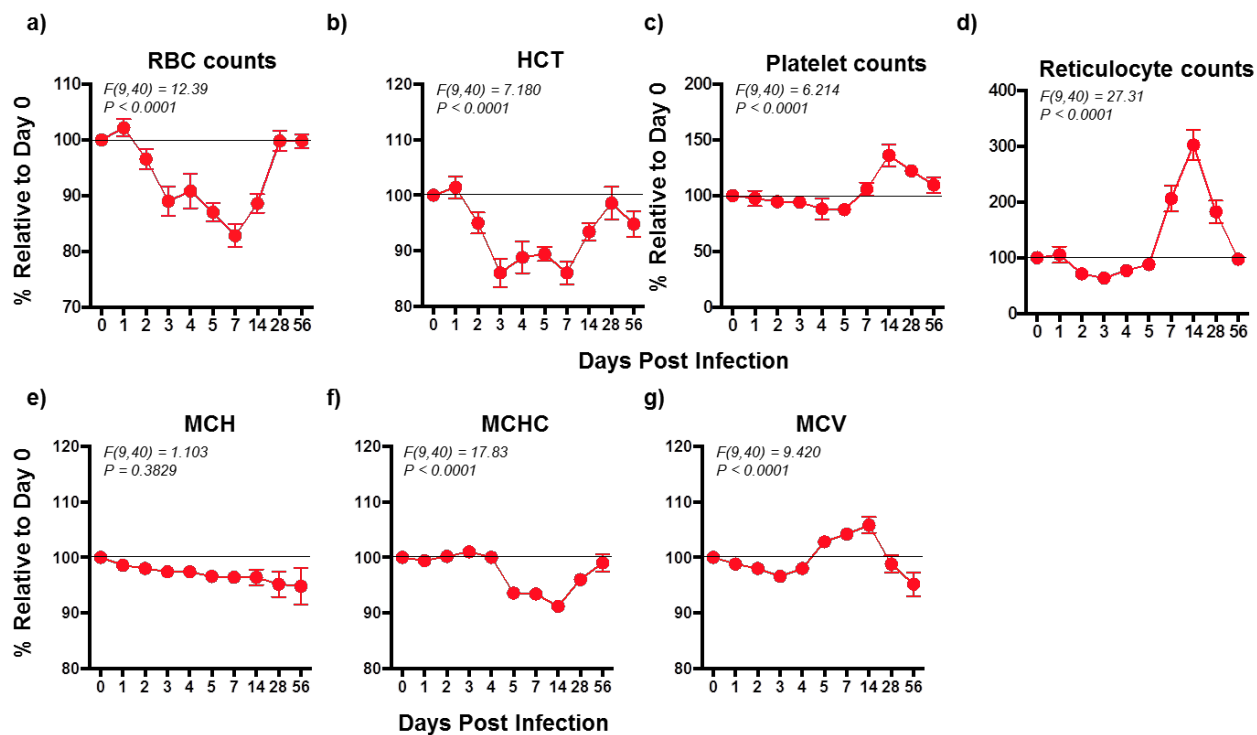


Figure 3: Red blood cell indices significantly decline during the acute phase of coinfection with ZIKV and DENV-2. Kinetics of (a) Red blood cell counts (RBC counts), (b) Hematocrit (HCT), (c) Platelet counts, (d) Reticulocyte counts, (e) Mean Corpuscular Hemoglobin (MCH), (f) Mean Corpuscular Hemoglobin Concentration (MCHC), and (g) Mean Cell Volume (MCV) in serum that was collected longitudinally from rhesus macaques ($n = 5$) coinfecting with ZIKV and DENV-2. Line represents day 0 values. Statistical differences were determined using One-way ANOVA and a $p < 0.05$ was considered significant. Error bars represent standard error.

2843 **Figure 3:**



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2845

Figure 4: Pro-inflammatory cytokine levels are significantly elevated during the acute phase of coinfection with ZIKV and DENV-2. Kinetics of (a) IL-17, (b) IL-4, (c) FGF, (d) Eotaxin, (e) RANTES, (f) TNF α , (g) MIG, (h) I-TAC, (i) MCP-1, and (j) MIP-1 in plasma that was collected longitudinally from rhesus macaques (n = 5) coinfecting with ZIKV and DENV-2. Statistical differences were determined using One-way ANOVA and $p < 0.05$ was considered significant. Error bars represent standard error.

Figure 4:

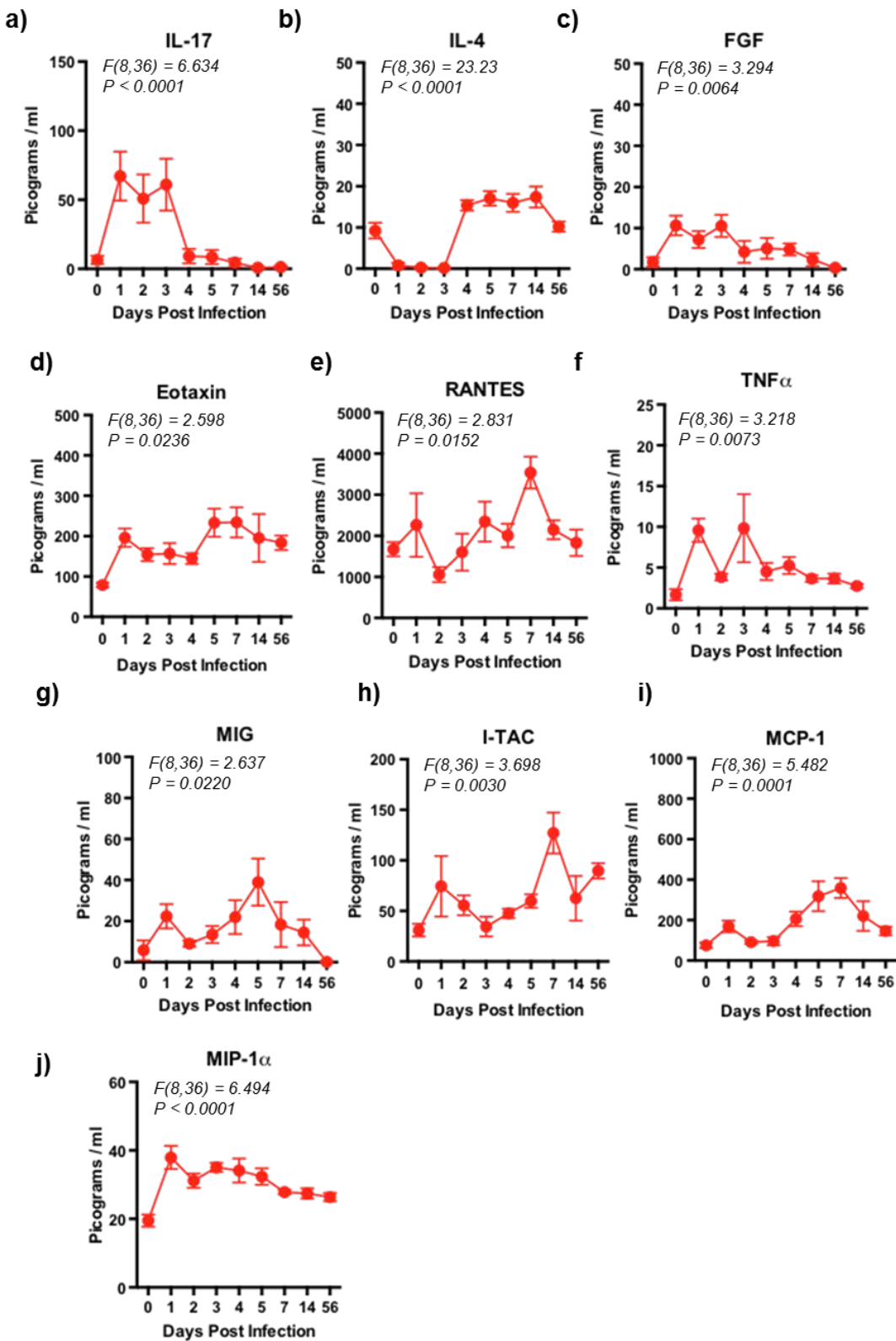
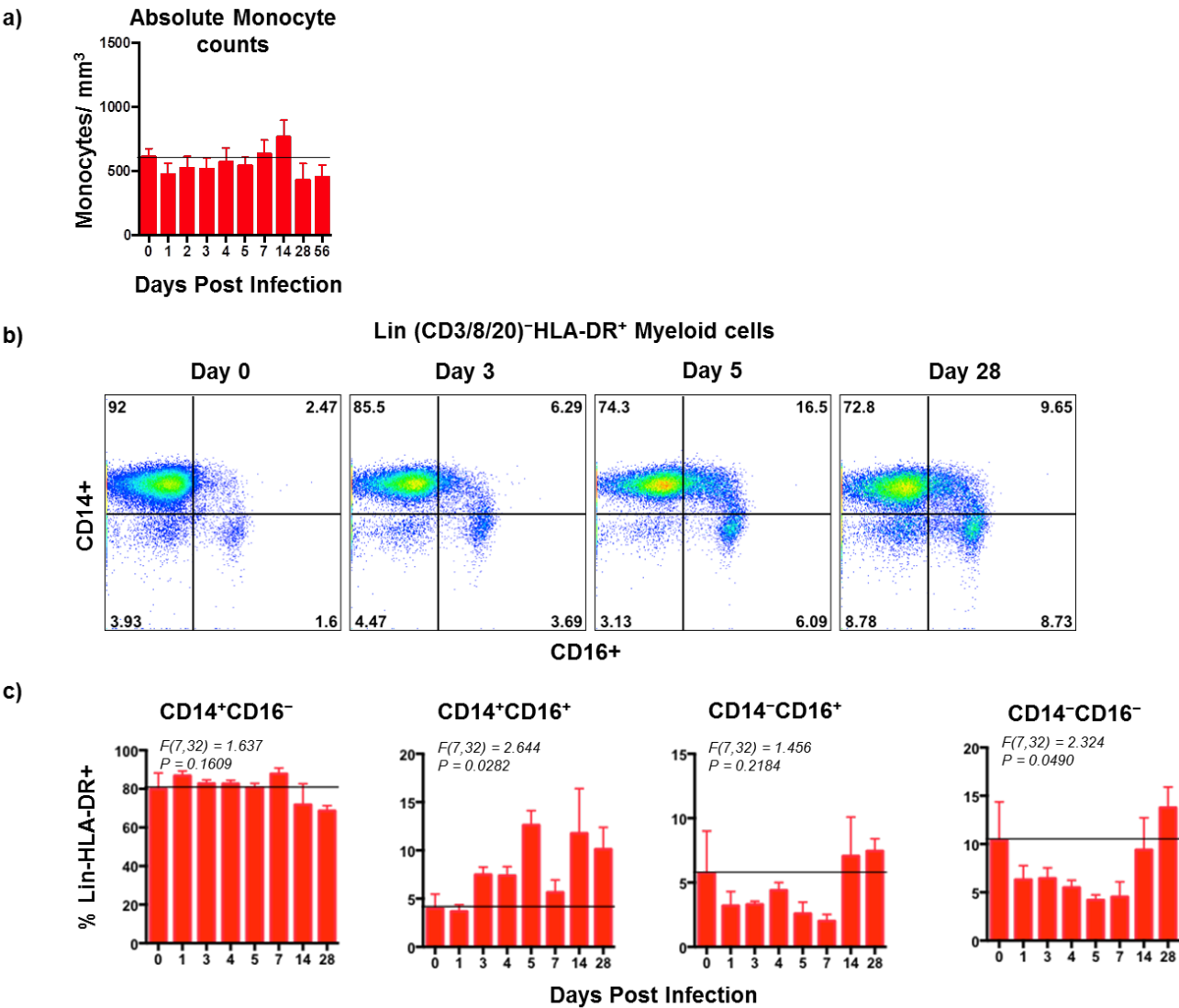


Figure 5: Proportions of pro-inflammatory monocyte subsets are significantly elevated during coinfection with ZIKV and DENV-2. (a) Absolute number of monocytes in peripheral blood, (b) representative dot plots showing changes in monocyte subsets in peripheral blood that was collected longitudinally from a single animal at day 0, 3, 5, and 28 after coinfection with ZIKV and DENV-2. Monocyte subsets were discriminated based on the expression of CD14 and CD16 on Lin (CD3/CD8/CD20)⁻HLA-DR⁺ myeloid cells. (c) Kinetics of peripheral blood monocyte subsets in rhesus macaques that were coinfectd with ZIKV and DENV-2. Line represents day 0 values. Statistical differences were determined using One-way ANOVA and $p < 0.05$ was considered significant. Error bars represent standard error.

Figure 5:



2872 **Chapter 5:**
2873
2874 **The Innate Response to Zika Virus Infection**
2875
2876 William G. Valiant
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Pathogenesis of ZIKV

The initial studies of Zika virus (ZIKV) found it to be neurotropic by its ability to infect neonatal mice through intracranial challenge¹. Since the 2014 outbreak in the Americas, further studies have elucidated the wide range of cells that ZIKV can infect. ZIKV, like Dengue virus (DENV) and other Flaviviruses, has been shown to infect cells of the skin and endothelial cells²⁻⁴, where the virus first enters the body from a mosquito bite, as well as within various immune cells such as monocytes and macrophages⁵⁻⁷. In addition to cell types generally associated with Flavivirus infection, ZIKV has also been shown to infect cells of the placenta, eye and brain⁸⁻¹². Along with cell culture-based tropism studies, rhesus macaques that are experimentally infected with ZIKV have detectable viral RNA in the brain, spinal cord lymph nodes, eyes, bladder, uterus, and seminal vesicles¹³. Taken together, these results show how ZIKV is able to infect a wide variety of cells in various tissues. To enter these cells, ZIKV is able to use a number of receptors such as AXL, DC-SIGN, TIM, and TAM^{2,3}. These molecules are expressed on a wide variety of cells such as astrocytes, endothelial cells, microglia, and monocytes³.

ZIKV is able to infect a wide variety of cells: ZIKV is able to infect skin and endothelial cells, at the site of infection, and immune cells as the virus becomes systemic. Additionally, cells of the reproductive tract and nervous system have been shown to be susceptible to ZIKV infection, facilitating the ability for ZIKV to be sexually transmitted and cause microcephaly in congenital zika syndrome respectively¹⁴⁻¹⁷. Additionally, ZIKV is able to infect, and cause damage to eyes. Adults infected with ZIKV have reported uveitis, an inflammation of the eye that can have serious complications including blindness^{18,19}. When ZIKV crosses the placenta, in addition to causing microcephaly, it can also cause eye related abnormalities. In two case series studies, which investigated rates of eye abnormalities, children with birth defects born to mothers

with suspected ZIKV infection in Brazil, 30% and 25% of infants had an ocular defect^{20,21}. The most common defects were focal pigment mottling and chorioretinal atrophy, both of which are a defect of the retina (Figure 1)²⁰. In a review of the data from the U.S. Zika Pregnancy and Infant Registry, 2% of children born from pregnancies with laboratory evidence of confirmed or possible Zika virus had an eye abnormality²².

Structure of the eye and retina

The eye is a complicated organ with a number of precise structures. The cornea, lens and iris compose the anterior section of the eye²³ (Figure 2A). Beneath the lens, the eye contains vitreous humor, which is composed of fine fibers intertwined that are attached to the surface of the retina. The retina consists of nerve cells and lines the back wall of the eye. It is responsible for sensing light and transmitting images to the optic nerve at the posterior of the eye. The retina is composed of many specialized cells (Figure 2B). Retinal pigment epithelium (RPE) cells make up the interior of the retina, and are responsible for supporting photoreceptors²⁴. The photoreceptors, rods and cones lie above the RPE and sense and respond to incoming photons. Bipolar, horizontal and retinal ganglion cells propagate the electrical signal generated by the photoreceptors²⁵. Amacrine cells are neural cells that interact with bipolar and ganglion cells to modulate their activity. Müller cells are a glial cell of the eye that span the length of the retina. Müller cells are responsible for maintenance of homeostasis, as well regeneration of photoreceptors following injury^{26,27}. The regenerative function of Müller cells is still under investigation, but Müller cells appear to have the ability to differentiate to a stem cell state and proliferate into other cell types, indicating their role in maintaining homeostasis and potential therapeutic use to treat retinal damage²⁷.

The innate immune response to ZIKV

Along with an adaptive response to ZIKV, the innate arm of the immune system also heavily responds to ZIKV infection. Following ZIKV infection, macaques experienced an increase in circulating NK cells²⁸. NK cells of CD16+ and CD16-CD56- lineage peaked between 2 and 4 days post infection²⁸. To combat this early rise in NK numbers, ZIKV is able avoid NK cell detection by increasing expression MHC Class I on infected cells²⁹. Similar trends were found in mice infected with DENV, where early activity of NK cells played a role in clearance of DENV infection. Along with an increase in NK cells during ZIKV infection, proinflammatory monocytes are significantly activated within circulating PBMC³⁰.

An interferon (IFN) response accompanies the cellular activation during ZIKV infection. When wild type mice are infected with ZIKV, they will have a transient to undetectable viremia and will not present with any symptoms^{31–33}. This is due to the lack of antagonism of the mouse IFN response by ZIKV proteins^{34,35}. Mice that are deficient in the interferon alpha receptor 1 (IFNAR1) will sustain a high viral burden of ZIKV following infection and mice will die about 6 days after infection^{32,33}. Similar effects are seen when IFNAR neutralizing antibody is injected into mice prior to infection³³. Beyond blocking the binding of IFN, if mice lack Irf3, Irf5 and Irf7 they will also succumb to ZIKV infection, even in the presence of IFN signaling³³. Similarly, STAT1 deficiency in mice will lead to death from ZIKV infection³⁶. These results are mirrored in mice infected with other species of Flaviviruses. Mice lacking IFNAR1 will support DENV viremia, and infection with mouse adapted DENV is lethal³⁷. Mice infected with a vaccine strain of YFV will succumb to infection in the absence of IFNAR1 or STAT1³⁸. Taken together, these studies show the importance of the IFN α response for the control of ZIKV within mice.

The importance of the IFN response to ZIKV infection is also highlighted by the interactions between ZIKV and proteins involved in the response to IFN. When cells are infected

with ZIKV, they have decreased ability to produce IFN stimulated genes (ISG) following stimulation³⁵. ZIKV accomplishes this by degrading STAT-2 within the cell^{34,35}. STAT2 is required for cellular signaling following binding of the IFN receptors on the surface of the cell^{39,40}. ZIKV NS5 degrades STAT2 within the cytoplasm, leading to the lack of IFN signaling³⁴. A number of ZIKV nonstructural proteins, NS2A, NS2B, NS4A, NS4B, and NS5, inhibit the activation of the IFN β promoter⁴¹. In addition, ZIKV NS1 has been found to interact with TBK-1, preventing its phosphorylation, leading to a decrease in IFN β production⁴¹.

Treatment of cells with IFN has been found to protect cells from ZIKV infection. Cells treated with IFN α , IFN β or IFN γ prior to infection did not produce detectable ZIKV in the supernatant following infection⁴². Another study found that cells treated with IFN α at concentrations greater than 100U/mL inhibited ZIKV replication when used to treat cells following infection⁴³. IFN α was found to prevent ZIKV replication in a dose-dependent manner, when Vero cells were treated prior to infection⁴⁴.

In addition to the IFN response, ZIKV also induces inflammasome activation. Tappe et al. detected that individuals in the recovery phase of ZIKV infection had higher levels of IL-1 β circulating within their plasma, which is a marker of inflammasome activation⁴⁵. He et al. identified that mice, like humans, have high levels of IL-1 β in their plasma following ZIKV infection, and that this was due to activation of the NLRP3 inflammasome⁴⁶. Interestingly, inactivated ZIKV is unable to stimulate an inflammasome response however cells transfected by ZIKV NS5 alone did produce IL-1 β ⁴⁶. This finding of inflammasome activation during ZIKV infection has been supported by other groups, identifying inflammasome activation in both human cell lines and mice^{47,48}.

Stimulation of the IFN response

As ZIKV enters and replicates within a cell, a number of pathogen associated molecular patterns (PAMP) are brought into the cell that are recognized by cellular pattern recognition receptors (PRR) (Figure 3). Binding of the PRRs can result in production of IFN, an innate immune mechanism that prevents viral replication. Since ZIKV is a positive sense RNA virus, PRRs that detect dsRNA and ssRNA are important to infection. TLR7 and TLR8 are able to recognize single stranded RNA, and are found within endosomes where ZIKV will initially enter cells, making them important for the recognition of ZIKV^{49,50}. PKR, RIG-I and TLR-3 are able to recognize double stranded RNA, which is produced as ZIKV RNA-dependent RNA polymerase replicates the negative strand of the genome during viral replication⁵¹⁻⁵⁴. When human dendritic cells were infected with DENV, IFN λ 1 was produced in a TLR3 dependent manner⁵⁵. PKR has also been identified as an important PRR in defense against other Flaviviruses⁵⁶.

