Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice

Angela M. Fowler,[1,5] William W. Tang,[1,5] Matthew P. Young,[1] Anila Mamidi,[1] Karla M. Viramontes,[1] Melanie D. McCauley,[1] Aaron F. Carlin,[2] Robert T. Schooley,[2] Jesica Swanstrom,[3] Ralph S. Baric,[3] Jennifer Govero,[4] Michael S. Diamond,[4] and Sujan Shresta[1,2,6,*]

[1]Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037, USA

[2]Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA

[3]Department of Epidemiology, University of North Carolina, Chapel Hill, NC 27599, USA

[4]Departments of Medicine, Molecular Microbiology, Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

[5]These authors contributed equally

[6]Lead Contact

*Correspondence: sujan@lji.org

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SUMMARY

Antibody (Ab)-dependent enhancement can exacerbate dengue virus (DENV) infection due to cross-reactive Abs from an initial DENV infection, facilitating replication of a second DENV. Zika virus (ZIKV) emerged in DENV-endemic areas, raising questions about whether existing immunity could affect these related flaviviruses. We show that mice born with circulating maternal Abs against ZIKV develop severe disease upon DENV infection. Compared with pups of naive mothers, those born to ZIKV-immune mice lacking type I interferon receptor in myeloid cells (*LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$) exhibit heightened disease and viremia upon DENV infection. Passive transfer of IgG isolated from mice born to ZIKV-immune mothers resulted in increased viremia in naive recipient mice. Treatment with Abs blocking inflammatory cytokine tumor necrosis factor linked to DENV disease or Abs blocking DENV entry improved survival of DENV-infected mice born to ZIKV-immune mothers. Thus, the maternal Ab response to ZIKV infection or vaccination might predispose to severe dengue disease in infants.

INTRODUCTION

Since the emergence of Zika virus (ZIKV) in dengue virus (DENV) endemic areas, a question that remains unanswered is how ZIKV and DENV immunity reciprocally affect each other in the context of sequential infections. DENV is the leading mosquito-transmitted viral infection globally (Guzman et al., 2010), with an estimated 390 million infections per year, and results in clinical symptoms ranging from unapparent to life threatening (Bhatt et al., 2013). DENV disease creates a substantial burden on public health resources, with more than 3 billion people at risk for infection worldwide (Shepard et al., 2016). DENV is a flavivirus and circulates as four different serotypes (DENV1–4) that vary by 25%–40% at the amino acid level. Although primary DENV infection usually manifests as a self-limiting febrile illness, secondary infections with a heterotypic serotype can result in dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), also referred to as severe dengue, which is associated with vascular leakage, hemodynamic shock, and death. One model for the pathogenesis of severe dengue involves the phenomenon of antibody (Ab)-dependent enhancement (ADE), where circulating cross-reactive Abs from the first DENV infection bind to the second DENV and facilitate its entry and replication in Fcγ receptor-expressing cells (Halstead, 2007).

ZIKV, the causal agent of congenital Zika syndrome (World Health Organization, 2016), is genetically and antigenically similar to DENV, with ~56% amino acid identity (Chang et al., 2017), and cross-reactivity between the two viruses at the Ab epitope level has been documented extensively (Bardina et al., 2017; Charles and Christofferson, 2016; Dejnirattisai et al., 2016; Kawiecki and Christofferson, 2016; Priyamvada et al., 2016; Stettler et al., 2016; Swanstrom et al., 2016). Indeed, studies have begun to evaluate the impact of the cross-reactive Ab response in protection against or pathogenesis of ZIKV and DENV infections. Although some cross-reactive monoclonal Abs generated against DENV protect against ZIKV (Barba-Spaeth et al., 2016; Fernandez et al., 2017), others generated against ZIKV can enhance DENV infection (Stettler et al., 2016). In the context of polyclonal Ab responses, prior ZIKV infection resulted in increased peak DENV viremia in macaques (George et al., 2017) and DENV-immune plasma enhanced ZIKV infection and disease severity in *Stat2*$^{-/-}$ mice (Bardina et al., 2017). These studies suggest that ADE can occur in different ZIKV and DENV infection scenarios.