After a PRR is activated by ZIKV, there is a signaling cascade that leads to IFN production with different signaling proteins used depending on the PRR. TLR7 and TLR8 will bind to MYD88, leading to phosphorylation of IRAK1 and IRAK4 proteins^{57,58}. The IRAKs will then phosphorylate IRF7, a transcription factor that leads to the production of IFN RNA^{57,59}. TLR3 interacts with TRIF to lead to phosphorylation of IRF3, which acts as a transcription factor⁶⁰. TLR3 associated TRIF is also able to activate the IKK pathway, leading to activation of NF κ B⁶¹. RIG-I and Mda-5 interact with IPS-1, I κ B kinase-related kinases to phosphorylate IRF3 and IRF7 which act as transcription factors, inducing the transcription of IFN genes^{52,62,63}. Once the IFNs are translated, they are secreted from the cell to signal in both an autocrine and paracrine manner⁶⁴.

Types of IFN

Generally innate IFNs can be divided into two categories, Type I and III (Table 1). Type I IFN consist of 7 different types, IFN β , IFN κ , IFN ϵ , IFN ω , IFN δ , IFN τ , and many subtypes of IFN α ⁶⁵. IFN γ is the only Type II IFN and there are three subtypes of IFN λ , the only Type III IFN⁶⁵. Each type of IFN has a separate receptor. Type I IFN bind to IFNAR1/2, Type II IFN bind to IFNGR1/2 and Type III IFN bind to IL10R and IL28R⁶⁵. IFNAR1/2 are found on almost all nucleated cells, while IL28R is preferentially expressed on epithelial cells⁶⁶. Both Type I and Type III IFN are involved in the antiviral response while Type II IFN is heavily involved in the M1 phenotype of macrophages and the Th1 response^{67,68}. Both IFN α and IFN λ have multiple subtypes. The 13 subtypes of IFN α each bind to their receptors at different affinities and have varied levels of antiviral activity⁶⁹. The three subtypes of Type III IFN, IFN λ 1, IFN λ 2 and IFN λ 3, despite having similar functions and activation pathways as Type I IFN are a distinct set of IFN^{68,70}. There is potentially a fourth subtype of IFN λ , IFN λ 4. Some humans have a mutation that allows production of IFN λ 4, an IFN that is associated with poor clearance of Hepatitis C Virus⁷¹. The expression of the different IFN subtypes is dependent on tissue and cell type and different IFN subtype expression profiles are produced, depending on the infecting virus^{72,73}.

The cellular response to IFN α

When IFN α binds to its receptor on the surface of the cell, transcription is induced through the Jak-STAT pathway (Figure 4). The kinases JAK1 and TYK2 are associated with IFNAR1 and IFNAR2 and upon binding of Type I IFN STAT1 and STAT2 are phosphorylated³⁹. Phosphorylated STAT1 and STAT2 will interact with IRF9 to form IFN-stimulated transcription factor (ISGF3)^{39,74}. ISGF3 binds to interferon-stimulated response elements (ISRE), leading to transcription of Interferon Stimulated Genes (ISG). Additionally Type I IFN binding can lead to ISG production through the mitogen-activated protein kinase p38

cascade⁷⁵. There are over 300 different ISG that are induced by Type I IFN binding, with production of these genes resulting in an antiviral state within the neighboring cells^{74,76}. Cells that are in an antiviral state can become immune to infection which can lead to prevention of the virus spreading from an infected, IFN producing cell.

A number of ISGs that are induced by Type I IFNs are involved in the propagation or induction of IFN signaling, sensitizing the cells to further IFN stimulation. These include additional PRRs, kinases and transcription factors. The up-regulation of genes such as STAT1 and IRF1 prevents viral proteins from inhibiting IFN signaling and also causes further IFN signaling to be more potent⁷⁶. In addition to up-regulation of IFN signaling genes, many proteins involved with the defense against virus infection are also up-regulated. These antiviral ISGs have a variety of functions and include deaminases including ADAR1 , exonucleases such as ISG20, translational regulators such as IFIT1 and IFIT2, and ribonucleases such as RNaseL^{74,76}. There are also ISGs that can induce apoptosis: CXCL10 is an ISG that acts as an apoptosis inducing chemokine⁷⁷. Overall, the expression of ISGs causes cells to enter an antiviral state where they are more sensitive to PAMPS and IFN signaling, and also express genes that directly have antiviral properties.

The cellular response to IFN λ

Similar to IFNAR1/2, IL10R and IL29R signal through JAK1 and TYK2, leading to production of ISGF3 (Figure 4)^{66,74,78}. Production of ISGF3, like in Type I IFN signaling results in transcription of many ISGs. Additionally, in some cell types, Type III IFN also activates the JAK2 signaling cascade, which is also activated by Type II IFN binding^{66,78,79}. Following phosphorylation of JAK2, a homodimer of STAT1 is phosphorylated and acts as a transcription factor. Additionally, there is evidence that Type III IFN binding can result in transcriptional

activation through STAT3 and Mitogen-Activated Protein Kinase signaling⁸⁰. The multiple signaling pathways can prevent inhibition of IFN activation by viral proteins.

Despite sharing a mechanism of ISG transcriptional activation, there are differences in the biological consequences of Type I and Type III IFN cellular activation due to differences in magnitude and kinetics⁸¹. Due to differential expression of receptors based on cell type, the antiviral effects of Type III IFN are more apparent at epithelial sites compared to Type I IFN. The ISGs induced by Type III IFN binding are a subset of the ISGs that are induced by Type I IFN^{66,82}. Similar to Type I IFN, ISGs with many different functions are produced following Type III IFN binding: translational regulators (IFIT1), apoptotic regulators (CXCL10) and proteins involved with IFN signaling (IRF7 and STAT2) are up-regulated in hepatocytes following treatment with IFN λ 2⁸³. Typically, type III IFN are less potent than Type I IFN, with higher levels of Type III IFN required for activation of cells, compared to Type I IFN⁶⁶. There are tissue specific differences, where studies have found that Type III signaling in epithelial cells results in transcription of some ISGs, including CXCL10 and IFIT3, at a higher level than Type I IFN⁸⁴.

The antiviral properties of IFN λ

Treatment of cells with IFN λ results in ISG production. These ISGs give the cells a detectable resistance to viral infection⁸⁵. Mice lacking IL28R experienced higher levels of influenza viremia⁸⁶. Mice were able to clear persistent norovirus infection following IFN λ treatment and mice lacking IL28R had higher viral loads within their stool⁸⁷. Macrophages treated with IFN λ had lowered levels of HIV reverse transcriptase activity⁸⁸. Treatment of T-cells prior to HIV infection was able to prevent infection and integration⁸⁹. The preventative effect of the IFN λ treatment was most potent if cells were treated prior to HIV infection,

although cells treated with IFN λ after infection with HIV still had lower levels of HIV reverse transcriptase activity⁸⁸. Conversely, when mouse keratinocytes and dendritic cells are treated with IFN λ then infected with West Nile virus (WNV) there was not a decrease in the level of viral replication⁹⁰. Despite the lack of protection *in vitro*, mice that lacked IL28R experienced higher levels of viremia and increased blood brain barrier permeability to WNV⁹⁰.

IFN λ and ZIKV infection

Due to the recent emergence of ZIKV there have been relatively few studies published on the viral interaction with IFN λ . Bayer et al. investigated ZIKV infection of primary human trophoblasts, finding that primary human trophoblasts were resistant to ZIKV infection while cell lines of trophoblast origin could be infected⁹¹. Primary human trophoblasts were shown to constantly secrete IFN λ , and when susceptible cells were cultured in conditioned media collected from primary human trophoblasts there was an increase in ISG activation accompanied by a decrease in ZIKV infection. Caine et al. demonstrated the relationship between hormones of the female reproductive tract and ZIKV susceptibility⁸⁴. These results indicated a role of IFN λ controlling ZIKV infection within the female reproductive tract, in a hormonal stage-dependent manner, where mice in the estrous phase are protected from ZIKV infection. Chen et al. infected IFNAR $^{-/-}$ mice with ZIKV during pregnancy, and showed lower viral loads within both fetus and mother if mice were treated with recombinant IFN λ at the time of infection⁹². This protection was implicated to be caused by ISG induction, with knockdown of MX1 resulting in an increase in ZIKV replication. Overall, treatment of cells with IFN λ at the time of or prior to infection has been shown to be resistant to ZIKV infection both *in vitro* and *in vivo*.

Conclusions

3084 Flavivirus infection is known to activate the innate immune response, through a variety of
3085 PRRs, such as RIGI and TLR, leading to the production of IFNs. The mechanisms of IFN
3086 production and ISG activation have been well described, although the interactions with ZIKV are
3087 currently under investigation. Studies have identified that ZIKV is able to inhibit the IFN
3088 response through a number of mechanisms. Despite the inhibition of the IFN response, Type I
3089 IFN have been shown to protect from ZIKV infection and initial evidence indicates that IFN λ
3090 may have the same effect. Further study of the dynamics of the IFN response to ZIKV and the
3091 effect that this IFN may have on ZIKV infection is required.

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3323 Zika Virus Infection in Mice. *Cell Host Microbe* **21**, 35–46 (2017).
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3326 **Table 1: Types of IFN and their receptors**
3327

Type		Subtype	Receptor
Type I	Alpha	1-13	IFNAR1/2
Type I	Beta	---	IFNAR1/2
Type I	Kappa	---	IFNAR1/2
Type I	Epsilon	---	IFNAR1/2
Type I	Omega	---	IFNAR1/2
Type I	Delta	---	IFNAR1/2
Type I	Tau	---	IFNAR1/2
Type II	Gamma	---	IFNGR1/2
Type III	Lambda	1-3	IL10R/IL28R

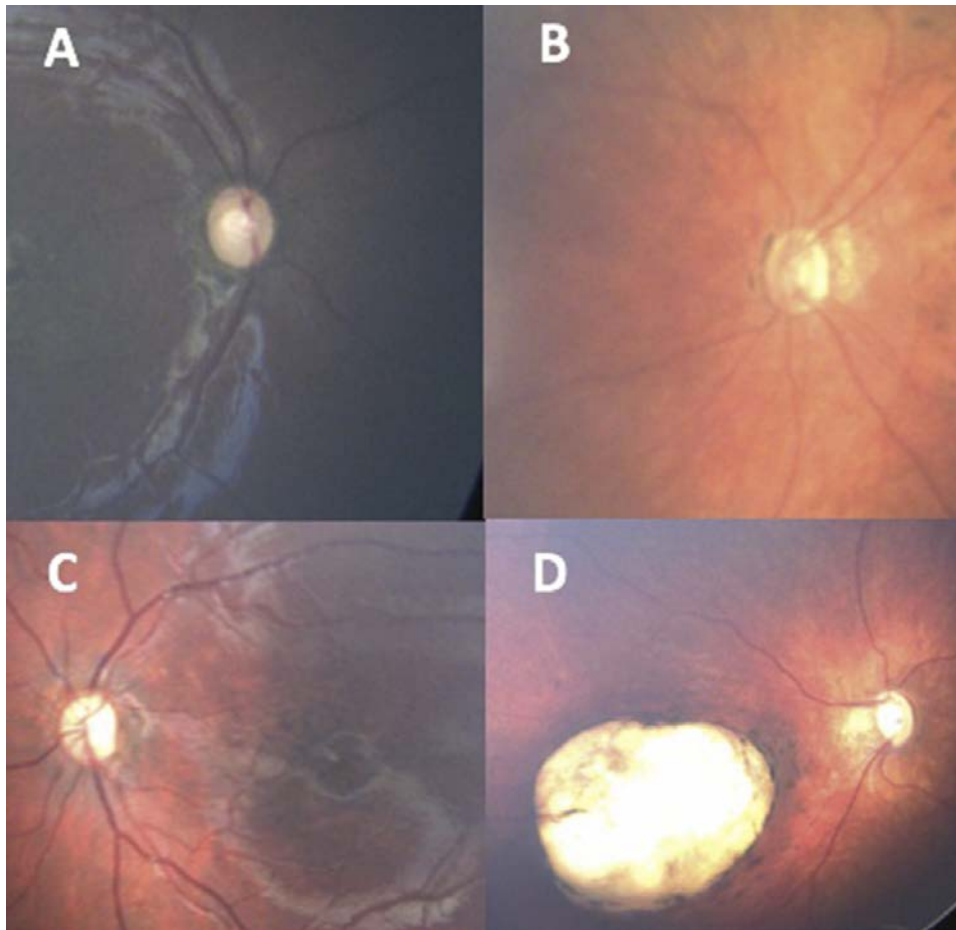
3328
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3330

Figure 1: Typical eye damage following ZIKV infection.

The eyes of a cohort of children born in Brazil to suspected ZIKV infected mothers were examined and monitored following birth. Representative images of damage to the optic nerve or retina are presented. A: atrophy of the optic nerve B: underdevelopment of the optic nerve C: pigment mottling of the retina with atrophy of the optic nerve D: chorioretinal scarring.

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3341 **Figure 1:**



3342
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3344 **Figure 2: Structure of the eye and retina**

3345 The structure of the eye (A) and cells of the retina (B). Within the retina, Müller cells,
3346 represented in green, span the length of the retina.

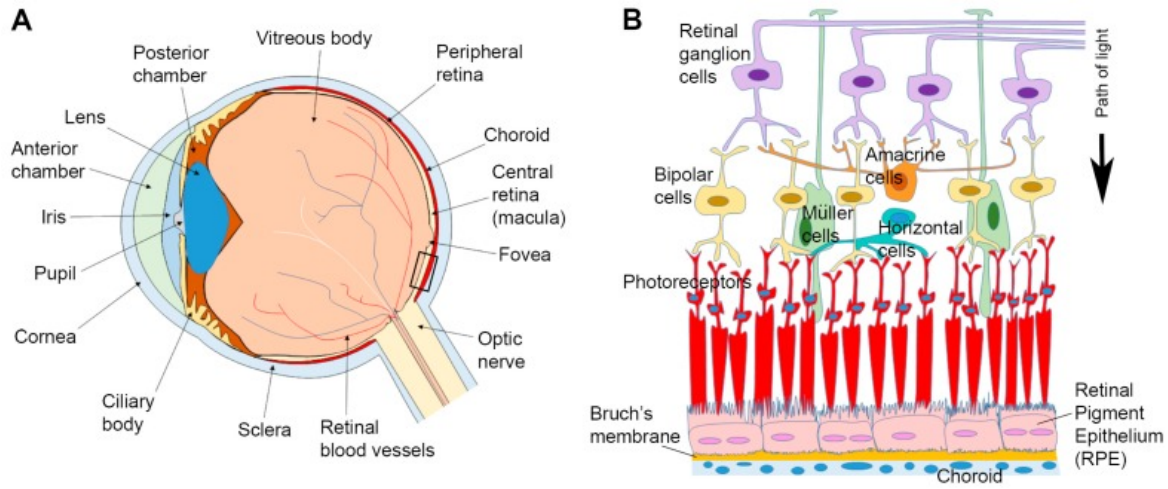
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3349 pigment epithelium (RPE) underlies irreversible blinding diseases." *Cells* 7.2 (2018): 16.

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3352 **Figure 2:**



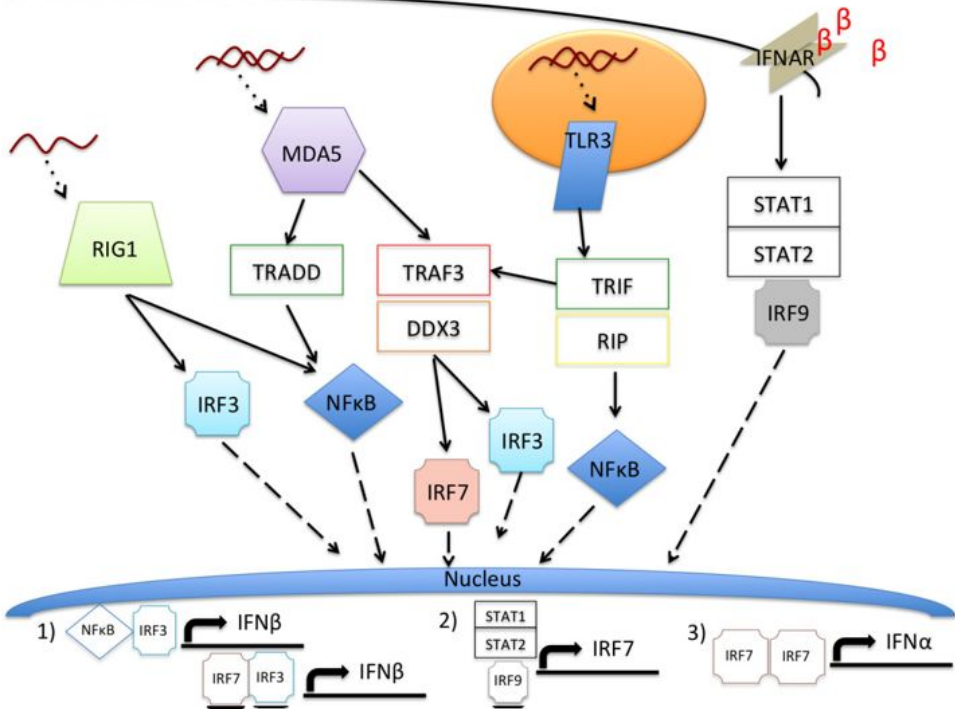
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Figure 3: Mechanisms of production of Type I IFN

PRRs such as RIG1, MDA5 and TLR-3 bind to viral RNA, leading to the eventual phosphorylation of transcription factors such as IRF3, NFκB and IRF7. These transcription factors initiate the transcription of IFN. As IFN are produced, they are secreted by the cell and go on to bind to receptors on the cell surface, or the surface of neighboring cells.

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3364 **Figure 3:**



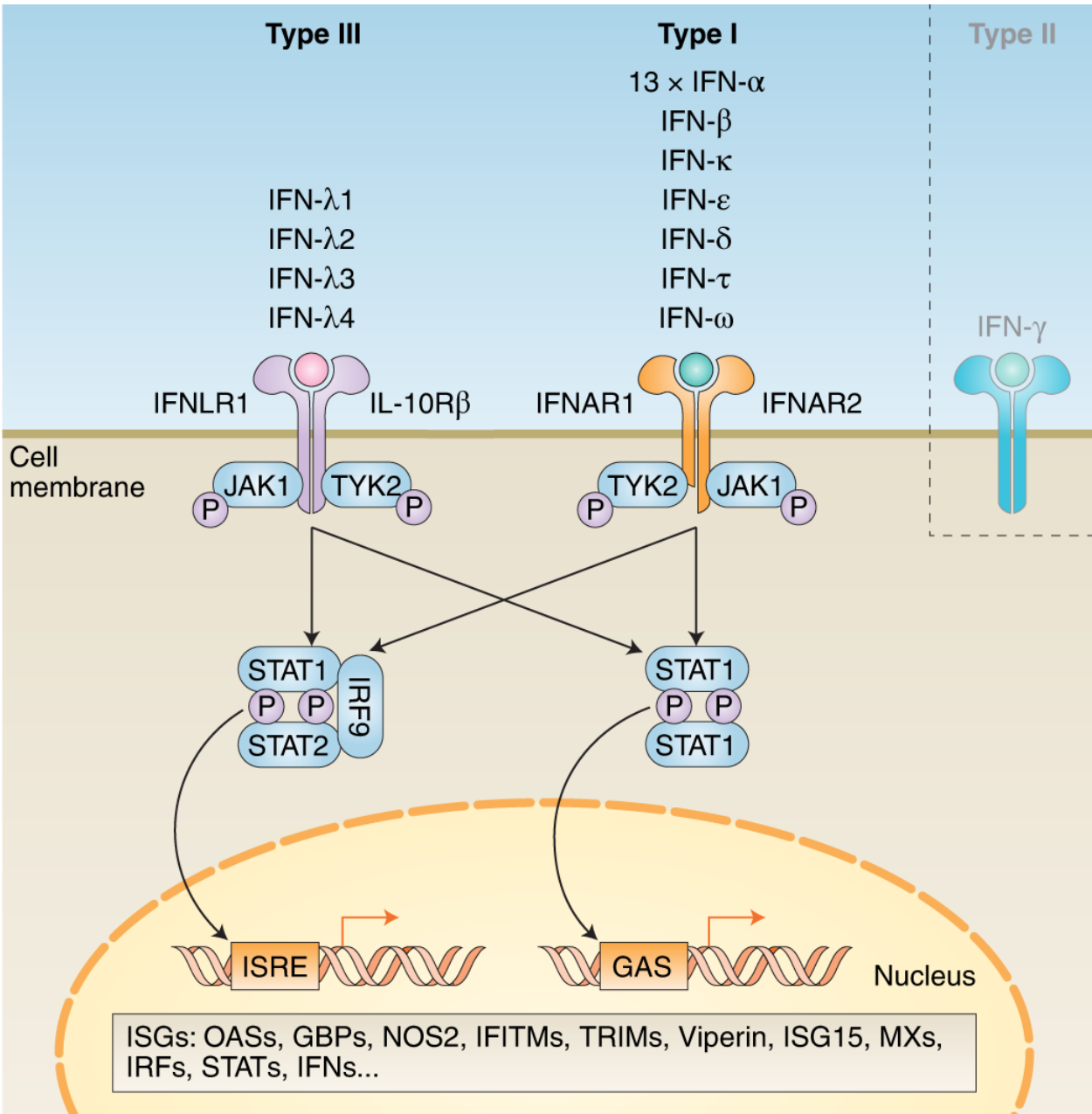
3365
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Figure 4: Mechanisms of ISG production

Type I IFN will bind to IFNAR1 and IFNAR2 and Type III IFN will bind to IFNLR1 (IL28R) and IL10R β . Following IFN binding, STAT1 and STAT2 will be phosphorylated and act as transcription factors to induce the expression of ISGs.