In humans, maternal Abs from DENV-immune mothers can provide protection, enhancement, or no effect when passively transferred to an infant (Chau et al., 2009; Elong Ngono and Shresta, 2018; Halstead et al., 2002; Simmons et al., 2007). As levels of maternal Abs in infants fall to subneutralizing levels, there is an increased risk of developing severe dengue (Halstead et al., 2002). As the geographic range of ZIKV expands, it will become possible for mothers to be exposed to ZIKV and their infants to be infected with DENV. It currently remains unknown whether ZIKV Abs can result in enhancement or protection when transferred passively to infants. Here, we develop a model of severe

dengue in mice born to ZIKV-immune mothers using established *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mouse models of ZIKV and DENV infection (Elong Ngono et al., 2017; Pinto et al., 2015; Tang et al., 2016). Our results demonstrate that maternally acquired ZIKV Abs can enhance DENV infection and disease severity in young mice.

RESULTS

Decreased Survival of Pups Born to ZIKV-Immune Mothers after DENV2 Infection

To investigate whether maternal Abs from ZIKV-immune mothers conferred protection or promoted severe dengue disease in young mice, we inoculated 4- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ pups born to ZIKV-immune or naive mothers with DENV2-S221 and monitored them for weight loss, clinical signs, and survival. Pups born to ZIKV-immune mothers had similar immune cell numbers and frequency in the spleen as those born to naive mothers (Figure S1). Animals born to long-term (8–12 months) ZIKV-immune mothers and challenged with DENV2 had increased clinical scores compared with naive *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice, although both groups exhibited similar weight loss (Figures 1A–1C). Most of the mice in the naive control group recovered from DENV infection, whereas 100% mice born to long-term (8–12 months) ZIKV-immune mothers died by day 6 post infection (p.i.) (Figures 1A, 1B, and 1D). To determine whether short-term ZIKV infection period in mothers also affected the outcome of DENV infection in their pups, we assessed clinical score, weight loss, and survival in pups born to short-term (2 months) ZIKV-immune mothers (Figures 1E–1H). Both naive and ZIKV-immune groups exhibited similar weight loss after DENV2 challenge (Figure 1G), yet mice born to ZIKV-immune mothers had increased clinical scores and decreased survival compared with those born to naive mothers (Figures 1E, 1F, and 1H). These results demonstrate that, at 4–5 weeks of age, pups born to ZIKV-immune but not naive mice develop lethal disease upon challenge with DENV2.

Increased DENV2 Burden in Pups Born to ZIKV-Immune Mice

To determine whether enhanced disease severity in pups born to long-term (6–13 months) ZIKV-immune mothers was associated with increased levels of DENV2 infection, we compared viral RNA levels in pups born to ZIKV-immune versus naive mothers at day 3 p.i. DENV2 RNA levels were increased significantly in the serum (5-fold, ***$p < 0.001$), spleen (13-fold, **$p < 0.01$), and liver (8-fold, **$p < 0.01$) in pups born to ZIKV-immune mothers relative to naive pups (Figure 1I). Thus, severe dengue disease manifestations correlated with increased DENV2 tissue burden in mice born to ZIKV-immune mothers.

To examine whether enhanced disease severity in mice born to short-term ZIKV-infected mothers was due to increased DENV infection, we measured levels of infectious DENV in pups born to short-term (2 months) ZIKV-immune or naive mothers at day 3 p.i. Infectious DENV levels were higher in the serum (25-fold, ***$p < 0.001$), spleen (3-fold, **$p < 0.01$), and liver (9-fold, **$p < 0.01$) in pups born to ZIKV-immune than to naive mothers (Figure 1J), confirming the correlation between severe dengue disease and increased DENV2 burden in mice born to ZIKV-immune mothers.

Improved Clinical Phenotypes and Decreased ZIKV Burden in Pups Born to ZIKV-Immune Compared with Naive or DENV-Immune Mothers

As we observed a negative impact of maternal ZIKV immunity in pups upon challenge with DENV, we next examined the reciprocal conditions by testing whether maternal ZIKV or DENV immunity influenced the outcome of subsequent challenge of pups with ZIKV. When pups born to ZIKV-immune mothers were challenged with ZIKV, they had significantly less infectious ZIKV in the serum, spleen, liver, brain, and eyes compared with pups born to naive or DENV2-immune mothers (Figure S2A). Similar levels of infectious ZIKV were detected in pups born to DENV2-immune and naive mothers, with the exception of the liver where maternal DENV immunity had a protective effect (Figure S2A). Consistent with these data, pups born to ZIKV-immune mothers and challenged with ZIKV had better clinical scores and less weight loss than those born to naive mice (Figures S2B, S2C, and S2F). In contrast, pups born to DENV2-immune mothers and challenged with ZIKV had clinical scores similar to those born to naive mice but had a slightly different weight change (Figures S2D, S2E, and S2G). These data suggest that ZIKV maternal immunity protects against ZIKV challenge in infancy whereas DENV maternal immunity has a more neutral effect. As expected (Ng et al., 2014), pups born to DENV2-immune mothers and then challenged with DENV2 exhibited better clinical scores and no weight loss compared with pups born to naive mothers (Figures S3A and S3B), indicating that maternal DENV immunity protects against homologous DENV challenge. Thus, at least under the conditions tested, the enhanced pathogenesis observed in pups from ZIKV-immune mothers that are challenged with DENV2 is unique and does not occur when pups born to ZIKV-immune or DENV2-immune mothers are challenged with ZIKV.

Maternally Acquired ZIKV Abs Bind but Do Not Neutralize DENV2

To begin defining the mechanism of maternal ZIKV Ab-mediated DENV pathogenesis, we tested maternal ZIKV Abs from the serum of 4- to 5-week-old pups from ZIKV-immune mothers for their capacity to bind and neutralize DENV2. Sera of pups born to ZIKV-immune but not naive mothers contained ZIKV- and DENV2-reactive Abs (Figures 2A and 2B). However, these sera neutralized ZIKV but not DENV2 infection in U937-DC-SIGN cells and even appeared to enhance DENV2 infection (Figures 2C and 2D). Levels of ZIKV-binding Abs in pups born to short-term (2 months) mothers was lower than the levels observed in pups born to long-term ZIKV-immune mothers, whereas DENV2-binding Ab levels were similar between the two groups of mice (Figures 2E and 2F). Thus, maternal Abs in 4- to 5-week-old pups born to ZIKV-immune mothers cross-react with but do not cross-neutralize DENV2 *in vitro*.

To understand the lack of enhancement of ZIKV pathogenesis in pups with maternally acquired DENV2 Abs, we analyzed the binding and neutralization capacity of sera from 4- to 5-week old pups born to DENV2-immune mothers. Serum samples from mice born to DENV2-immune mothers bound to DENV2, but not ZIKV (Figures S3C and S3D). Additionally, these sera could neutralize DENV2 (Figure S3E). Thus, the absence of enhanced ZIKV pathogenesis in pups born to DENV2-immune