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Figure 4:



3382 **Chapter 6:**
3383
3384 **Zika Virus Infection of Müller Glia cells Leads to Production of Interferon Lambda,**
3385 **causing increased Viral Replication**
3386
3387 William G. Valiant

Abstract:

Ocular birth defects have been identified in children born with congenital zika syndrome, including damage to the retina and the optic nerve. Within the retina, Müller glial cells are responsible for maintenance of homeostasis and are implicated in regeneration following damage, and previous studies have identified Müller glial cells as the target of Zika virus (ZIKV) within the retina. The innate immune response has been shown to be induced following ZIKV infection, and is important for control of viremia, however within the eye the immune response is associated with the pathogenesis of many retinal diseases. We hypothesized that the innate immune response of Müller glial cells to ZIKV infection is unable to control viremia and could potentially contribute to the pathology observed in congenital zika syndrome. To understand the consequences of the innate immune response following ZIKV infection within the retina, MIO-M1 cells, an immortalized cell line of Müller glial cells, were infected with ZIKV. The MIO-M1 cells were productively infected and there was evidence of cellular death following infection. Following infection with ZIKV, MIO-M1 cells produced a large number of Interferon Stimulated Genes, along with a high level of Interferon lambda (IFN λ). The produced IFN λ was unable to suppress ZIKV infection, and blocking of the IFN λ response with neutralizing antibodies led to a decrease in ZIKV replication. These results implicate the potential therapeutic effect of blocking the IFN λ response and future studies will be required.

Introduction

Zika virus (ZIKV) was first identified in a sentinel monkey in 1947¹. Since its identification, ZIKV has emerged into the Americas as a serious public health threat². ZIKV is transmitted by the *Aedes spp.* mosquito and infection typically results in a mild febrile illness. ZIKV has also been associated with severe outcomes such as birth defects and Guillain-Barré Syndrome^{3,4}. A number of studies have investigated the connection between ZIKV infection and microcephaly, finding that ZIKV has tropism for cells of neural and placental origin. *In vitro* studies have found that cell lines of placental origin, such as HTR-8 and Jeg-3, are able to be infected by ZIKV⁵. Other groups have identified that ZIKV RNA can be found within tissues of the brain and placenta⁶.

In addition to being associated with microcephaly and neurological damage in fetal infections, ZIKV infection has also been associated with a number of eye-related abnormalities. Case reports have described adults infected with ZIKV experiencing uveitis, a condition that can lead to complications including retinal detachment, optic nerve damage or vision loss⁷. In a case series in Brazil, following children born with microcephaly assumed to be caused by ZIKV, over 30% of children had an eye defect⁸. The most common defect was focal pigment mottling and chorioretinal atrophy. In a large CDC study, 2% of all children evaluated for congenial zika syndrome within the United States had at least one eye abnormality⁹. When mice are injected with ZIKV intravitreally, they will develop retinal lesions¹⁰. The severity of the retinal lesions, as well as the viral load, was increased in mice that lacked ISG15¹⁰. Although ZIKV infection is more likely to result in microcephaly than result in damage to the eye, ZIKV complications involving the eye are still of great public health significance and warrant further study.

3430 The delicate and complex cellular mechanisms of the eye are vulnerable to damage by the
3431 immune system, and the eye is considered to be an immune privileged site¹¹. We sought to
3432 further study if ZIKV infection of the eye could cause an activation of the immune response,
3433 leading to ocular damage. The immune responses within the eye have been identified as an
3434 underlying factor of pathogenesis of many retinal diseases, and even appropriate levels of
3435 inflammation within the eye can be considered sight-threatening^{12,13}. Within the innate response,
3436 interferons (IFN) have been known to be heavily involved in ZIKV infection. Wild type mice
3437 will survive ZIKV infection, while mice that lack IFN α signaling, either through IFN α receptor
3438 (IFNAR) knockout or neutralizing antibody treatment, will die to ZIKV challenge^{14,15}. ZIKV has
3439 been shown to inhibit STAT-2 signaling to inhibit the response to IFNs^{16,17}. Although Type I
3440 IFN (α/β) have been heavily implicated in protection from ZIKV, additional studies have
3441 investigated the effect of Type III IFN (λ) on ZIKV infection. A cell line of human trophoblasts,
3442 when cultured in conditioned media containing IFN λ , were found to be resistant to ZIKV
3443 infection due to activation of IFN stimulated genes (ISG) such as MX1, CXCL11, GCA and
3444 PHF11¹⁸. Similarly, mice that are treated with IFNAR neutralizing antibody, when treated with
3445 recombinant IFN λ prior to infection were protected from ZIKV infection¹⁹. Beyond infection of
3446 adults, it was found that fetal outcomes improved in pregnant mice infected with ZIKV if they
3447 were treated with IFN λ prior to infection²⁰. The studies that have found IFN λ mediated
3448 protection from ZIKV share a number of similarities: IFN λ is found to be protective if cells or
3449 animals are treated prior to infection with ZIKV, and IFN λ activates ISGs, especially MX1.
3450 Interestingly, IFN λ is not naturally sufficient for the protection against ZIKV, as IFNAR^{-/-} mice,
3451 which are highly susceptible to ZIKV infection, still are able to produce and respond to IFN λ ²¹.

Since the eyes are an important target of ZIKV infection, a number of studies have investigated the tropism of ZIKV in cell lines generated from the eye. By florescent microscopy, ZIKV has been shown to infect cell lines of retinal pigment epithelium (RPE), retinal vascular endothelial, retinal Müller, choroidal endothelial, and retinal cone photoreceptor origin¹⁰. In general, cells of the eye express an innate immune response with upregulation of ISGs following ZIKV infection. The effect of ZIKV infection of retinal pigment epithelium cells (RPE) has been well described, and results in an innate immune response^{10,22}. Singh et al. identified production of Type I and Type II IFN, CXCL10, CCL5, MX1 and ISG15 by RPE and human retinal vascular endothelial cells following ZIKV infection¹⁰. RPE cells infected with ZIKV were shown to have increased permeability following infection²². *In vivo* infections of mouse eyes have found that Müller cells are infected within the retina and implicates Müller cells as the primary target of ZIKV within the retina²³. Additionally, primary Müller cells isolated from mouse eyes were able to be infected with ZIKV²³. Müller cells are glial cells of the retina that are responsible for support of the nervous cells and for the maintenance of homeostasis within the retina^{24,25}. MIO-M1 cells are a cell line that was spontaneously immortalized from human Müller cells²⁶. MIO-M1 cells have been shown to express markers associated with Müller cells and have basic electrophysiological properties of Müller cells²⁶.

Although the damaging effects of ZIKV infection within the eye are known, the mechanisms that result in either damage or protection have not been fully elucidated. We sought to understand how the innate immune response within the retina interacts with ZIKV, focusing on MIO-M1 cells as a model for Müller cells, the target of ZIKV within the retina. Müller cells have a role for supporting the other cells of the retina, and have also been shown to have the capacity to facilitate regeneration of the nervous tissues within the retina²⁴. Damage caused by

ZIKV infection of Müller cells may lead to a decrease in the retina's ability to recover, which could lead to ocular birth defects that are observed as part of congenital zika syndrome. Our goal was to understand the innate immune response to ZIKV infection within MIO-M1 cells. We have identified the production of IFN λ , but not IFN α , by MIO-M1 cells following ZIKV infection, which leads to induction of many ISGs and increased ZIKV replication.

Results:

ZIKV infection of MIO-M1 cells results in productive replication and cell death

Previous studies have identified that ZIKV infection of MIO-M1 cells leads to production of ZIKV proteins as detected by immunofluorescence. To determine if ZIKV could productively replicate within MIO-M1 cells, monolayers of cells were infected with ZIKV of the Asian lineage (ZIKVFSS13025) at a multiplicity of infection (MOI) of 0.5 or 0.01. Over the course of infection, supernatants were collected and the viral load was determined by plaque forming unit (PFU) assay in Vero cells (Figure 1A). Infection at either MOI resulted in an initial decrease in detectable PFU, indicating the eclipse phase, with a rise in detected virus at 24 hours post infection (hpi). The rise in detectable virus indicates that ZIKV is able to fully replicate within MIO-M1 cells and produce infectious virions. When monolayers of MIO-M1 cells were infected with ZIKV and overlaid with methylcellulose, plaques were observed 4 days post infection (Figure 1B,C). The plaques were large, with 2 plaques spanning the width of a 6-well plate, with an oblong shape. Monolayers of MIO-M1 cells, if infected with a high MOI of ZIKV will display CPE 48 hpi (Figure 1D,E). While uninfected MIO-M1 cells form a monolayer with few gaps and rectangular shaped cells, ZIKV infected MIO-M1 cells will become rounded and lose contact with the neighboring cells or detach. Taken together, these results show that MIO-M1

cells were productively infected by ZIKV, and ZIKV causes damage and death within these cells.

ZIKV infection induces ISG and IFN λ production in MIO-M1 cells

Since the eye is a predominantly protected immune site we were interested in the capacity of the innate immune pathways of Müller cells to react to ZIKV infection. MIO-M1 cells were infected with ZIKV, and 48 hpi, cellular RNA was collected from infected and mock infected cells. By 48 hpi, approximately 10^7 PFU/mL ZIKV was detected in the supernatant by plaque assay on Vero cells (Figure 2A). The collected RNA was used in a Nanostring assay, the nCounter® Human Immunology v2 Panel. This high throughput panel allows for a non-biased assessment of the innate immune activation of cells by measuring the expression of 594 general immunology genes. We found that many of the genes included in the panel were upregulated following ZIKV infection, with over 255 of the tested genes increasing more than 2.5 fold (Figure 2B). A number of the genes that are activated in response to ZIKV infection are ISG, such as CXCL10, and IFIT2 (Table 1). We additionally detected the production of IFN. The Nanostring assay did not identify induction of Type I, but did identify production of Type III IFN, with IFN λ 2 being upregulated by 390 fold and IFN λ 1 being upregulated 1600 fold following ZIKV infection (Table 2).

The Nanostring assay only had coverage of a limited set of the different subtypes of IFNs, so the expression of multiple IFN subtypes was determined by relative qPCR. MIO-M1 cells were infected with ZIKV, and 48 hpi, cellular RNA was collected from infected and mock infected cells. qRT-PCR was performed on the isolated RNA, and fold induction of various IFN subtypes was determined compared to uninfected cells and normalized to Beta Actin expression.

3520 Low levels of IFN α 1, IFN α 2a, IFN α 14 and IFN α 16 expression was detected, in contrast to
3521 100,000 to 10,000,000 fold induction of each subtype of IFN λ (Figure 3A).

3522 While the relative qPCR is able to identify the transcriptional state of cells, it is not able
3523 to identify the presence of secreted IFN protein. To measure the signaling capacity of the
3524 secreted IFN within the supernatant we used the HEK-Blue assay (Invivogen). The HEK-Blue
3525 assay uses either HEK-Blue α / β cells, which are able to detect the presence of only Type I IFNs,
3526 or HEK-Blue λ cells, which are able to detect the presence of only Type III IFN. These are cell
3527 lines, of a Human Endothelial Kidney lineage, that are transfected to express STAT-2 to have a
3528 complete signaling pathway downstream of the IFN receptors, and also have Secreted
3529 Embryonic Alkaline Phosphatase (SEAP) inserted downstream of ISG54. When IFNs bind to
3530 the receptor of these cells, SEAP is secreted into the supernatant and can be used in quantitative
3531 colorimetric assays. As supernatant collected from HEK-Blue cells is incubated with the
3532 substrate of SEAP, there will be a detectable colorimetric change proportional to the amount of
3533 IFN that bound to the receptors of the HEK-Blue cells. HEK-Blue α / β cells are transfected to
3534 express IFNAR1/2 at high levels but do not express IL28R, the receptor for Type III IFN, so
3535 SEAP will be produced in the presence of Type I IFN. HEK-Blue λ cells have IFNAR1/2
3536 knocked out and express IL28R, as well as IL10R, so SEAP will be produced in the presence of
3537 Type III IFN.

3538 HEK-Blue α / β cells produced a slight increase in SEAP when exposed to supernatants
3539 collected from ZIKV infected MIO-M1 cells: there was no detected increase in the level of Type
3540 I IFN production, except for a slight but significant increase at 48 hpi (Figure 3B). Despite the
3541 statistically significant increase, there was less than a 0.04 increase in the OD, which is likely not
3542 biologically relevant compared to a 1.15 increase in OD when HEK-Blue α / β cells are exposed to

recombinant IFN α . When this assay was repeated with HEK-Blue λ cells, supernatant collected 3 hours post ZIKV infection resulted in a significant increase in produced SEAP (Figure 3C). The magnitude of produced SEAP increased 24 and 48 hpi. Additionally, a commercially available ELISA was used to detect IFN λ in the supernatant, and 24 and 48 hpi IFN lambda was detected (Figure 3D). IFN α was not detected within the supernatant of ZIKV infected MIO-M1 cells by ELISA (data not shown). Taken together, these results show that ZIKV infection of MIO-M1 cells results in the induction of an IFN response, which is primarily IFN λ .

MIO-M1 cells respond to IFN λ with an ISG response.

Subsequently, we sought to determine the consequence of IFN λ production by MIO-M1 cells outside of the context of ZIVK infection. First, to confirm that MIO-M1 cells are able to respond to secreted IFN, the level of expression of IFN receptors was measured. Receptors for both Type I (IFNAR1 and IFNAR2) and Type III (IL10R and IL28R) IFN were detected by qPCR in uninfected and ZIKV infected MIO-M1 cells, although there was not a significant change in the level of expression following 48 hours of ZIKV infection (Figure 4A).

Additionally, when MIO-M1 cells are treated with individual subtypes of recombinant IFN λ , an ISG response is detected. There was a modest increase in CXCL10, IFIT-2 and MX1 expression 3 hours post treatment with each subtype of IFN λ , although IFN λ 2 induced CXCL10 expression at the highest magnitude (Figure 4B). The magnitude of induction increased by 6 hours post treatment, with the largest magnitude of induction caused by IFN λ 2 treatment (Figure 4C).

Similar to treatment with IFN λ , when MIO-M1 cells are infected with ZIKV, expression of CXCL10, IFIT-2 and MX1 is induced (Figure 4D). These results show that MIO-M1 cells are able to produce an ISG response following exposure to IFN λ , and that each subtype of IFN λ is able to activate the ISG production. Like IFN λ , treatment of MIO-M1 cells with IFN α induces

ISG expression indicating that these cells respond as efficiently to IFN α as IFN λ (Supplementary Figure 1, Supplementary Table 1).

Treatment of cells with IFN prior to ZIKV infection leads to a decrease in viral production

Previous studies have identified the protective effects of IFN in combatting Flavivirus infections^{27,28}. To identify the effects of IFN signaling on ZIKV infection, MIO-M1 cells were treated with increasing concentrations of IFN α and IFN λ for 24 hours prior to ZIKV infection. The IFN α used was an undefined mix of the subtypes of IFN α diluted between 0.01ng/mL and 10ng/mL. The IFN λ was an equal mixture of the three subtypes, with a total activity between 0.1U and 1000U. Cells treated with 1ng/mL IFN α and above had a significant drop in ZIKV RNA within the supernatant 48 hpi (Figure 5A). There was not a significant decrease in detected ZIKV when MIO-M1 cells were treated with IFN λ prior to infection, however there was a decrease in detected virus in cells treated with 1000U/mL IFN λ that trended towards significance (Figure 5A). Staining of cells treated with IFN prior to ZIKV infection supported the detected viral loads (Figure 5B). There was no detectable infection in cells treated with 1ng/ml IFN α , or above, while there was a decrease in the number of infected cells following IFN λ treatment, there was a detectable infection in cells treated with 1000U/mL.

Blocking IFN λ during ZIKV infection leads to a decrease in viral replication

Treating cells with IFN prior to ZIKV infection allows for the induction of an ISG response by a specific IFN, but once the cells are infected they will produce a natural IFN response. Since ZIKV-infected MIO-M1 cells did not produce Type I IFN following ZIKV infection, it is possible to directly study the impact of Type III IFN on the cells and ZIKV production. Neutralizing monoclonal antibodies against IFN λ 1, IFN λ 2 and IFN λ 3 were tested for their ability to prevent ISG induction when MIO-M1 cells were treated with recombinant IFN λ .

Each of the three neutralizing antibodies was able to prevent the induction of ISGs by the recombinant IFN subtype they are designed to neutralize (Supplementary Figure 2A-C). If all three neutralizing antibodies are added to the supernatant of ZIKV infected MIO-M1 cells at a concentration of 10ug/mL, at the time of infection, then there is not a detected SEAP response in HEK-Blue λ cells when incubated with supernatant collected 12 or 48 hpi (Supplementary Figure 2D). Since the HEK-Blue assay measures IFN binding to the cellular receptor, though the MIO-M1 cells may be producing IFN λ , the IFN will not be able to stimulate the cells in the presence of the neutralizing antibody. However, if only a single neutralizing antibody is added there is still a detectable IFN λ response, as the MIO-M1 cells produce each subtype of IFN λ following ZIKV infection (Supplementary Figure 2D).

Twelve hours post ZIKV infection in the presence of each IFN λ neutralizing antibody, MIO-M1 cells produced IFN λ at levels comparable to the absence of antibody (Figure 6A). In the presence of 1ug/mL, or less, IFN λ neutralizing antibody there was not a change of ISG expression following ZIKV infection, and in the presence of 10ug/mL IFN λ there was a drop in CXCL10 and IFIT-2 induction, although this drop was not significant (Figure 6B). Interestingly, there was a slight, but significant, increase in MX1 expression. Further investigation may be required to identify what pathways are inducing MX1 expression in the absence of IFN λ , although the slight activation could be induced by the low levels of detected IFN α . Treatment of MIO-M1 cells with 10ug/mL IFN λ neutralizing antibody is sufficient to decrease the signaling capacity of secreted IFN λ , as measured by the stimulation of HEK-Blue λ cells (Figure 6C).

When the IFN λ response to ZIKV is blocked, there is a decrease in viral replication. In the presence of 10ug/mL IFN λ neutralizing antibody, 12 hpi, there was a significant decrease in the number of copies of ZIKV RNA detected in the supernatant (Figure 6D). There was not a

decrease in ZIKV RNA when lower levels of IFN λ neutralizing antibody were used that do not fully neutralize the secreted IFN λ . Over the course of ZIKV infection, the presence of 10ug/mL of IFN λ neutralizing antibody was able to efficiently neutralize secreted IFN λ at 12, 24 and 48 hpi (Figure 7A). This neutralization resulted in a significant decrease in detected ZIKV RNA at 24 and 48 hpi (Figure 7B).

Discussion:

Since ZIKV emerged in the Americas, it has been associated with severe birth defects. ZIKV has been shown to cross the placenta and infect a developing fetus, potentially resulting in microcephaly or eye defects. Our results show that Müller cells were productively infected with ZIKV. These glial cells are important for maintaining homeostasis within the eye and are also implicated in regeneration of damaged retinal cells. ZIKV infection has been shown to induce a potent innate response within infected animal models characterized by cellular activation and IFN production^{14,15,29}. We investigated the innate immune response of MIO-M1 cells to ZIKV. ZIKV infection of MIO-M1 cells resulted in an induction of innate immune response-associated genes. A number of the genes that are upregulated within the MIO-M1 cells following infection are ISGs. The presence of these ISGs in a single cell culture indicates that the MIO-M1 cells produced IFN following ZIKV infection, leading to signaling within the cells. High levels of IFN λ , but no IFN α , were detected in the supernatant of ZIKV infected MIO-M1 cells by ELISA and HEK-Blue assay, as well as within the cells by qRT-PCR. Previous studies have identified Müller glial cells as a target for ZIKV infection within the eye²³.

Other groups have identified the interactions of IFN and ZIKV infection^{17,30,31}. Mice that lack IFN α signaling are susceptible to lethal ZIKV infection^{14,15}. These IFN α deficient mice

possess functional IFN λ signaling, and yet still die to ZIKV challenge. Our findings in the MIO-M1 cells seem to mirror previous findings in mice: IFN α treatment of cells makes them resistant to ZIKV infection, and treating cells prior to infection with high concentrations of IFN λ results in a decrease in ZIKV replication. We also found that despite the ability to produce IFN λ during infection, MIO-M1 cells are still susceptible to ZIKV infection, like IFN α deficient mice. We did not detect IFN α production by these cells and found that IFN λ treatment was sufficient for production of ISGs. Together these findings show that the MIO-M1 cells are undergoing an IFN λ mediated response to ZIKV infection that is not protective or able to control the viral infection.

Treatment of ZIKV infected MIO-M1 cells with neutralizing antibody resulted in a decrease in the level of ZIKV production, coinciding with a lack of ISG activation. While pre-treatment of MIO-M1 cells with IFN λ can provide a low level of protection from ZIKV infection, the production of IFN λ over the course of ZIKV infection results in an increase in viral production, suggesting that IFN λ stimulation of infected MIO-M1 cells generates increased overall transcription and protein production leading to an increase in ZIKV replication. Other studies that have investigated the effects of IFN λ on ZIKV have not focused on the impacts of IFN λ that is produced during infection, instead focusing on treatment of cells with IFN λ before or at the time of infection. Chen et al. treated mice with recombinant IFN λ 24 hours prior to and following ZIKV infection, observing a decrease in infection severity with IFN treatment²⁰. Interestingly, Bayer et al. found that the protective effects of IFN λ treatment of trophoblasts was lost if cells were treated after establishment of ZIKV infection supporting the need for IFN λ exposure before infection¹⁸. Caine et al. was able to investigate the effects of IFN λ produced during infection by infecting mice lacking IFN λ receptor¹⁹. Mice lacking an IFN λ response had

3658 slightly increased viral load within the female reproductive tract, but only in the absence of IFN α
3659 signaling and progesterone treatment¹⁹. While this finding conflicts with our results, the
3660 differences in IFN λ response may be due to the different body sites being studied, or the
3661 interaction between the additional hormones needed to establish ZIKV infection and IFN λ .
3662 Further studies comparing the IFN λ response within the eye *in vivo* will be required to fully
3663 understand potential differences.

3664 In conclusion, our results show that Type III IFN produced during ZIKV infection could
3665 contribute to ocular pathology seen in congenital zika syndrome. IFN λ stimulation of MIO-M1
3666 cells resulted in production of many genes associated with innate immunity, although this study
3667 was unable to identify a specific mechanism through which IFN λ causes increased ZIKV
3668 replication. Further studies investigating the mechanism of IFN λ mediated ZIKV replication
3669 could provide insight into how ZIKV leads to damage within the eye. Additionally, blocking of
3670 the IFN response could be utilized as a potential therapeutic to prevent ocular damage in
3671 congenital zika syndrome. Future studies using *in vivo* models of ocular damage could determine
3672 the potential therapeutic relevance of blocking IFN λ .

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3674

3675 **Methods:**

3676 Culture of cells and virus:

3677 Zika virus (ZIKVFSS13025, Asian Lineage) stocks were produced by collection of
3678 supernatant from infected Vero cells. Titer was determined by plaque forming unit assay on
3679 monolayers of Vero cells. MIO-M1 cells were cultured in complete DMEM-10 and were
3680 cultured at 37C, 5%CO₂. MIO-M1 cells were infected with ZIKV at an MOI of 0.5: media was
3681 removed from cells, and replaced with a low volume of DMEM-1 containing virus for one hour.
3682 Following infection, additional DMEM-1 was added to each well to maintain cells. Following
3683 infection, cells were treated with trypsin, collected and pelleted for RNA collection.

3684 Plaque assays:

3685 MIO-M1 cells were set to full confluence in 6-well plates. 300uL of serially diluted
3686 stocks of ZIKV was added to each well. After incubating the plates at 37 °C in 5% CO₂ for
3687 1 hour, 1 ml of 2x EMEM containing 10% FBS mixed with an equal volume of 2%
3688 methylcellulose, warmed to 37 C, was added to each well. The plates were incubated for a period
3689 of 4 days, fixed with 10% formalin and stained with hematoxylin and eosin then imaged.

3690 Detection of CPE:

3691 MIO-M1 cells were set to full confluence in 6-well plates. Media was removed and cells were
3692 infected with ZIKV at an MOI of 3. 48 hpi cells were imaged by light microscopy.

3693 Determination of PFU/mL:

3694 Vero cells were set to full confluence in 24-well plates. Supernatant collected from infected cells
3695 was serially diluted 10-fold, then used to infect the Vero cells. After incubating the plates at
3696 37 °C in 5% CO₂ for 1 hour, 1 ml of 2x EMEM containing 10% FBS mixed with an equal
3697 volume of 2% methylcellulose, warmed to 37 C, was added to each well. The plates were

3698 incubated for a period of 5 days, fixed with 10% formalin and stained with hematoxylin and
3699 eosin then the number of plaques per well was determined.

3700 Detection of ZIKV RNA:

3701 Viral loads were determined using real-time quantitative RT-PCR assay. RNA was extracted
3702 from the supernatant using the QIAamp MinElute Virus spin kit (Qiagen) and reverse transcribed
3703 using a mixture of random hexamers and anchored oligo-dT primers. Synthesized cDNA was
3704 PCR amplified using Zika (forward: GGAAAAAAGAGGCTATGGAAATAATAAAG, reverse:
3705 CTCCTTCCTAGCATTGATTATTCTCA, probe: AGTTCAAGAAAGATCTGGCTG) specific
3706 primers and probes. The PCR reaction was set up in duplicate using Taq-polymerase (Bioline
3707 USA, Inc., Taunton, MA) and assayed in the 7500 Taqman instrument (Applied Biosystems)
3708 using the following conditions: 48 °C for 30 minutes, 95 °C for 10 minutes followed by 40 cycles
3709 of 95 °C for 15 seconds and 1 minute at 60 °C. The number of ZIKV viral copies was determined
3710 using standards.

3711 Detection of cellular genes by qPCR:

3712 MIO-M1 cells infected with ZIKV were treated with trypsin, pelleted and frozen. Cellular RNA
3713 was collected with the RNeasy kit (Qiagen), quantified by nanodrop and cDNA was produced, as
3714 above. cDNA was amplified using Quantitect SYBR Green (Qiagen) according to
3715 manufacturer's instructions using primer sets listed in Supplementary Table 2. 100ng cDNA was
3716 used in each qPCR reaction. Fold induction of genes was determined by $\Delta\Delta CT$ against
3717 uninfected or untreated cells, using Beta Actin as a RNA standard.

3718 Nanostring assay:

3719 MIO-M1 cells were set in a 6-well plate to full confluence and infected with ZIKV at an MOI of
3720 0.5. 48 hpi, cells were treated with 0.25% trypsin, pelleted and frozen. Alternatively MIO-m1

3721 cells were treated with 100ng/mL recombinant IFN α for 24 hours (PBL assay bioscience), or
3722 untreated and uninfected for 48 hours. Cellular RNA was collected from the pellets using the
3723 Qiagen RNeasy kit. RNA concentration was determined by nanodrop. The nanostring assay was
3724 performed according to manufacturer's specifications. The absolute copies per reaction were
3725 calculated, compared to internal controls. The fold expression was determined by dividing the
3726 copy number of a given gene from infected cells by the copy number of that gene from
3727 uninfected cells.

3728 Treatment of cells with recombinant IFN

3729 MIO-M1 cells were seeded to full confluence in a 24 well plate. DMEM was removed and
3730 replaced with DMEM-1 containing IFN. IFN α (PBL assay science) was used in ng/mL, and
3731 contained an undefined mixture of the various subtypes of recombinant human IFN α . IFN λ (PBL
3732 assay science) was used in units/mL, using either a single subtype or an equal mixture of each of
3733 the three subtypes of IFN λ . After 24 hours of IFN treatment at 37C 5%CO₂, the media was
3734 removed and replaced with a low volume of media containing virus at an MOI of 0.5. After one
3735 hour of infection, media was added to the wells. Supernatant and cell pellets were harvested over
3736 the course of infection. Viral RNA and cellular RNA were collected and analyzed as above.

3737 Detection of IFN by ELISA:

3738 IFN λ was detected by a commercial ELSIA (PBL Assay Science). Manufacturer's instructions
3739 were followed. Briefly, half-area 96-well plates were bound with a capture antibody, blocked,
3740 then undiluted cell culture supernatant. Detection antibody was added, and the colorimetric
3741 change of TMB was determined at 450nm.

3742 Detection of IFN by HEK-Blue:

3743 HEK-Blue IFN α / β cells and HEK-Blue IFN λ cells (InvivoGen) were cultured according to
3744 manufacturer's instructions. Cells were grown to full confluence, treated with trypsin, and then
3745 diluted to 280,000cells/mL. 180uL of cells was mixed with 20uL of undiluted supernatant
3746 collected from infected MIO-M1 cells. The cells and supernatant were added to a 96 well plate
3747 and cultured for 24 hours at 37C 5%CO₂. After incubation, 20uL of media was removed from
3748 the HEK-Blue cells, mixed with 180uL Quantibblue (InvivoGen) detection media in a 96-well
3749 plate, then incubated for one hour at 37C 5%CO₂. After one hour, the plates were read at 655nm
3750 with a plate reader.

3751 Data Analysis:

3752 Statistical analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Prism
3753 Software, Inc. San Diego, CA). Differences between test condition and uninfected, or untreated
3754 conditions, were determined by 2-way nonparametric t test. A $p < 0.05$ was considered
3755 significant. Error bars represent standard error.

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3830 **Figure 1: MIO-M1 cells are susceptible to ZIKV infection and damage**

3831 A: MIO-M1 cells were infected with ZIKV at an MOI of 0.01 (red) or an MOI of 0.5 (blue).

3832 Supernatant was collected over the course of infection and the PFU/mL was determined by
3833 infection of Vero cells.

3834 B, C: Monolayers of MIO-M1 cells were infected with serially diluted ZIKV. 5 days post
3835 infection, cells were fixed with formalin and stained with hematoxylin and eosin. After staining,
3836 monolayers of uninfected (B) and plaques of ZIKV infected (C) cells were imaged.

3837 D, E: Monolayers of fully confluent MIO-M1 cells were infected with ZIKV at an MOI of 3. 48
3838 hpi cells were imaged by light microscopy to observe CPE.

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3841 **Figure 1:**

A

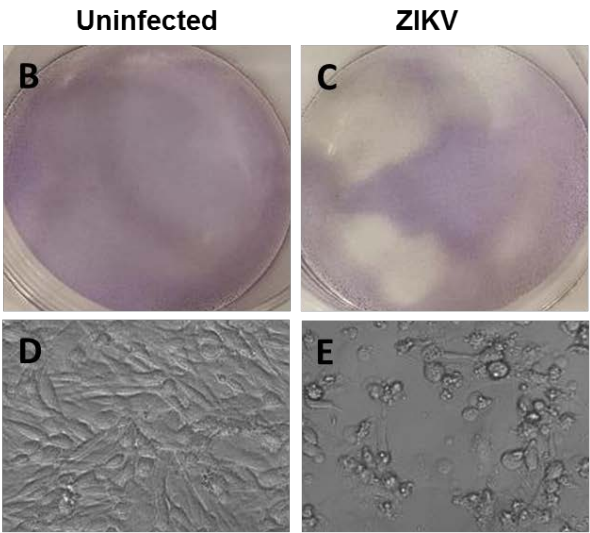
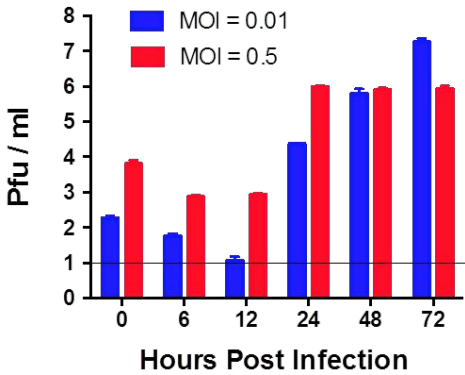


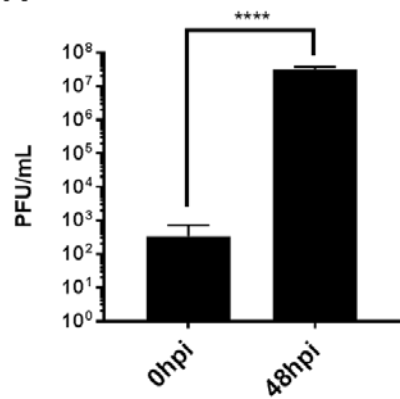
Figure 2: ZIKV infection of MIO-M1 cells results in induction of ISGs

A: MIO-M1 cells were infected with ZIKV and supernatant was collected at the time of infection (0hpi), to determine initial dose, and 48hpi. PFU/mL of supernatant was determined by infection of Vero cells. ***: $p < 0.001$

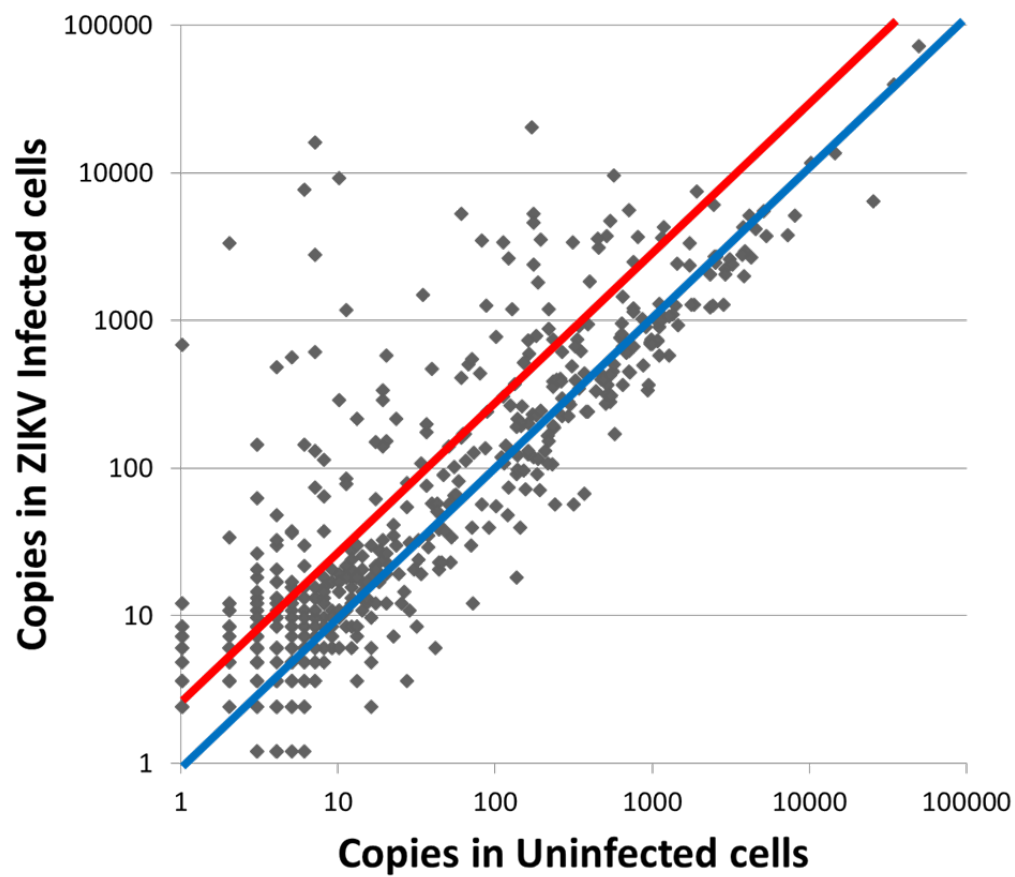
B: RNA was isolated from MIO-M1 cells 48 hpi infected with ZIKV or mock infection. This RNA was used in a nanostring assay, the nCounter® Human Immunology v2 Panel, giving absolute expression of over 500 innate-immune associated genes. Each gene in the panel was plotted, with the number of copies present in the uninfected condition on the x-axis and the number of copies present in the ZIKV-infected condition on the y-axis. The blue line indicates equal expression under both conditions. A point above the red line is expressed at least 2.5 fold greater following ZIKV infection, compared to uninfected cells.

Figure 2:

A



B



3860 **Figure 3: IFN λ but not IFN α is produced by MIO-M1 cells following ZIKV infection**

3861 A: RNA was collected 48 hours post ZIKV infection and the level of expression of IFN genes
3862 was determined compared to uninfected cells, relative to Beta Actin. ND: not detected

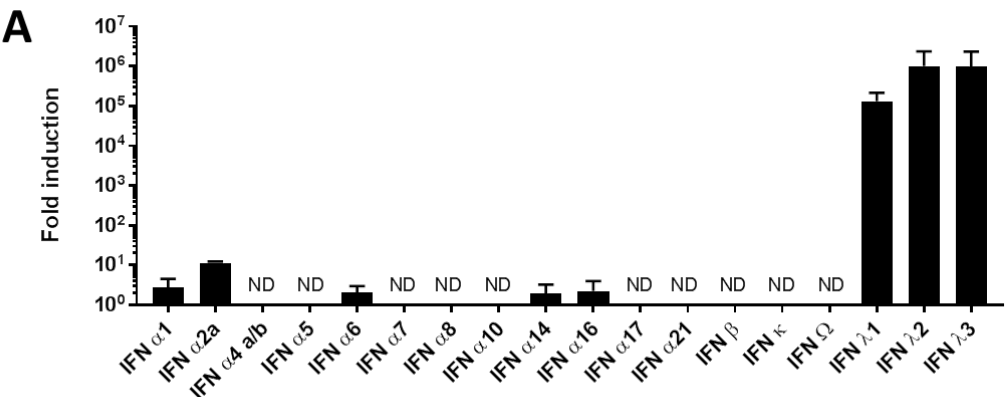
3863 B: HEK-Blue α/β assay of MIO-M1 cell supernatant collected over the course of ZIKV infection.
3864 HEK-Blue α/β cells were exposed to 500pg/mL of recombinant IFN α as a positive control. *:
3865 $p < 0.05$

3866 C: HEK-Blue λ assay of MIO-M1 cell supernatant collected over the course of ZIKV infection.
3867 ***: $p < 0.001$

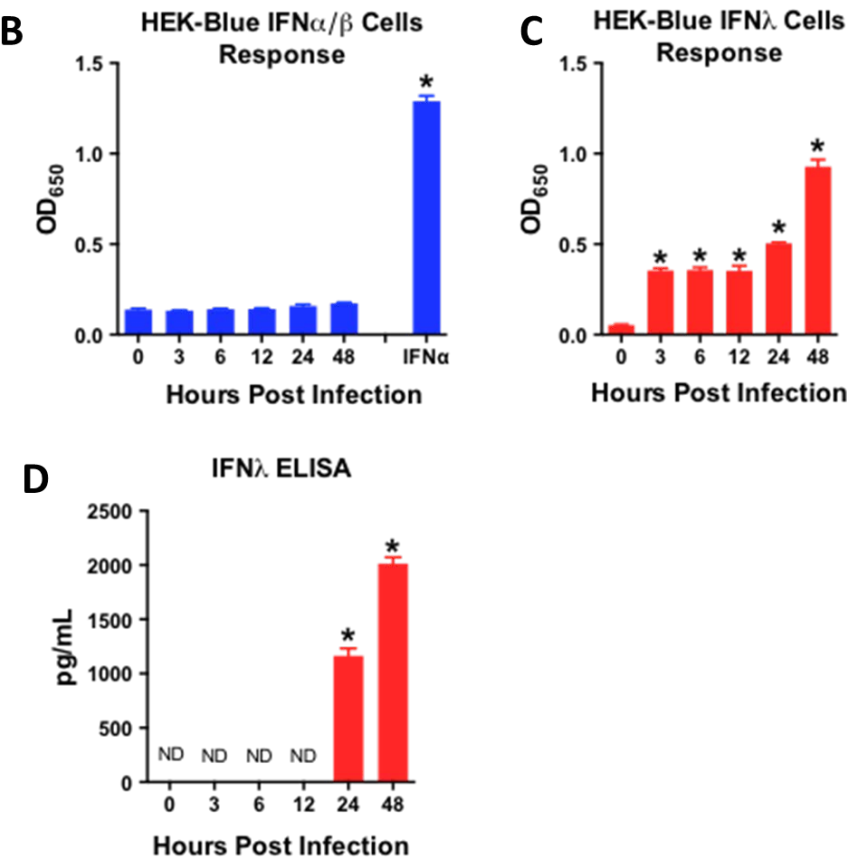
3868 D: IFN λ as detected by ELISA from of MIO-M1 cell supernatant collected over the course of
3869 ZIKV infection. ND: not detected

3870

3871 **Figure 3:**



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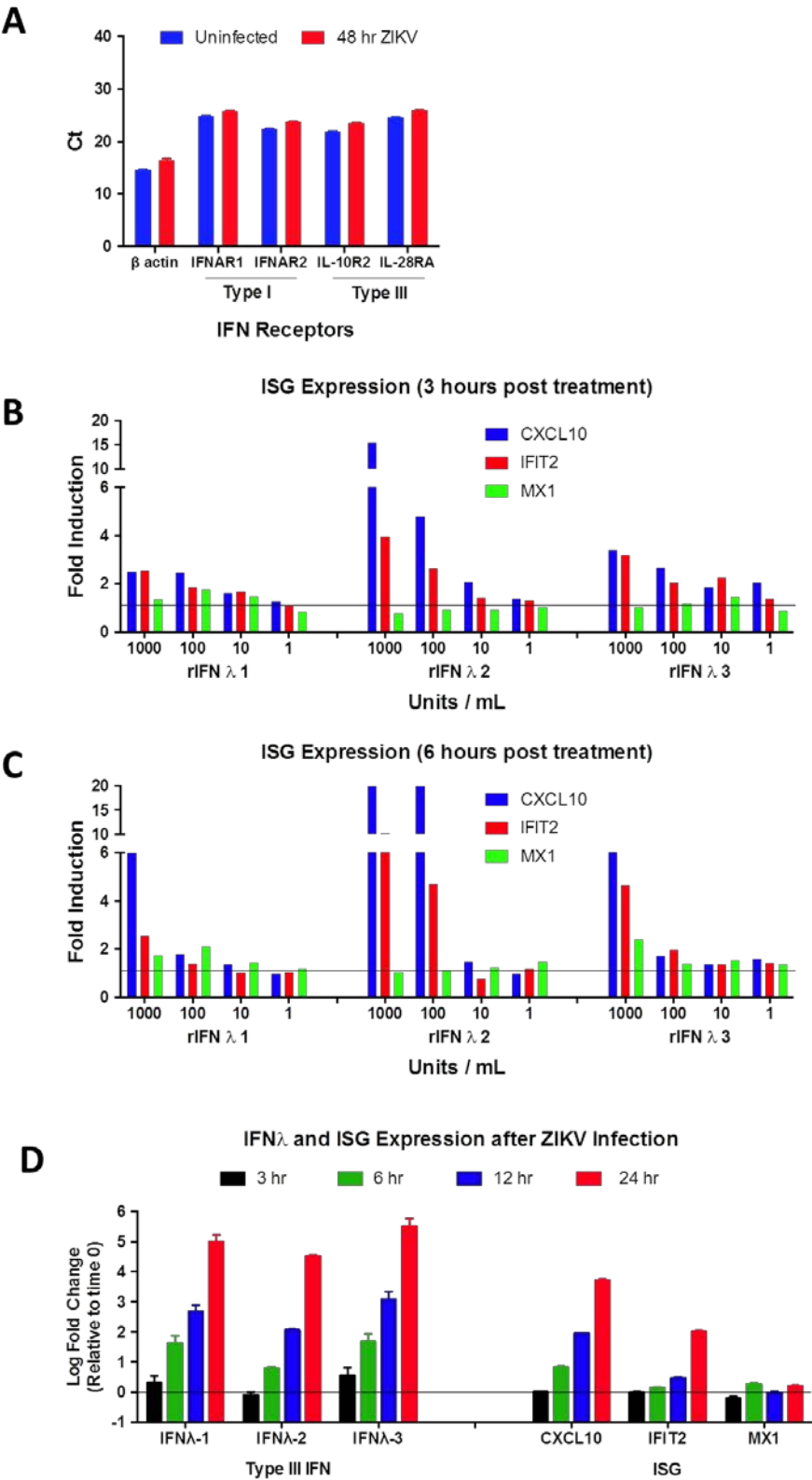
Figure 4: IFN λ alone results in induction of ISGs

A: RNA was isolated from MIO-M1 cells 48hpi (red) with ZIKV or mock infection (black) and the level of expression of Beta Actin and IFN receptor was determined by qRT-PCR. The cycle of detection is plotted for each gene of interest.