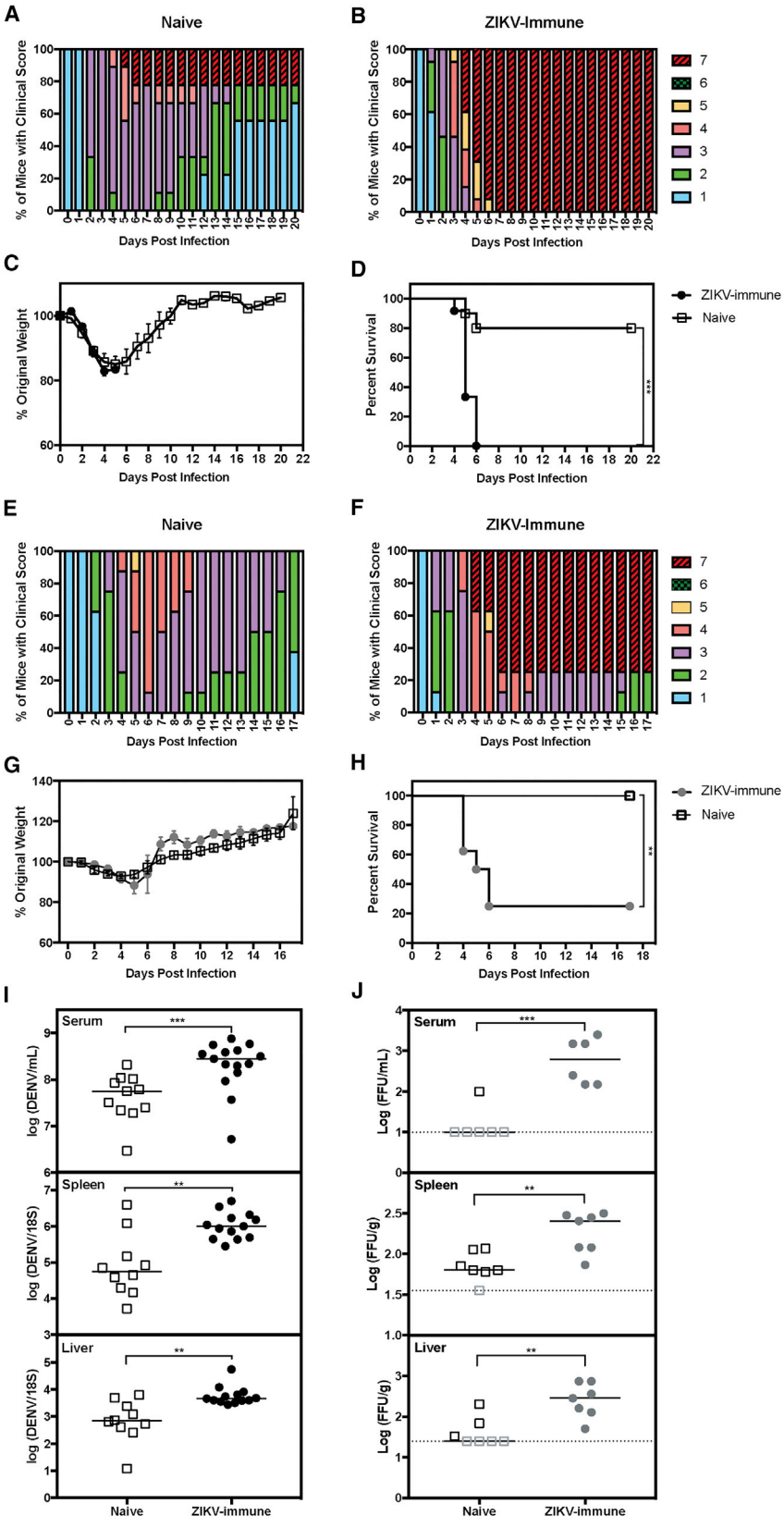

Figure 1. Mice Born to ZIKV-Immune Mothers Have Increased Dengue Disease Severity and DENV Burden

Four- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice born to mothers previously infected with ZIKV strain SD001 (10^6 focus-forming units [FFU] via retro-orbital route) or to naive mothers were challenged with DENV2 strain S221 (10^6 FFU via tail vein).

(A) Clinical scores of infected mice (n = 11) from naive mothers (n = 2).

(B) Clinical scores of infected mice (n = 15) from ZIKV-immune mothers infected for 8–12 months (n = 3).

(C and D) Weight loss and survival data of infected mice from naive (open squares) versus ZIKV-immune mothers infected for 8–12 months (black circles).

(E) Clinical scores of infected mice (n = 8) from naive mothers (n = 2).

(F) Clinical scores of infected mice (n = 8) from ZIKV-immune mothers infected for 2 months (n = 2).

(G and H) Weight loss and survival data of infected mice from naive (open squares) versus ZIKV-immune mothers infected for 2 months (gray circles). To determine DENV viral burden, we euthanized mice at 3 days p.i. and harvested serum, spleens, and livers.

(I) The levels of DENV RNA from each tissue were measured via qRT-PCR. Viral RNA levels in the serum, spleen, and liver of DENV2-infected pups (open squares, n = 11) from naive mothers (n = 2) were compared with pups (black circles, n = 13) from ZIKV-immune mothers infected for 6–13 months (n = 4).