B,C: MIO-M1 cells were treated with a mix of each subtype of recombinant IFN λ between 1U/mL and 1000U/mL and RNA was collected 3 (B) and 6 (C) hours post treatment. The level of expression of CXCL10 (black), IFIT-2 (red) and MX1 (blue) was determined compared to untreated cells, relative to Beta Actin.

D: MIO-M1 cells were infected with ZIKV and RNA was collected between 3 and 24 hpi. The level of expression of CXCL10 (black), IFIT-2 (red) and MX1 (blue) was determined compared to untreated cells, relative to Beta Actin.

3886 **Figure 4:**



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Figure 5: Treatment of cells with IFN prior to ZIKV infection results in a reduction of viral production

A: MIO-M1 cells were treated with between 0.01ng/mL and 100ng/mL IFN α or between 0.1U/mL and 1000U/mL IFN λ for 24 hours. Media containing IFN was removed and cells were infected with ZIKV. The viral load was determined 48hpi by qPCR of RNA collected from the supernatant. *: p<0.05, compared to cells treated with no IFN prior to infection.

B: MIO-M1 cells were treated with between 0.01ng/mL and 100ng/mL IFN α or between 0.1U/mL and 1000U/mL IFN λ for 24 hours. Media containing IFN was removed and cells were infected with ZIKV. Cells were fixed with methanol ant stained for ZIKV NS3 (green) and DAPI (blue) 48hpi:

- i. Uninfected
- ii. ZIKV infected, untreated
- iii. ZIKV infected, 0.01ng/mL IFN α
- iv. ZIKV infected, 0.1ng/mL IFN α
- v. ZIKV infected, 1ng/mL IFN α
- vi. ZIKV infected, 10ng/mL IFN α
- vii. ZIKV infected, 100ng/mL IFN α
- viii. ZIKV infected, 0.1 U IFN λ
- ix. ZIKV infected, 1 U IFN λ
- x. ZIKV infected, 10 U IFN λ
- xi. ZIKV infected, 100 U IFN λ
- xii. ZIKV infected, 1000 U IFN λ

3912 **Figure 5:**

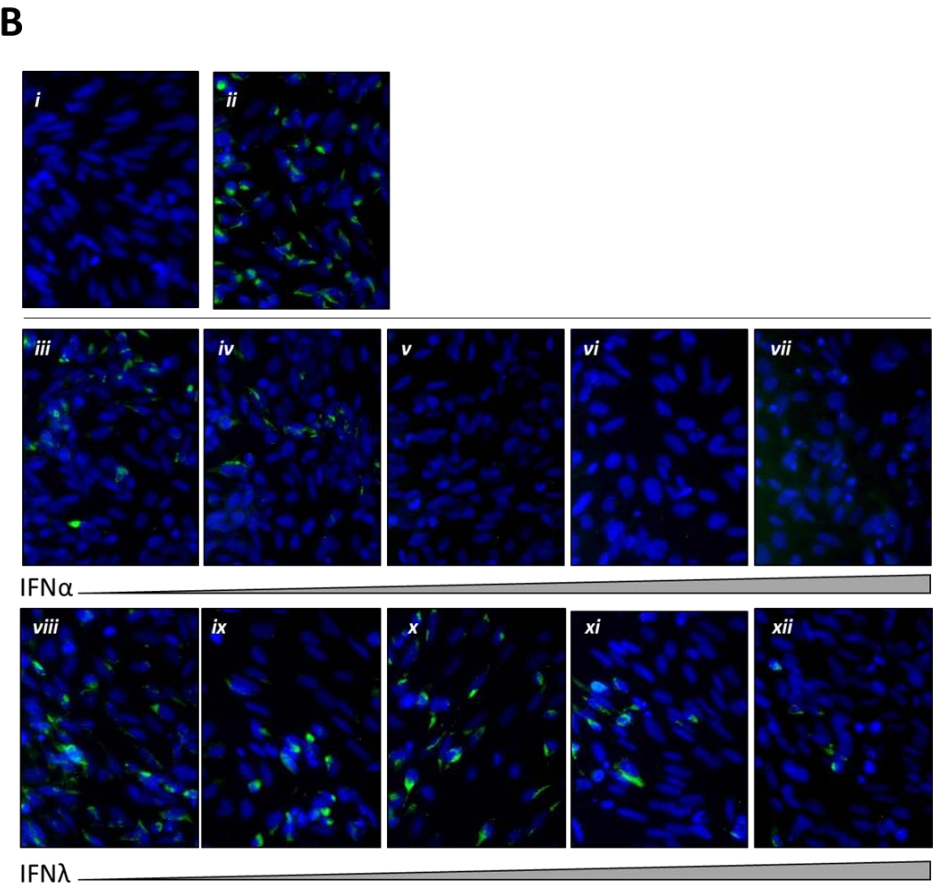
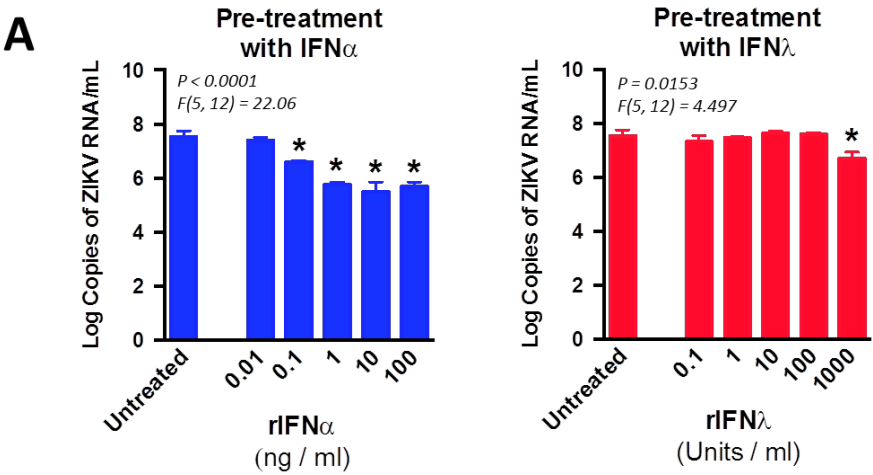


Figure 6: Blocking IFN λ binding following ZIKV infection leads to a decrease in viral production

A,B: MIO-M1 cells were infected with ZIKV and treated with IFN λ 1-neutralizing , IFN λ 2-neutralizing , and IFN λ 3-neutralizing antibody at the time of infection at concentrations between 10ng/mL and 10 μ g/mL. 12 hpi cellular RNA was collected and expression of each subtype of IFN λ (A) and select ISGs (B) was determined compared to uninfected, untreated cells relative to Beta Actin. *: p<0.05

C: MIO-M1 cells were infected with ZIKV and treated with equal concentrations of IFN λ 1-neutralizing , IFN λ 2-neutralizing , and IFN λ 3-neutralizing antibody at the time of infection at concentrations between 10ng/mL and 10 μ g/mL. 12 hpi supernatant was collected and the signaling capacity of IFN λ within the supernatant was determined by HEK-Blue λ assay. *: p<0.05 **: p<0.01 ***: p<0.001

D: MIO-M1 cells were infected with ZIKV and treated with IFN λ 1-neutralizing , IFN λ 2-neutralizing , and IFN λ 3-neutralizing antibody at the time of infection at concentrations between 10ng/mL and 10 μ g/mL. 12 hpi viral load of the supernatant was determined by qPCR. ***: p<0.001

3932 **Figure 6:**

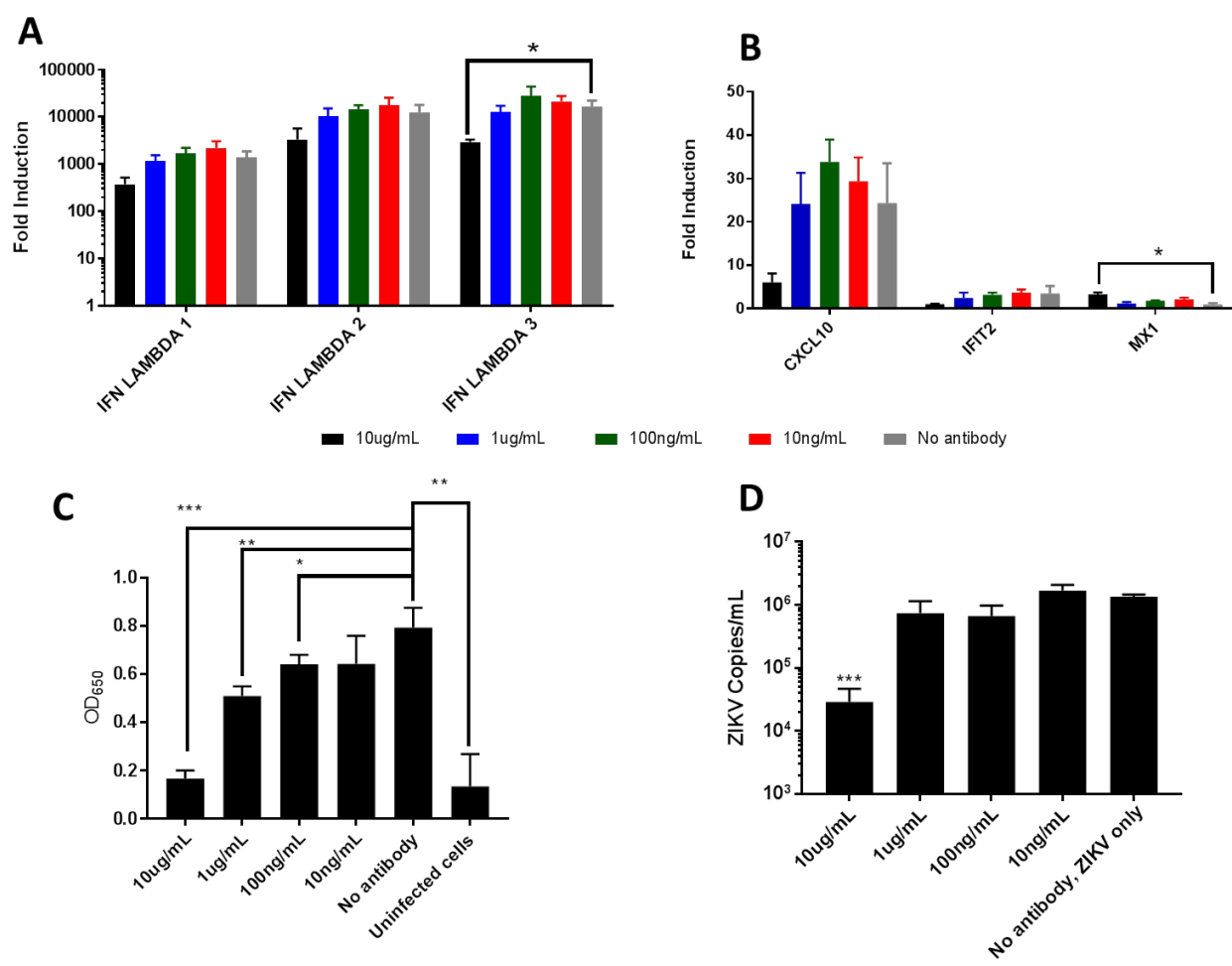


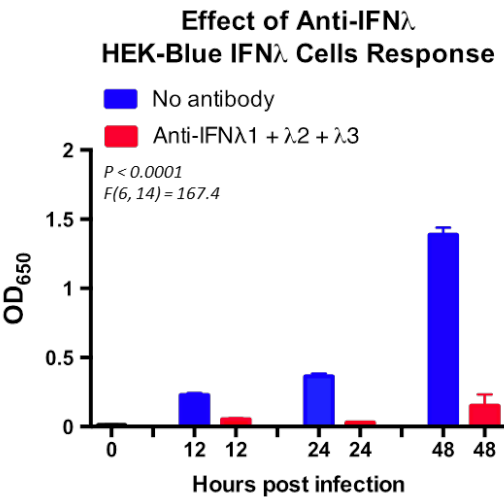
Figure 7: A high concentration of IFN λ neutralizing antibodies decreases ZIKV replication over the course of infection

A: MIO-M1 cells were infected with ZIKV and treated with 10 μ g/mL of IFN λ 1-neutralizing , IFN λ 2-neutralizing , and IFN λ 3-neutralizing antibody at the time of infection. 12, 24 and 28 hpi supernatant was collected and the signaling capacity of IFN λ within the supernatant was determined by HEK-Blue λ assay. ***: p<0.001

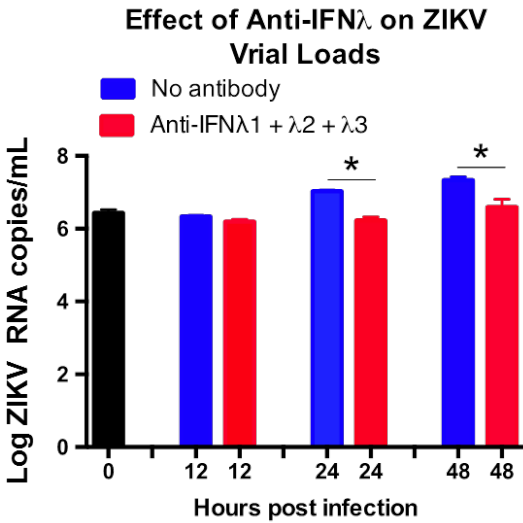
B: MIO-M1 cells were infected with ZIKV and treated with 10 μ g/mL of IFN λ 1-neutralizing , IFN λ 2-neutralizing , and IFN λ 3-neutralizing antibody at the time of infection. 12, 24 and 28 hpi viral load of the supernatant was determined by qPCR. ns: not significant *: p<0.05 **: p<0.01

3945 **Figure 7:**

A



B



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Table 1: Expression of selected innate immune-associated genes following ZIKV infection

Probe Name	Copies in Uninfected cell	Copies in ZIKV infected cell	Fold induction
CCL5	6.13	7745.42	1263.53
CXCL9	1.02	680.76	667.41
CXCL10	7.15	16089.53	2250.28
CXCL11	10.21	9189.01	900.00
IFIT2	170.58	20251.32	118.72
IRF1	311.54	3373.61	10.83
IRF7	19.41	141.22	7.28
MX1	449.43	3558.28	7.92
STAT1	759.94	1210.64	1.59
STAT2	1447.37	2443.00	1.69
TNF	5.11	566.09	110.78

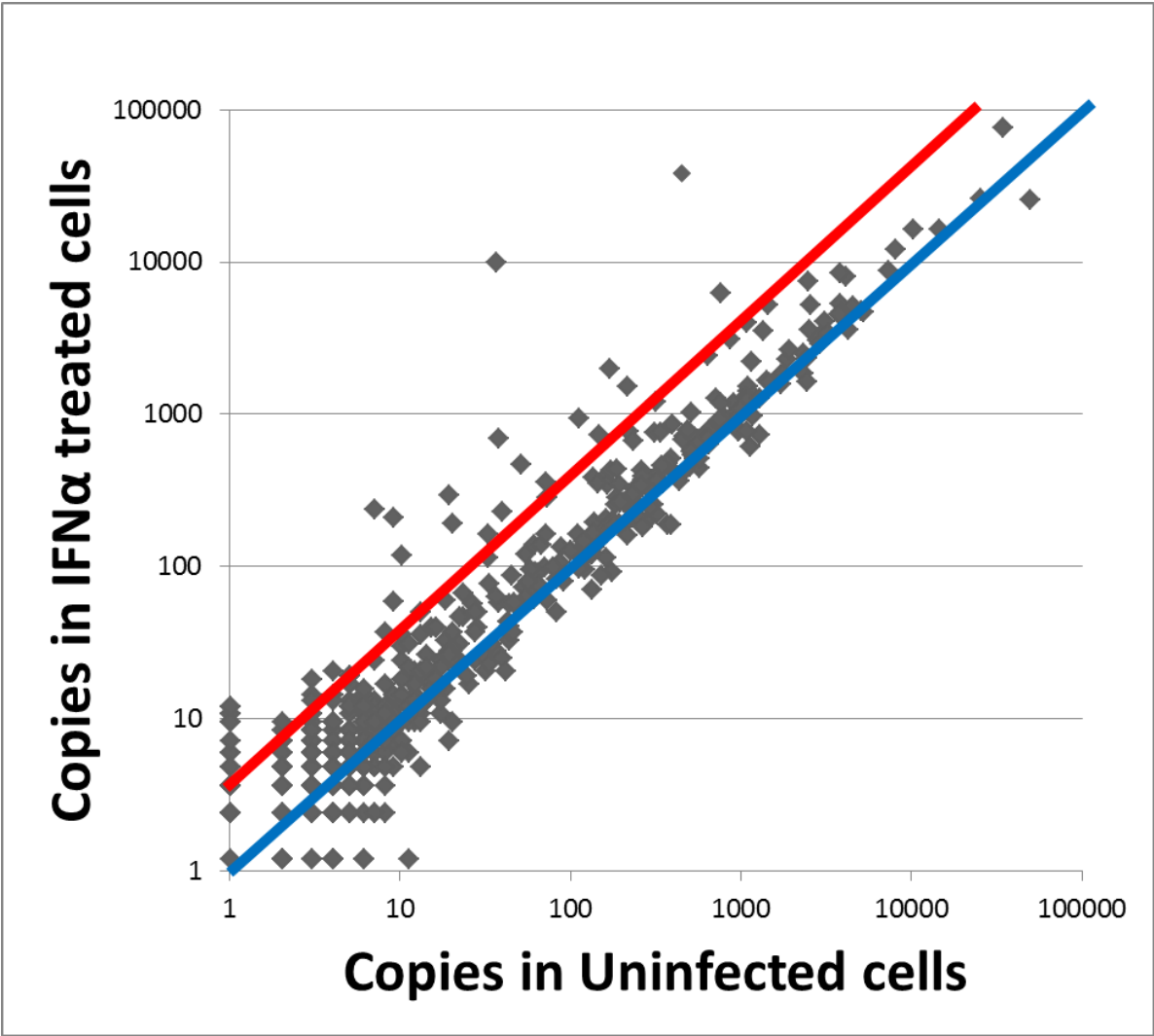
Table 2: Expression of selected IFN genes following ZIKV infection

Probe Name	Copies in Uninfected cell	Copies in ZIKV infected cell	Fold induction
IFN α 1/13	2.04	3.62	1.77
IFN α 2a	7.15	10.86	1.52
IFN β	4.09	12.07	2.95
IFN γ	5.11	4.83	0.95
IFN λ 1	2.04	3338.61	1636.57
IFN λ 2	7.15	2793.04	390.63
IFN λ 2/3	4.09	486.43	118.93

Supplementary Figure 1: IFN α treatment of MIO-M1 cells results in immune activation and ISG production

RNA was isolated from MIO-M1 cells 24 hpi treatment with 100ng.mL recombinant IFN α or mock treatment. This RNA was used in a nanostring assay, the nCounter® Human Immunology v2 Panel, giving absolute expression of over 500 innate-immune associated genes. Each gene in the panel was plotted, with the number of copies present in the uninfected condition on the x-axis and the number of copies present in the ZIKV-infected condition on the y-axis. The blue line indicates equal expression under both conditions. A point above the red line is expressed at least 2.5 fold greater following ZIKV infection, compared to uninfected cells.

3967 **Supplementary Figure 1:**



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Supplementary Figure 2: IFN λ neutralizing antibodies prevent ISG activation

A: MIO-M1 cells were treated with 1000U/mL IFN λ 1. At the time of treatment IFN λ 1-neutralizing monoclonal antibody was added to the media at concentrations between 0.01 and 10 μ g/mL. 6 hours after treatment, RNA was collected from the cells and expression of CXCL10 (black), IFIT2 (light gray) and MX1 (dark gray) was determined compared to untreated cells, relative to Beta Actin.

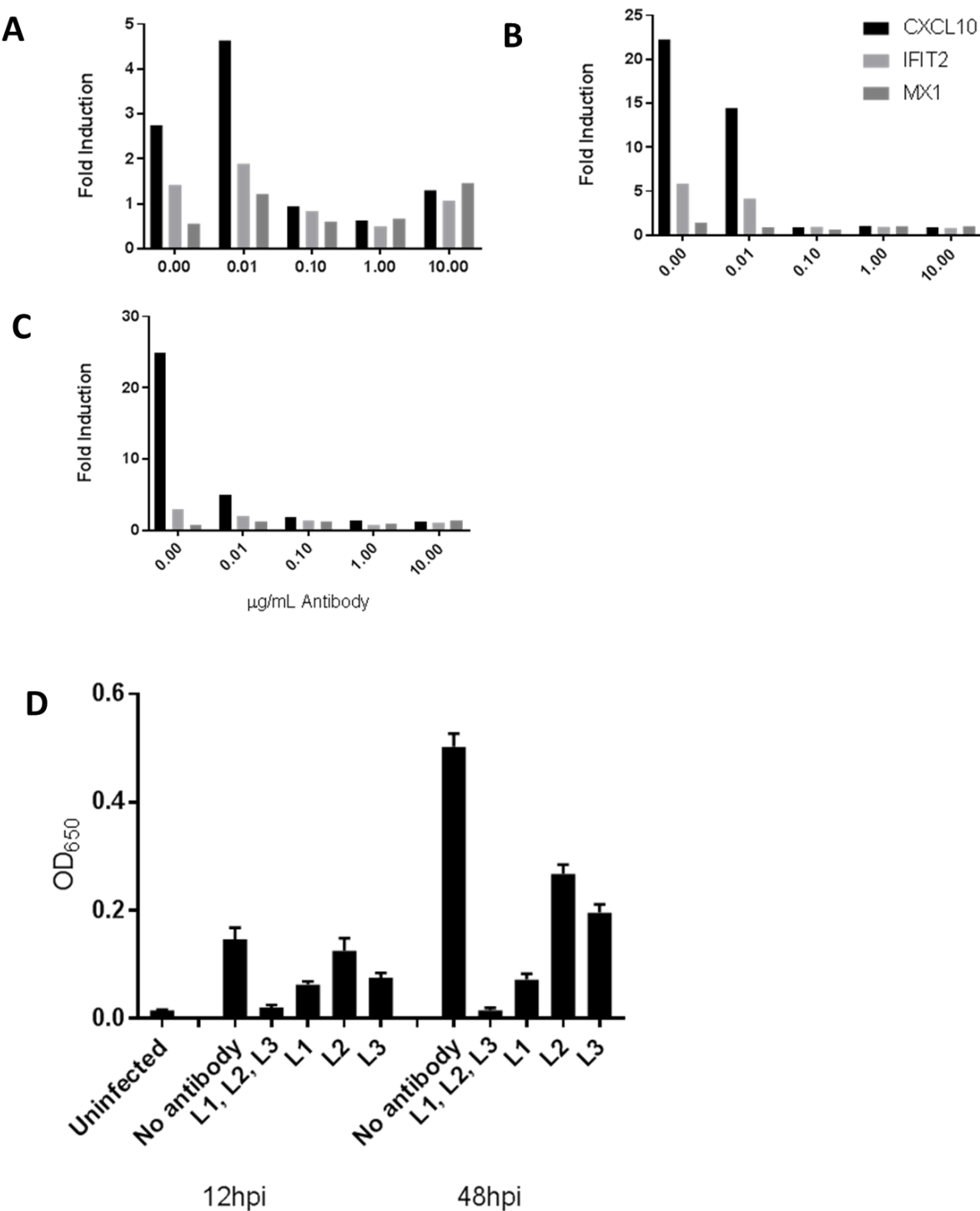
B: MIO-M1 cells were treated with 1000U/mL IFN λ 2. At the time of treatment IFN λ 2-neutralizing monoclonal antibody was added to the media at concentrations between 0.01 and 10 μ g/mL. 6 hours after treatment, RNA was collected from the cells and expression of CXCL10 (black), IFIT2 (light gray) and MX1 (dark gray) was determined compared to untreated cells, relative to Beta Actin.

C: MIO-M1 cells were treated with 1000U/mL IFN λ 3. At the time of treatment IFN λ 3-neutralizing monoclonal antibody was added to the media at concentrations between 0.01 and 10 μ g/mL. 6 hours after treatment, RNA was collected from the cells and expression of CXCL10 (black), IFIT2 (light gray) and MX1 (dark gray) was determined compared to untreated cells, relative to Beta Actin.

D: MIO-M1 cells were infected with ZIKV. At the time of infection, 10 μ g/mL of IFN λ -neutralizing antibody was added. 12 and 48 hpi supernatant was collected and the level of IFN λ signaling capacity was determined by HEK-Blue λ assay.

No antibody: untreated ZIKV infection L1, L2, L3: 10 μ g/mL of each IFN λ 1-neutralizing, IFN λ 2-neutralizing, and IFN λ 3-neutralizing antibody L1: 10 μ g/mL of IFN λ 1-neutralizing antibody only L2: 10 μ g/mL of IFN λ 2-neutralizing antibody only L3: 10 μ g/mL of IFN λ 3-neutralizing antibody only

3993 **Supplementary Figure 2:**



3996 **Supplementary Table 2: Expression of selected innate immune-associated genes following**
3997 **recombinant IFN α treatment**

3998

Probe Name	Copies in Uninfected cell	Copies in IFN α treated cell	Fold induction
CCL5	6.13	14.39	2.35
CXCL9	1.02	27.58	27.04
CXCL10	7.15	237.39	33.20
CXCL11	10.21	118.7	11.63
IFIT2	170.58	1999.84	11.72
IRF1	311.54	760.13	2.44
IRF7	19.41	296.14	15.26
MX1	449.43	38331.5	85.29
STAT1	759.94	6181.77	8.13
STAT2	1447.37	5183.04	3.58
TNF	5.11	4.8	0.94

3999

4000 **Supplementary Table 2: Primer sequences used**

4001

Gene Target	F Primer Sequence	R Primer Sequence
IFN α -1/13	TAGACAAATTCTGCACCGAAC	AGATGGAGTCCH-ICATTCATC
IFN α -2a/b	GGTAGCAGGAGGACCTTGATG	GGAGGACAGGGATGGTTTCAG
IFN α -4a/b	TCATTTCTCCTGCCTGAAGG	GAGGACAGAGATGGCTTGAG
IFN α -5	CACTTCTAGACAAATTCTACACTG	GGATAGAGTCCACATTCATCAG
IFN α -6	TGATTCAGCAGACCTTCAATC	TGCTGGTAAAGTTCAGTATAGAG
IFN α -7	CAGACCCACAGCCTGCGT	AAACTCCTCCTCTGGGAATCTG
IFN α -8	GATGATAAACAGTTCCAGAAGG	AAGTTCGATGTAGAATTCATCTAG
IFN α -10	GGGACAAATGGGAAGAATCTC	AACTGGTTGCCATCAAACCTC
IFN α -14	AGGAGGAATTTGATGGCAAC	AGCAGCAGATGAGTTCTTTG
IFN α -16	ATTGAACITTTCCAGCAACTG	TTCATCAGGGCAATCTCTTC
IFN α -17	AAGAATCTCTCCTTTCTCCTGCCTG	CTTGAGTCTTCTGGAAGTGG
IFN α -21	TCATCTGCTACTTGGGAACAG	CACATTCATCAGGGGAGTCTC
IFN β	TTGACATCCCTGAGGAGATTAAGC	TTAGCCAGGAGGTTCTCAACAATAG
IFN ω	CCTTGAGGAGGTACTTCC	CCAGGTCTCTATCTTTACTTC
IFN κ	CTGGACTGTAACITACTGA	TGCTCATACTACTCAGAAG
IFN λ 1	GTTCAAATCTCTGTCCACCAC	TTCAGCTTGAGTGACTCTTC
IFN λ 2	GCCAAAGATGCCTTAGAAGAG	CAGAACCTTCAGCGTCAGG
IFN λ 3	GCCAAAGATGCCTTAGAAGAG	CAGAACCTTCAGCGTCAGG
IL-10R β	GGCTGAATTTGCAGATGAGCA	GAAGACCGAGGCCATGAGG
IL-28R	ACCTATTTTGTGGCCTATCAGAGCT	CGGCTCCACTTCAAAAAGGTAAT
IFNAR1	GTGATACACATCTCTCCTGG	GTATAATCCCATTAAAGAACATAG
IFNAR2	GAGTAAACCAGAAGATTTGAAG	CGTGTTTGGAATTAACITGTC

4002

4003

4004	Chapter 7
4005	Summary and Conclusions
4006	
4007	William Valiant
4008	

Introduction:

Due to the antigenic similarities between Zika virus (ZIKV) and Dengue virus (DENV) we hypothesized that ZIKV-induced antibodies would be capable of enhancing DENV infection. The research performed in this thesis was the first report of *in vivo* enhancement of DENV by previous ZIKV infection. The polyclonal antibody response to ZIKV infection does not neutralize DENV and is sufficient for the enhancement of DENV *in vitro*. Further research has shown that the presence of DENV-neutralizing antibodies in ZIKV convalescent serum increases the peak enhancing dilution of serum. Since publishing these findings, further studies have demonstrated that maternally transferred ZIKV-induced antibody causes lethal DENV infection in newborn mice, adding further evidence that ZIKV-induced antibodies are able to cause enhancement of DENV³. Additionally, two studies have been published on the enhancement of Dengue virus (DENV) by Zika virus (ZIKV) induced antibodies^{1,2}. Both ZIKV-induced monoclonal antibodies and polyclonal serum from wild type mice infected with ZIKV were able to enhance DENV infection of monocytes *in vitro*. The findings reported here have implications for both public health responses to future DENV outbreaks, as well as ZIKV vaccine design.

Summary of results:

Enhanced DENV infection following ZIKV infection

Rhesus macaques experienced enhanced DENV infection when challenged 2 months following ZIKV infection (Chapter 2). The duration of DENV viremia in ZIKV-naïve and ZIKV-convalescent macaques was the same, however ZIKV-convalescent macaques experienced significantly higher peak viremia 5 days post infection. The enhanced viremia was also accompanied by enhanced production of monocyte and macrophage associated cytokines

compared to ZIKV-naïve animals. The cytokines produced, including I-TAC, MCP-1, and IL-8, are often associated with severe dengue infections of humans^{4,5}. Additionally, while there was not a significant change of body temperature of ZIKV-naïve animals during DENV infection, ZIKV-convalescent macaques experienced fever but not signs of rash or hemorrhage. Rhesus macaques do not experience severe dengue like humans, and although all the animals survived, the clinical signs observed are consistent with enhanced secondary DENV infection that has been used as a model of DHF^{6,7}.

The antibody response to ZIKV

Primary ZIKV infection resulted in a significant response in experimentally infected rhesus macaques (Chapter 3). ZIKV-binding IgM was detected in serum 7 days post infection, peaking at 14 days post infection. ZIKV-binding IgM levels were slightly elevated above baseline 56 days post infection. ZIKV-binding IgG was first detected in the serum 14 days post infection, with the levels remaining high 56 days post infection. The serum collected from ZIKV convalescent animals 56 days post infection was able to neutralize ZIKV at a 10⁴ dilution by PRNT. At each point where ZIKV-binding antibodies were detected, DENV-binding, cross-reactive antibodies were detected as well. The DENV-2-binding IgM response following ZIKV infection had similar kinetics as the ZIKV-binding IgM response, peaking 14 days post infection. The DENV-2-binding IgG response following ZIKV infection initially rose to high levels at 14 days post infection, although decreased in magnitude out to 56 days post infection. The DENV-2-binding and ZIKV-binding IgG was primarily the IgG1 subtype, with low levels of IgG2 and IgG3 detected. Serum collected 56 days post infection did not neutralize DENV-2 by PRNT.

Serum collected both 56 and 112 days post ZIKV infection enhanced infection of each of the 4 DENV serotypes *in vitro* (Chapter 2, Appendix chapter 1). The serum maximally enhanced

at either a neat or 1:10 dilution, with between 20-fold and 250-fold enhancement, depending on the serotype. Serum was able to enhance DENV-2 infection 14 days post ZIKV infection (Chapter 3). The magnitude of enhancement increased over time, peaking at 56 days post infection.

Similar to the results observed in macaques, serum collected from individuals previously infected with ZIKV contained IgM and IgG that bound to both ZIKV and DENV-2 (Appendix Chapter 2). Interestingly, of the two individuals tested, the serum from one individual was not able to neutralize DENV-2 while the serum from the other individual neutralized DENV-2 and ZIKV, indicating a previous DENV infection. Serum collected from both individuals was able to enhance DENV-2 infection of FcR expressing cells *in vitro*.

The antibody response to DENV

Similar to ZIKV infection, experimental DENV-2 infection of rhesus macaques results in a cross-reactive antibody response (Chapter 3). DENV-2 infection resulted in a DENV-2-binding and ZIKV-binding IgM response that is first detected 14 days post infection and decreases to near baseline levels by 56 days post infection. The DENV-2-binding IgG response is significantly more long-lasting than the ZIKV-binding IgG response, with the ZIKV-binding IgG response decreasing in magnitude 28 and 56 days post infection while the DENV-2-binding IgG response continued to increase. Serum collected 56 days post DENV-2 infection was able to neutralize DENV-2 by PRNT at a 10^3 dilution, while no neutralization of ZIKV was detected.

The antibody response during enhanced DENV

When ZIKV-convalescent macaques were infected with DENV-2 an anamnestic antibody response was detected (Chapter 3). A slight increase in both DENV-2-binding and ZIKV-binding IgM was detected 7 days post infection, while a significant increase in DENV-2-binding IgG was

4078 detected 7 days post infection. An IgG response in naïve animals infected with DENV-2 was not
4079 detected until 14 days post infection, indicating a DENV-specific anamnestic antibody response.
4080 In the absence of a previous ZIKV infection, this neutralizing response was not detected until 14
4081 days post DENV infection. The increase in DENV-2-binding IgG coincided with the detection of
4082 DENV-neutralizing antibodies. This neutralizing anamnestic antibody response occurred after
4083 peak DENV-2 viremia, indicating that the response was too late to protect from enhanced
4084 viremia. During convalescence, 56 days post infection with DENV-2, serum from ZIKV-
4085 convalescent macaques was able to potently neutralize both ZIKV and DENV-2.

4086 **The consequences of ZIKV and DENV co-infection**

4087 Rhesus macaques simultaneously challenged with ZIKV and DEN-2 at the same site of
4088 infection experienced ZIKV and DENV-2 viremia that was not significantly different from single
4089 infected naïve animals (Chapter 5). ZIKV viremia peaked 3 days post infection while DENV-2
4090 viremia peaked 5 days post infection. During infection there was a biphasic activation of immune
4091 cells and production of inflammatory cytokines. Serum collected 56 days post neutralized both
4092 ZIKV and DENV-2 at a magnitude similar to single infected animals (Chapter 4).

4093 **The presence of DENV-neutralizing antibodies alters the dynamics enhancement mediated** 4094 **by ZIKV-convalescent serum**

4095 As described above, a number of studies were performed on serum that contained
4096 neutralizing antibodies against both ZIKV and DENV-2. Serum from macaques co-infected with
4097 ZIKV and DENV-2, as well as macaques sequentially infected with ZIKV then DENV-2,
4098 neutralized both ZIKV and DENV-2 (Chapter 3). Additionally, one of the ZIKV-convalescent
4099 human serum samples tested neutralized both ZIKV and DENV-2 (Appendix Chapter 2). Serum
4100 collected from co-infected or sequentially infected macaques enhanced DENV-2 infection *in*

vitro, with a peak enhancing dilution of 1:100. This is a 10-fold shift from the 1:10 maximally enhancing dilution of ZIKV-convalescent serum. A similar trend was observed between the two human serum samples tested where serum that neutralized ZIKV only maximally enhanced DENV-2 at a dilution of 1:10 while serum that neutralized ZIKV and DENV-2 maximally enhanced DENV-2 at a dilution of 1:10,000. The larger difference in peak enhancing titer between the single neutralizing and co-neutralizing serum of the human samples may be due to the more potent neutralizing response to Flavivirus infection in humans compared to macaques.

ZIKV infection induces Interferon lambda production in Müller cells

MIO-M1 cells, an immortal cell line of human Müller cells, are susceptible to infection with ZIKV (Chapter 6). Following infection, many genes associated with innate immunity were induced, including Interferon Lambda (IFN λ). All three subtypes of IFN λ were produced within 6 hours of ZIKV infection, but no production of Type I IFN was detected. MIO-M1 cells treated with IFN λ had a similar up-regulation of ISG, and MIO-M1 cells treated with high levels of IFN λ prior to infection were protected from ZIKV infection. Additionally, when the signaling ability of IFN λ was blocked with the addition of neutralizing antibodies following ZIKV infection, there was a decrease in the level of ZIKV infection.

Conclusions:

These studies demonstrate the ability of ZIKV-induced antibodies to enhance DENV infection. ZIKV-induced antibodies are able to enhance DENV infection *in vitro*, and ZIKV infection results in a DENV-binding IgG1 antibody response that is not neutralizing, which is typically associated with risk of enhancement^{8,9}. While the *in vitro* enhancement model of DENV infection of monocytes is far removed from natural infection, the rhesus macaque model

of DENV infection and enhancement is close to natural human infection. Given that ZIKV-convalescent macaques experience enhanced DENV-viremia, accompanied by enhanced immune activation, it is possible that ZIKV-convalescent humans may experience enhanced DENV infection as well. The ability of human serum from ZIKV-convalescent individuals to enhance DENV infection indicates this risk as well.

Despite our consistent laboratory findings, there have been no confirmed reports of enhanced DENV-infection in people that are ZIKV convalescent. This could be for a number of reasons. The macaques used in these studies were initially Flavivirus naïve; this is not the case for most individuals living in areas of the ZIKV epidemic. DENV is prevalent within Brazil, and many individuals are also vaccinated for yellow fever. Previous Flavivirus immunity has been shown to augment the immune response to subsequent infection and the findings in naïve macaques may not accurately represent populations of South America that were infected with ZIKV¹⁰. The macaques do, however, represent naïve populations such as travelers or deployed members of the military. Additionally, the lack of detection of enhanced DENV following ZIKV infection may be due to a lack of surveillance. Epidemiological studies that are able to identify risk of enhancement related to presence of DENV-binding antibodies require cohorts of thousands of children with up to a decade of measurement to detect differences in outcomes, and with how recent the ZIKV epidemic took place there may not have been enough time to detect a difference in rates of enhancement¹¹. In early 2019, there were large outbreaks of dengue fever in areas where the ZIKV epidemic took place in South America. Jamaica reported a 4.5 times increase in dengue fever cases in 2018 compared to 2017¹².

Our studies on the innate immune response to ZIKV infection have identified the importance of IFN λ . Late in ZIKV infection there are high levels of inflammatory genes

4147 produced, and IFN λ is, at least in part, responsible for the production. Despite the high levels of
4148 ISG up-regulation in MIO-M1 cells, these cells will still support ZIKV replication, as viral load
4149 increases even in the presence of ISGs. This highlights the importance of timing of the IFN
4150 response: exposure to IFN λ prior to infection will cause cells to resist ZIKV infection, while
4151 exposure following ZIKV infection will lead to increased viral replication. The finding that
4152 blocking IFN λ signaling in ZIKV infected MIO-M1 cells lead to a decrease in viral replication
4153 indicates that IFN λ neutralizing antibodies could have therapeutic potential. Further testing in
4154 animal models of ZIKV infection will be required.

4156 **Future studies:**

4157 While *in vitro* and animal models of DENV enhancement have been able to show the
4158 ability of ZIKV-induced antibodies to enhance DENV infection, these models do not fully
4159 replicate human infection. To fully understand if ZIKV infection predisposes individuals to a
4160 later severe dengue infection, large scale epidemiologic studies will be required. These studies
4161 will need to monitor individuals who are both ZIKV-naïve and ZIKV-convalescent and compare
4162 the rates of severe dengue between them, potentially in a case-control design. Clinical trials of
4163 ZIKV vaccines will need to be designed in a way that identifies the risk of severe dengue
4164 following vaccination. The results of these studies would inform the realistic risk of severe
4165 dengue in areas where there were ZIKV outbreaks.

4166 We identified that DENV neutralizing antibodies delayed the peak enhancing titer of
4167 serum, although due to funding constraints we were unable to challenge the co-infected animals
4168 with DENV to determine if they were protected from enhancement. Future studies investigating

4169 the susceptibility of co-infected or sequentially infected animals to enhanced DENV infection
4170 would allow for a further understanding of the consequence of the shift in enhancing titer.

4171 While these studies have found a connection between ZIKV replication and IFN λ in
4172 MIO-M1 cells, future studies can understand the role IFN λ plays in other cells targeted by ZIKV
4173 infection. Elucidating the role of IFN λ on ZIKV replication within cells of the placenta and the
4174 fetal nervous system would identify potential further applications of these results. To understand
4175 the impact of IFN λ in the eye as a whole, *in vivo* studies using IFNAR^{-/-} mice infected with
4176 ZIKV and treated with IFN λ neutralizing antibody could find evidence of *in vivo* activity.

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4220 **Appendix Chapter 1**

4221 **A Simple Flow Cytometry Based Assay to Determine *In Vitro* Antibody Dependent**

4222 **Enhancement of Dengue Virus Using Zika Virus Convalescent Serum**

4223 William G. Valiant and Joseph J. Mattapallil

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4226 **Published as:**

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4228 vitro antibody dependent enhancement of dengue virus using Zika virus convalescent

4229 serum. *JoVE (Journal of Visualized Experiments)*, (134), e57371.

4230

4231 **Abstract:**

4232 Antibody dependent enhancement of infection has been shown to play a major role in
4233 Dengue viral pathogenesis. Traditional assays that measure the capacity of antibodies or serum to
4234 enhance infection in impermissible cell lines have relied on using viral output in the media
4235 followed by plaque assays to quantify infection. More recently, these assays have examined
4236 Dengue virus (DENV) infection in the cell lines using fluorescently labeled antibodies. Both
4237 these approaches have limitations that restrict the widespread use of these techniques. Here, we
4238 describe a simple *in vitro* assay using Dengue virus reporter viral particles (RVP) that express
4239 green fluorescent protein and K562 cells to examine antibody dependent enhancement (ADE) of
4240 DENV infection using serum that was obtained from rhesus macaques 16 weeks after infection
4241 with Zika virus (ZIKV). This technique is reliable and involves minimal manipulation of cells,
4242 does not involve the use of live replication competent virus, and can be performed in a high
4243 throughput format to get a quantitative readout using flow cytometry. Additionally, this assay
4244 can be easily adapted to examine antibody dependent enhancement (ADE) of other flavivirus
4245 infections such as Yellow Fever virus (YFV), Japanese Equine Encephalitis virus (JEEV), West
4246 Nile virus (WNV) etc. where RVP are available. The ease of setting up the assay, analyzing the
4247 data, and interpreting results makes it highly amenable to most laboratory settings.

4248

4249

Introduction:

Antibody dependent enhancement (ADE) of infection is a process whereby partially cross-reactive antibody responses induced by a serotype of virus enhances uptake of another serotype of virus, leading to increased viral replication and viremia. ADE has been extensively documented in Dengue virus (DENV) infections where four major serotypes are prevalent. In a subset of patients, ADE is associated with Dengue hemorrhagic fever (DHF). We have recently shown that Zika virus (ZIKV) infection induced significantly high levels of DENV cross-reactive antibody responses that caused ADE of DENV *in vitro* and likely contributed to the enhancement of DENV viremia *in vivo*^{1,2}. Antibody dependent enhancement assays are a valuable tool to assess the capacity of antibodies to enhance secondary infection with related viruses and provide valuable insights into the pathogenesis of flavivirus infections and inform the development of vaccines.

The assay described here uses DENV reporter viral particles (RVP) along with K562 cells that are normally impermissible to infection. The RVP are structurally intact replication incompetent DENV viral particles that encode a sub-genomic green fluorescent protein (GFP) replicon that is expressed after a single round of replication³. As such, cells that become infected with RVP fluoresce green and can be readily detected using flow cytometry or microscopy. The RVP used in this assay were obtained from commercial sources. They can, however, be generated against other viruses and used in the assay described in this manuscript. The K562 cells are an FcγIII-receptor expressing Leukemia cell line that bind to the Fc region of antibodies and become infected in the presence of sub-neutralizing concentrations of antibody^{4,5}.

ADE assays have been extensively used in studies investigating the risk factors for severe dengue and to delineate the mechanisms of *in vitro* ADE⁶⁻⁸. The ADE assay described here can be quickly and easily used to determine the capacity of serum to enhance *in vitro* infection using RVP and flow cytometry as compared to other assays used currently, which require either the determination of plaque forming units (pfu) in Vero cells or antibody staining of infected cells⁶⁻¹¹ both of which are time consuming and labor intensive.

The serum samples used to demonstrate the protocol described in this manuscript were obtained from rhesus macaques that were housed and cared for in accordance with local, state and federal policies in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility. All animal experiments were reviewed and approved by Institutional Animal Care and Use Committee and samples were acquired through a tissue sharing protocol.

Protocol:

1. Day 1

Note: Perform all the steps described below in a sterile laminar flow biosafety cabinet used for tissue culture in a BSL-2 laboratory.

1.1. Thaw the serum samples at room temperature and transfer 100 uL of each serum sample to a sterile tube. Heat inactivate for 30 minutes at 56 °C either in a water bath or a temperature adjustable thermomixer. Make 10-fold serial dilutions of the serum sample ranging from 1:1 to 1:1000 using cold RPMI-10 (RPMI with 10% fetal bovine serum).

4296 1.2. Transfer 10 μ L of serially diluted serum sample to each well of a sterile 96-well V-bottom
4297 plate. Include two sets of control wells, RPMI-10 with RVP only and without serum sample, and
4298 RPMI-10 only without RVP and without serum sample. Set up each serum sample and RPMI-10
4299 controls in triplicates.

4300 1.3. Remove the Dengue-1, 2, 3 and 4 Reporter Viral Particles (RVP) from the -80 °C freezer
4301 and thaw them in a 37 °C water bath. Transfer the thawed RVP immediately to ice.
4302 Approximately 170 μ L RVP is required for each serum sample (10 μ L of RVP x 3 wells for each
4303 serum dilution (1:1, 1:10, 1:100, 1:1000)) and 10 μ L of RVP x 3 wells for each of the RPMI-10
4304 with RVP only control wells.

4305 1.4. Pipette 10 μ L of RVP into each well of the 96-well V-bottom plate containing the serum
4306 sample, and to the RPMI-10 with RVP (no serum) control wells. Do not add RVP to the RPMI-
4307 10 only (no serum sample and no RVP) wells. Mix thoroughly by pipetting up and down 5 - 10
4308 times. Add 10 μ L cold RPMI-10 in lieu of RVP to each cold RPMI-10 only (no serum sample
4309 and no RVP) control wells.

4310 1.5. Transfer the 96-well V-bottom plate to an incubator and incubate the plate for 1 hour at 37
4311 °C in the presence of 5% CO₂. While the 96-well V-bottom plate is incubating, clean the surface
4312 of the biosafety cabinet with 70% Ethanol and run the UV light for 15 minutes.

4313 1.6. Remove a fully confluent T75 flask of K562 cells from the incubator, mix the cells well
4314 using a sterile 5 mL pipet, and transfer 5 mL of cells to a sterile 15 mL conical tube. The K562
4315 cells are maintained in RPMI-10 and sub-cultured at 1×10^6 / mL.

4316 1.7. Count the number of cells by removing 10 μ L cells from the sterile 15 mL conical tube and
4317 mix with 10 μ L trypan blue. Count the cells using a hemocytometer to determine the total
4318 number of cells in the 15mL conical tube.

4319 1.8. Centrifuge the 15 mL conical with cells at $\sim 1200 \times g$ for 10 minutes, decant the supernatant,
4320 and resuspend the cells in warm RPMI-10 at a concentration of 80,000 cells/30 μL of media
4321 (2.66×10^6 cells /mL).

4322 1.9. Remove the 96-well V-bottom plate from the incubator (step 1.6), transfer 30 μL of K562
4323 cells to each well of the 96-well V-bottom plate, and mix thoroughly by pipetting up and down 5
4324 - 10 times. Transfer the 96-well V-bottom plate to an incubator and incubate the plate for 1 hour
4325 at 37 °C in the presence of 5% CO_2 .

4326 1.10. After incubating for 1 hour, remove the 96-well V-bottom plate from the incubator and
4327 centrifuge at $\sim 1200 \times g$ for 5 minutes. After centrifugation, decant the media from the wells by
4328 turning the plate upside down into a container containing 10% bleach.

4329 1.11. Wash the cells in each well by resuspending them in 125 μL of warm RPMI-10, mix
4330 thoroughly with a pipette, and centrifuge the plate at $\sim 1200 \times g$ for 5 minutes. Decant the media
4331 by turning the plate upside down into a container containing 10% bleach. Repeat this wash step
4332 two times.

4333 1.12. After washing, add 100 μL of warm RPMI-10 to each well, mix up and down with the
4334 pipet and incubate the plate for 48 hours at 37 °C in the presence of 5% CO_2 .

4335

4336 **2. Day 3**

4337 2.1. Remove the 96-well V-bottom plate containing 100 μL of cells and media/well from the
4338 incubator and move it to a biosafety cabinet. Using a multichannel pipette, mix the contents of
4339 each well and transfer the cells and media to a 96 well U-bottom plate or to pre-labeled 5 mL
4340 polypropylene tubes.

4341 2.2. Rinse each well in the 96-well V-bottom plate with 100 μ L of 1% Paraformaldehyde (PFA)
4342 in 1x Phosphate Buffered Saline (PBS) and transfer to the respective wells in the 96 well U-
4343 bottom plate or labeled 5 mL polypropylene tubes from step 2.1 to yield a final concentration of
4344 0.5% PFA/well or tube. Mix thoroughly using a multichannel pipette, cover the plate with
4345 aluminum foil, let it sit in the incubator for 30 minutes to fix the cells.

4346 2.3. Prepare the flow cytometer by running unstained K562 cells to calibrate the side and
4347 forward scatter along with the fluorescence settings. The only fluorescence channel required is
4348 FL1, as GFP is the only fluorescence emitted from the cells. Acquire ~30 - 50,000 cells from
4349 each sample.

4350 Note: Any flow cytometer capable of reading a single fluorescence can be used for acquiring the
4351 data as cells infected with RVP will only emit green fluorescence due to the presence of GFP,
4352 and no other fluorescence channel are needed.

4353

4354 **3. Data Analysis**

4355 3.1. Analyze data acquired on the flow cytometer using any flow cytometry analysis software.

4356 3.1.1. Set the first gate based on FSC-A (forward scatter – area) vs FSC-H (forward scatter –
4357 height) to include single cells and exclude auto-fluorescent doublets from analysis¹². Then, gate
4358 singlet-gated cells based on SSC-A (side scatter – area) vs FSC-A to exclude any autofluorescent
4359 debris.

4360 3.1.2. Analyze SSC-A vs FSC-A gated cells for the expression of GFP based on SSC-A vs GFP-
4361 A. Determine the percentage of GFP+ cells for each dilution of the serum and control samples
4362 by setting a gate around GFP+ cells.

4363

3.2. Determine the average percentage of GFP+ cells for each serum sample dilution and control wells by dividing the total percentage of GFP+ cells in the triplicate wells by 3.

3.3. Calculate fold-enhancement of infection by dividing the average percentage of GFP+ cells in the serum samples for each dilution divided by the average percentage of GFP+ cells in the RPMI-10 with RVP (no serum sample) control wells.

3.4. Graph fold-enhancement (y-axis) versus dilution (x-axis), and perform statistical analysis using ANOVA followed by Tukey's post-hoc test for multiple comparisons.

Representative Results:

Sera from 4 rhesus macaques were collected 16 weeks after infection with ZIKV and tested for their potential to enhance DENV-1, 2, 3 and 4 infection in K562 cells. The animals had peak viremia of $\sim 1 \times 10^5$ copies of ZIKV RNA/mL of plasma by day 3 after infection that declined to levels that were below the limit of detection by 7 days after infection. No viremia was detected in the plasma at 16 weeks post-infection. The assay was performed as described in the above protocol and the collected data were analyzed using the Flowjo 9.8 software.

The gating strategy used to identify GFP+ cells are shown in Figure 1. The initial gate was set to exclude autofluorescent doublets and only include single cells based on FSC-A vs FSC-H. The next plot shows only cells from the single cell gate and a gate was drawn based on SSC-A vs FSC-A to exclude any autofluorescent debris. These gated cells were then displayed in a third plot based on SSC-A vs FL1-A to identify GFP+ cells. Cells infected with RVP express GFP. The percentage of GFP+ cells was determined by setting a gate around these cells. Approximately 12.2% of GFP+ cells are detectable in the ZIKV infected serum sample as

4386 compared to no GFP+ cells in the control sample. This gating strategy is followed for each serum
4387 dilution and control sample included in the assay.

4388 The percentage of GFP+ cells was determined for each sample dilution and control
4389 sample as described above (Figure 1). Data from a representative animal using DENV-1 RVP
4390 are shown in Figure 2. As compared to the no serum control sample that had 0.253% GFP+ cells,
4391 the percentage of GFP+ cells were 2.58% in undiluted sample, 12.8% at 1:10, 0.735% at 1:100,
4392 and 0.022% at 1:1000 dilution. The percentage of GFP+ cells will change as the serum is diluted
4393 due to the decreasing concentrations of antibody in the sample.

4394 As enhancement of infection is associated with the antibody concentrations that is ideal
4395 to increase uptake of virus, we observed a dramatic increase in the percentage of GFP+ cells at
4396 1:10 dilution as compared to either undiluted sample or other dilutions, suggesting that the
4397 undiluted sample had higher concentrations of antibody whereas the 1:100 and 1:1000 dilutions
4398 had lower levels of antibody that was not sufficient to induce ADE of infection. If antibody
4399 concentrations are significantly high, then additional dilutions of serum should be included to
4400 determine the dilution at which ADE becomes apparent. In the serum samples we examined, we
4401 observed ADE at a dilution of 1:10. On the other hand, ADE may be observed at lower dilutions
4402 if the antibody concentration in the serum is quite high.

4403 Next, we calculated the fold-enhancement of infection by dividing the average percentage
4404 of GFP+ cells in the triplicate wells for each dilution with the average percentage of GFP+ cells
4405 in the triplicate wells for no serum control. The kinetics of ADE against DENV-1, 2, 3 and 4
4406 serotypes were determined by graphing fold-enhancement on the y-axis and serum dilution on
4407 the x-axis (Figure 3). The data shows that the serum collected at 16 weeks post infection

4408 significantly enhanced infection of K562 cells by DENV-1, 2, 3 and 4 serotypes at a dilution of
4409 1:10.

4410

4411 **Discussion:**

4412 Flaviviruses such as DENV and ZIKV share significant evidence of homology in both
4413 their structural and non-structural proteins that generate antibodies that cross-react with each
4414 other^{13,14}. These cross-reactive antibody responses have been shown to enhance of infection both
4415 *in vivo* and *in vitro* with either a heterologous serotype or other related flaviviruses^{1,2}. The
4416 potential to cross enhance infection has significant implications for management of the disease
4417 and also for vaccine development.

4418 Traditional culture based assays use non-permissive cells that are infected with infectious
4419 live DENV in the presence of serum. Enhancement of infection is measured by quantifying the
4420 amount of virus in the supernatant using standard plaque assays¹¹. This assay requires culture of
4421 virus in two cell lines and requires significantly long periods of time to perform. Additionally,
4422 not all the DENV serotypes plaque the same way, adding another level of complexity to these
4423 assays.

4424 Other assays have modified the plaque based assay protocol to measure infection of cell
4425 lines by combining intracellular staining for DENV using fluorescently labeled antibodies and
4426 flow cytometry^{9,10,14}. Though this reduces the time required to determine the level of infection as
4427 compared to plaque based assays there are a number of optimization steps required for these
4428 assays to work consistently. Intracellular staining protocols are time consuming, as they require
4429 fixing and permeabilizing of cells followed by intracellular staining and require well-titrated
4430 DENV specific antibodies that can clearly discriminate each serotype. Additionally, these assays

4431 are not easily amenable to a high throughput format and suffer from high levels of intra-assay
4432 variability.

4433 In contrast to the above described assays, the DENV RVP based assay is easy to set up,
4434 does not use infectious virus, and is highly amenable to high throughput that can be readily used
4435 to measure the potential of serum to enhance infection. Additionally, this assay is not as labor
4436 intensive as other assays and can be completed within 2 - 3 days, which offers a major advantage
4437 over current ADE assay protocols. Though the assay described in the manuscript examined the
4438 capacity of ZIKV infected serum to enhance infection of DENV-1, 2, 3 and 4 serotypes, the
4439 assay can be readily adapted to examine serum from both experimental and clinical samples
4440 obtained from other flavivirus infections such as Yellow Fever virus (YFV), Japanese Equine
4441 Encephalitis virus (JEEV), and West Nile virus (WNV) if RVP are available, and with other cell
4442 lines. It is, however, critical to optimize the assay by titrating the volume of RVP, number of
4443 cells/ well, and the duration of incubation to allow optimal staining of cells without background
4444 staining.

4445 Titrations need to be performed by incubating a defined number of target cells (for ex:
4446 80,000 K562 cells or a cell line being tested) with variable volumes of RVP (for ex: 2.5 μ L, 5
4447 μ L, 7.5 μ L, 10 μ L, 20 μ L etc). Once the correct titer for each RVP is obtained, the duration of
4448 incubation can be determined using the obtained titer along with the defined number of cells and
4449 incubating them as described in the protocol for variable periods of time (for ex: 24 hours, 48
4450 hours, 72 hours etc.).

4451 We observed ADE of infection by all 4 serotypes of DENV at a dilution of 1:10
4452 suggesting that antibody levels in the serum needed to be diluted 10-fold to detect enhancement
4453 of infection. On the other hand, studies have reported enhancement at lower dilutions, which

would indicate that the concentrations of antibody in the serum sample were likely too high needing lower dilutions to detect ADE *in vitro*. As ADE depends on the right concentration of antibody in the serum sample, most samples are likely to show ADE at dilutions lower than the undiluted serum.

In conclusion, the protocol described in this manuscript is simple and easy to adopt, and allows for scaling up to enable high throughput screening of a large number of samples in a short period time frame to yield high quality data that can easily be analyzed and interpreted.

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WGV performed all the experiments and analyzed the data; JJM designed and supervised the study; WGW and JJM wrote the paper.

DISCLOSURES

The authors have nothing to disclose.

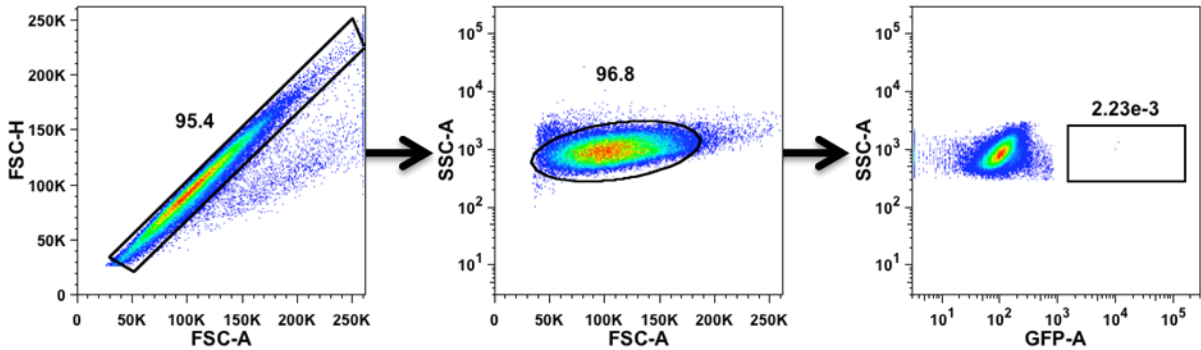
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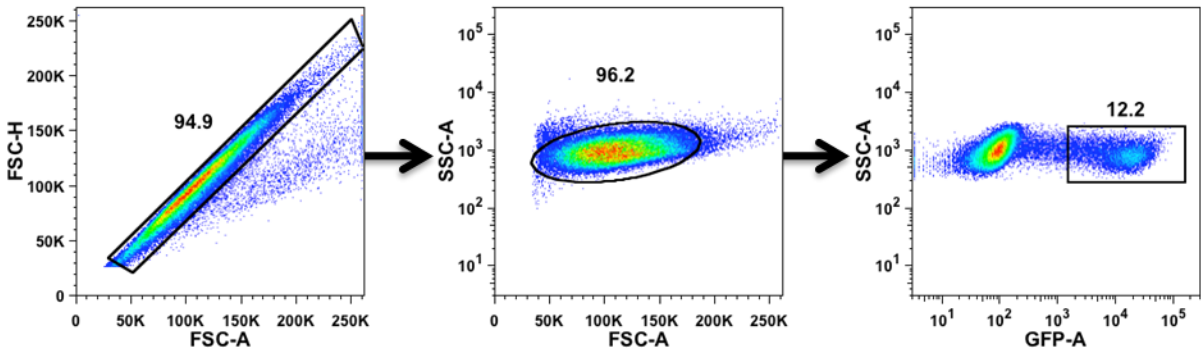
Figure 1: Gating strategy for acquiring and analysis by flow cytometry. K562 cells infected with DENV RVP express GFP that was analyzed using a flow cytometer. (A) No serum control and (B) 16 week post-ZIKV infected serum. Cells were first gated based on forward scatter area (FSC-A) vs forward scatter height (FSH-H) to exclude doublets followed by a side scatter (SSC-A) vs FSC-A to exclude debris. The SSC-A vs FSC-A gated cells were then gated based on SSC-A vs GFP-A (Green Fluorescence Protein-area). The black rectangles and ovals represent the gates, and the numbers above the gates are the percentage of cells falling within that gate. The black arrow between the FACS plots shows the sequence of gates used.

Figure 1:

A

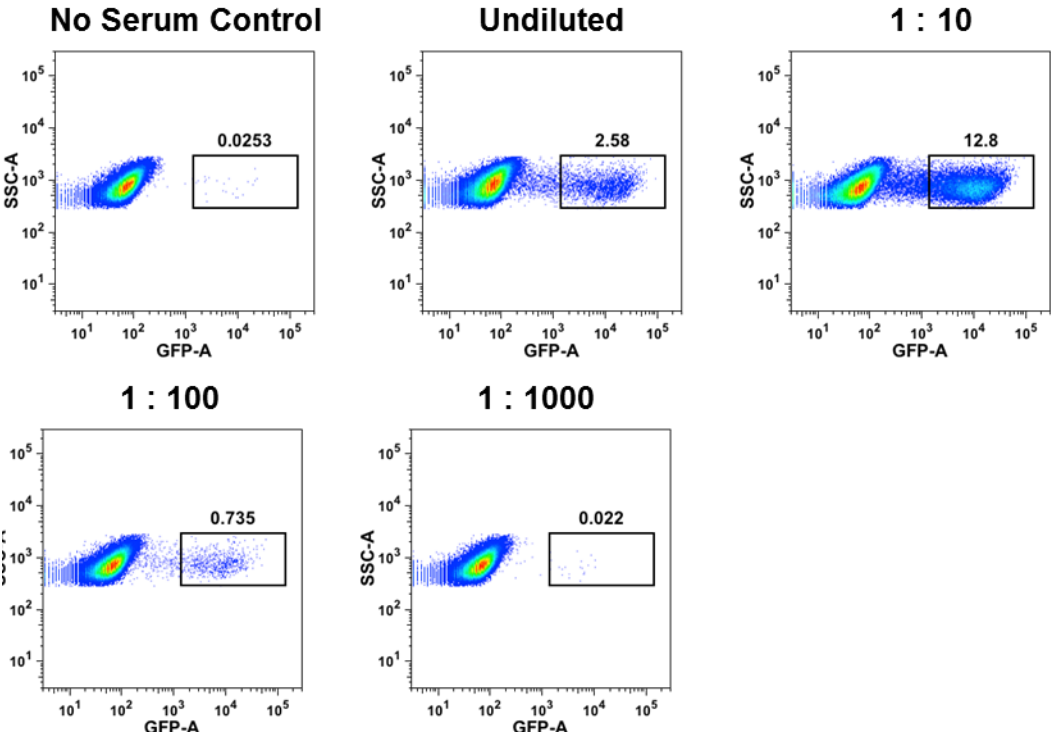


B



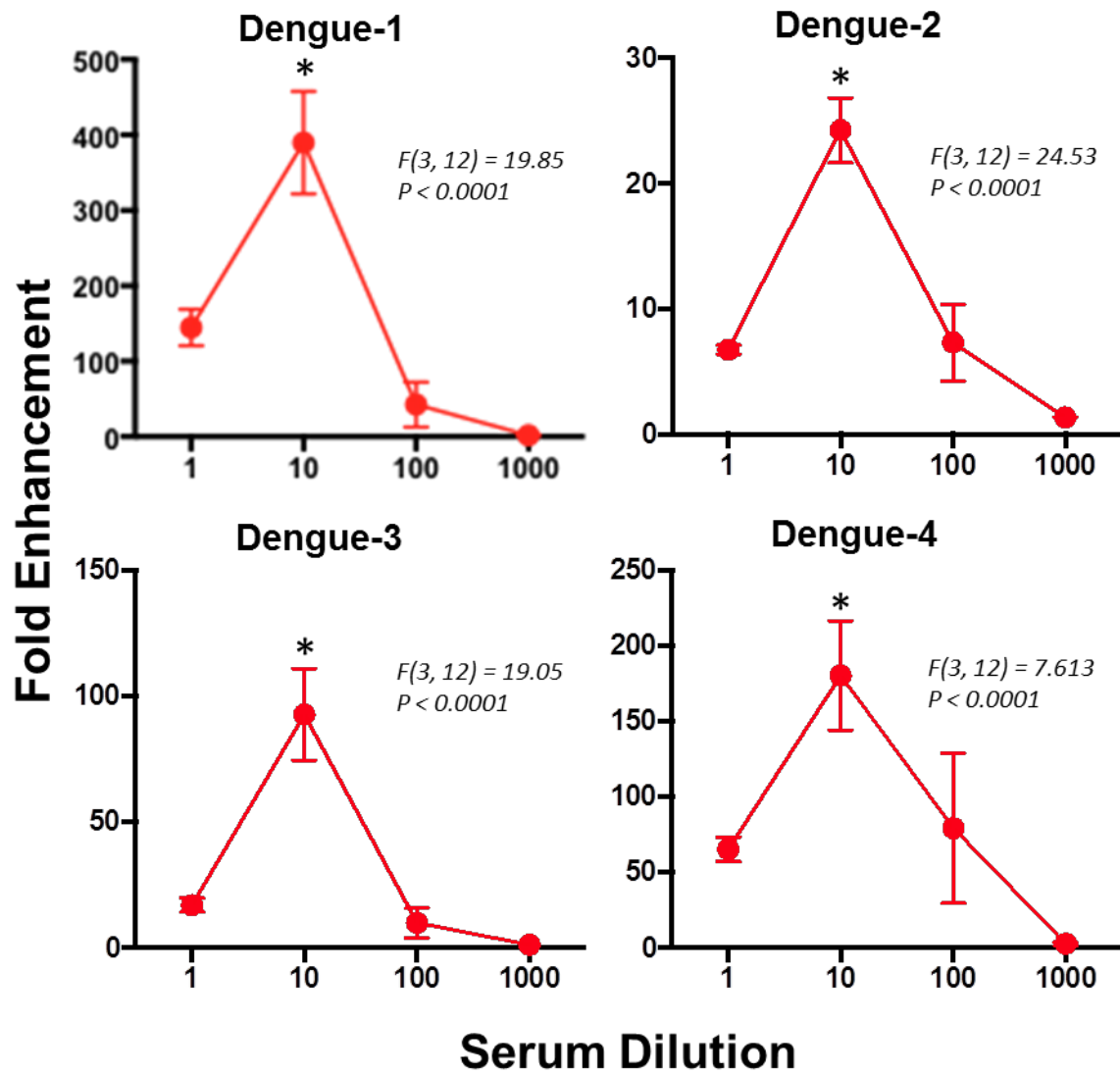
4528 **Figure 2: Representative dot plots showing frequency of DENV RVP+ cells.** The percentage
4529 of K562 cells that were infected with DENV-1 RVP was determined by flow cytometry. Serum
4530 collected from a representative Rhesus macaque at 16 weeks post infection was incubated with
4531 DENV-1 RVP either undiluted or at a 1:10, 1:100, and 1:1000 dilutions and compared to no
4532 serum control samples. The percentage of GFP+ cells at each concentration of serum is shown.
4533
4534

Figure 2:



4538 **Figure 3: Significant enhancement of Dengue-1, 2, 3 and 4 infection is observed at 1:10**
4539 **dilution of serum.** Antibody dependent enhancement of Dengue infection was examined for the
4540 4 DENV serotypes by plotting serum dilutions on the x-axis and fold-enhancement (relative to
4541 no serum sample controls) on the y-axis. Sera collected from 4 ZIKV immune rhesus macaques
4542 at 16 weeks after infection was used in this assay. Error bars represent standard error and *
4543 represents $p < 0.05$.
4544

Figure 3:



4549 **Appendix Chapter 2:**

4550 **Human Serum with High Neutralizing Antibody Titers Against Both Zika and Dengue**

4551 **Virus Shows Delayed *In Vitro* Antibody Dependent Enhancement of Dengue Virus**

4552 **Infection**

4553 William G. Valiant, Tahaniyat Lalani, Heather C. Yun., Anjali Kunz., Timothy H. Burgess &

4554 Joseph J. Mattapallil

4555

4556 **Published as:**

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4561

Abstract

Zika virus infection in a dengue virus naïve subject was associated with the induction of high levels of cross-reactive binding antibodies. These responses were however, largely non-neutralizing and displayed a capacity to enhance dengue infection *in vitro* at significantly low dilution (1:10). In contrast, a subject who had high levels of neutralizing antibodies against both dengue and Zika viruses enhanced infection at a dilution of 1:10,000. These results suggest that high levels of dengue cross-neutralizing antibodies could potentially prevent the enhancement of dengue infection in Zika virus convalescent individuals.

Introduction

Zika virus (ZIKV) and Dengue virus (DENV) are related Flaviviruses that share significant structural similarities leading to the induction of cross-reactive antibody responses[1-3]. Previous studies have shown that ZIKV infection induces high levels of DENV cross-reactive antibodies with little or no cross-neutralization[2, 4-7] suggesting that these responses are less likely to protect from DENV infection. On the other hand, induction of cross-reactive antibodies in the absence of cross-neutralization was associated with significant enhancement of DENV infection *in vivo* in rhesus macaques[5]. Likewise, polyclonal serum from wild type mice injected with ZIKV as well as monoclonal antibodies isolated from a human previously infected with ZIKV was shown to enhance DENV infection of monocytes *in vitro*[8, 9].

Enhancement of infection has been documented in humans infected with heterologous serotypes of DENV[10, 11]. Though the exact mechanisms for enhanced disease are not clear, numerous studies have implicated a role for cross-reactive antibodies that form immune complexes with virions and mediate infection of Fc-receptor expressing cells leading to enhancement of infection. Studies have reported higher rates of DENV infection in monocytes in the presence of diluted serum from DENV convalescent subjects[11, 12], and passive transfer of

4587 DENV binding antibody to nonhuman primates was associated with enhanced DENV
4588 viremia[13]. There is limited evidence to show that human subjects infected with ZIKV can
4589 similarly enhance DENV infection either *in vivo* or *in vitro*. The primary objective of this study
4590 was to characterize DENV specific cross-reactive antibodies in two US travelers following
4591 infection with ZIKV and examine the capacity of sera from these individuals to enhance DENV
4592 infection *in vitro*.

4593 **Materials and Methods**

4594 **Samples**

4595 Serum samples were obtained from subjects enrolled in an observational cohort study
4596 (TravMil - Deployment and Travel Related Infectious Disease Risk Assessment, Outcomes, and
4597 Prevention Strategies Among Department of Defense [DoD] Beneficiaries) that prospectively
4598 evaluates infectious disease risks, and the effectiveness of prevention and treatment strategies, in
4599 DoD beneficiaries traveling outside the continental United States for ≤ 6.5 months. This protocol
4600 was reviewed and approved by the Infectious Disease Institutional Review Board at the
4601 Uniformed Services University, and informed consent was obtained from each subject prior to
4602 sample collection. In addition, the investigators have adhered to the policies for protection of
4603 human subjects as prescribed in 45 CFR 46.

4604 Subject #1: A 30 year-old female who traveled to Dominican Republic for 2 weeks in
4605 May of 2016. No prior history of Japanese Encephalitis or Yellow Fever vaccination was
4606 reported. The subject experienced an illness that began with fever, night sweats, and retro-orbital
4607 headache, followed by diffuse maculopapular rash, myalgia, small joint polyarthralgia, mild sore
4608 throat, and mild conjunctivitis in the right eye during travel. Her symptom complex lasted
4609 approximately 8 days prior to the time of assessment in the Infectious Disease Clinic following

her return. Serum was negative by Triplex Real-time RT-PCR assay for ZIKV, DENV and Chikungunya virus but ZIKV RNA was detected in the urine by RT-PCR on day 8 after onset of illness. Serology was confirmed using CDC Zika MAC-ELISA. A convalescent serum sample for the research study was obtained 3.5 weeks after illness onset and return from travel.

Subject #2: A 28 year-old male who traveled to Puerto Rico for 2 weeks in July of 2016. The subject was vaccinated against Japanese Encephalitis in 2015 and no prior Yellow Fever vaccination was reported. He experienced fever, fatigue, itching, headache, rash, diarrhea, arthralgia, conjunctivitis, and enlarged cervical lymph nodes, starting at 2 days prior to his return. His symptoms were ongoing at the time of presentation to the Infectious Disease Clinic one day following his return from travel. ZIKV RNA was detected in both serum and urine by Triplex Real-time RT-PCR assay at day 3 after the onset of illness. Serology was confirmed using CDC Zika MAC-ELISA. A convalescent serum sample for the research study was obtained 4 weeks after illness onset and return from travel.

Serum samples collected from four subjects enrolled in TravMil study whose sera was negative for ZIKV and DENV antibodies by PRNT were included as negative controls in the present study. Triplex Real-time RT-PCR assay was not performed as the negative control subjects were asymptomatic and serum was collected after return from travel.

ELISA

Relative levels of ZIKV and DENV-2 specific IgM and IgG in acute serum were determined using whole ZIKV and DENV-2 in a sandwich ELISA based in house assay. Briefly, ELISA plates were coated with anti-Flavivirus monoclonal antibody (clone 4G2) overnight at 4⁰C. After washing 3x with 1x PBS-Tween, the plates were blocked with 1x PBS-BSA overnight at 4⁰C. Whole ZIKV or DENV-2 virus diluted in 1x PBS-Tween was added to each well after

washing 3x and incubated for 1 hour at 37°C. Serum samples diluted 1:100 in 1x PBS-Tween was added to each well and incubated for 1 hour at 37°C. No serum control wells were setup simultaneously as negative controls and all samples were setup in duplicates. The plates were washed 3x and Horseradish peroxidase (HRP)-conjugated anti-IgG or IgM detection antibody (IBL-America, Minneapolis, MN) was added to each well. After incubating at room temperature for 30 minutes the plates were developed with Tetramethylbenzidine (TMB). The reaction was stopped after 15 minutes with TMB stop solution (IBL-America, Minneapolis, MN) and the plates were analyzed at 450 nm using an ELISA plate reader. The relative O.D. was determined after subtracting the O.D. of the no serum negative controls from the O.D of the samples. The assay cutoff was set at an O.D. of 0.1 for both IgM and IgG based on the O.D. of serum samples from flavivirus negative subjects (n = 4).

Plaque reduction neutralization test (PRNT)

PRNT assays were performed as described previously[5].

Antibody dependent enhancement (ADE) assay

ADE assays were performed as described previously[5, 14].

Data analysis

Enhancement of DENV infection was examined by flow cytometry and the data was analyzed using Flowjo 9.8 software.

Results

DENV cross- reactive antibodies are readily detectable in serum after ZIKV infection

We first examined the relative levels of ZIKV and DENV-2 binding antibodies using whole virus with the intent of determining envelope specific antibody responses (Figure 1a and

1d). Subject#1 had similar levels of anti-ZIKV and DENV-2 IgM where anti-ZIKV IgG levels were higher than anti-DENV IgG. In contrast, both anti-ZIKV and DENV-2 serum IgM levels are 2x lower in Subject#2 as compared to Subject#1. Interestingly, though anti-ZIKV IgG levels were similar to that of Subject#1, anti-DENV-2 serum IgG levels were higher than Subject#1 suggesting that Subject#2 induced higher levels of DENV cross-reactive antibodies.

Subject# 1 induced high levels of ZIKV specific neutralizing antibodies that did not cross neutralize DENV

To determine if the differences in DENV specific IgG levels between the two subjects were due to differences in induction of cross-reactive responses or prior DENV infection, we assessed their ZIKV and DENV-2 specific neutralizing antibody responses using the PRNT assay (Figure 1b and 1e). Our results showed that Subject#1 had a ZIKV PRNT₅₀ titer of 1:1000 with no cross-neutralizing antibody responses against DENV-2. In contrast, Subject#2 had higher PRNT₅₀ titres against both ZIKV and DENV-2 suggesting that this subject was exposed to both ZIKV and DENV.

Serum from Subject#1 and Subject#2 display differential capacity for *in vitro* enhancement of DENV infection

We have previously reported that prior exposure to ZIKV significantly enhanced DENV viremia in rhesus macaques that was associated with high levels of DENV cross-reactive antibody responses. To assess if ZIKV infection in humans show a similar phenotype, we examined the capacity for serum from both subjects to enhance DENV infection *in vitro* using K562 cells, a cell line that expresses the low affinity FcγRII receptor that binds to IgG immune complexes.

Serum from Subject#1 was found to enhance infection of K562 cells by DENV-2 at a dilution of 1:10 (Figure 1c). In contrast, Subject#2 demonstrated enhancement at a dilution of 1:10,000 (Figure 1f) suggesting that presence of higher levels of DENV specific neutralizing antibodies likely prevent enhancement at lower dilutions.

Discussion

Structural similarities between ZIKV and DENV have been associated with the induction of highly cross-reactive antibodies[1-3]. Though studies in non-human primates[5] and mice[8] have shown the potential for ZIKV induced cross-reactive responses to enhance DENV infection there is limited data to support these findings in humans. Our results show that serum from ZIKV infected human subjects was capable of mediating ADE of DENV infection *in vitro*. Large epidemiological studies are needed to determine if these findings directly translate to the potential for higher risk of severe dengue but they add to the evidence from animal studies that ZIKV induced antibodies could potentially enhance DENV infection; monoclonal antibodies[9] or polyclonal serum[8] has been shown to enhance DENV infection *in vitro* whereas ZIKV convalescent nonhuman primates experienced enhanced DENV viremia[1-3].

As has been reported previously, serum from DENV naïve ZIKV infected human subject showed little cross-neutralizing antibody responses against DENV[2, 4-7]; PRNT50 titres were <1:10 (Fig. 1b) suggesting that the *in vitro* enhancement of infection of DENV was likely associated with cross-reactive binding antibodies in the absence of detectable neutralizing activity against DENV. Data from Subject#2 were found to support this argument as enhancement of DENV infection occurred at a dilution of 1:10,000 in the presence of high levels of DENV specific neutralizing antibodies as compared to a dilution of 1:10 for serum from

4701 Subject#1 who had no detectable cross-neutralizing antibodies against DENV. Previous studies
4702 using heterologous DENV challenge in non-human primates have reported an increase in the
4703 titer of maximally enhancing dilution of serum that correlated with higher levels of neutralizing
4704 activity against DENV[15]. These findings raise the exciting possibility that high levels of
4705 neutralizing antibodies if induced against both ZIKV and DENV simultaneously could
4706 potentially prevent enhancement of DENV infection and at the same time protect from ZIKV
4707 infection. Additional studies are needed to test this hypothesis.

4708 Taken together, these data provide additional evidence that DENV cross-reactive
4709 antibodies induced by ZIKV could potentially enhance DENV infection. Additional
4710 epidemiological evidence is needed to determine if prior exposure to ZIKV in DENV naïve
4711 humans enhances subsequent DENV infection *in vivo*.

4712

4713 **Notes**

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4738 United States Government work as a work prepared by a military service member or employee
4739 of the United States Government as part of that person’s official duties.

4740 **Author contributions:** WGV performed the experiments and analyzed the data; WJV,
4741 TL, THB and JJM designed the study and wrote the manuscript. HCY and AK supervised the
4742 collection of samples and edited the manuscript.

4743 **Conflicts of interest:** The authors declare no financial conflict of interest.

4744

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Figure 1: Zika virus infection induces DENV cross-reactive IgM and IgG in serum that enhances DENV infection. Relative levels of anti-Zika and anti-Dengue-2 IgM and IgG in serum from (A) Subject#1 and (D) Subject#2 were determined using Zika and Dengue-2 virus specific ELISA. The assay cutoff was set at an O.D. of 0.1 for both IgM and IgG based on the O.D. of serum samples from flavivirus negative subjects (n = 4). Zika and Dengue-2 virus specific PRNT₅₀ titres in serum from (B) Subject#1 and (E) Subject#2 were determined using Plaque reduction neutralization test. Antibody dependent enhancement of Denvgue-2 infection in K562 cells using sera from (C) Subject#1 and (F) Subject#2.