(J) The levels of infectious DENV2 were measured via focus-forming assay in the serum, spleen, and liver of DENV2-infected pups (open squares, n = 7) from naive mothers (n = 2) and pups (gray circles, n = 7) from ZIKV-immune mothers infected for 2 months (n = 2).

Data in (C) and (G) were pooled from two independent experiments and are expressed as mean ± SEM; unpaired Student's t test of groups for each day. In (D) and (H) the log-rank test (***$p \leq 0.001$, **$p < 0.01$) was used. Data in (I) and (J) were pooled from two independent experiments. Mann-Whitney test (***$p < 0.001$, **$p < 0.01$).

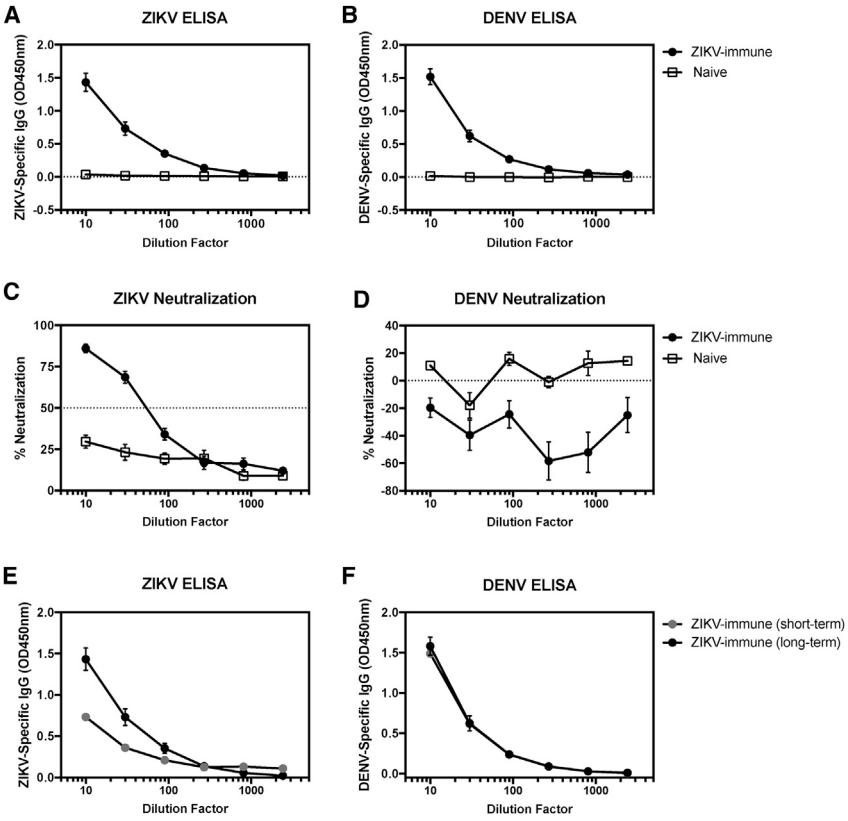

Figure 2. Sera from Mice Born to ZIKV-Immune Mothers Neutralize ZIKV but Not DENV2 Infection

Serum was collected from 4- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice born to mothers previously infected with ZIKV strain SD001 (10^6 FFU via retro-orbital route) or naive mothers. Serum of pups (open squares, n = 10) from naive mothers (n = 2) were compared with pups (black circles, n = 13) from ZIKV-immune mothers infected for 6–13 months (n = 4).

(A–D) Anti-ZIKV IgG (A) and anti-DENV IgG (B) were detected via ELISA. Neutralization capacity was assessed against (C) ZIKV SD001 and (D) DENV2 S221 via U937-DC-SIGN cells and a flow cytometry-based assay (Wen et al., 2017). Dotted line indicates limit of detection for ELISA and 50% or 0% neutralization line for the neutralization assay. Serum of pups (black circles, n = 13) from ZIKV-immune mothers infected for 6–13 months (n = 4) were compared with serum of pups (open squares, n = 10) from ZIKV-immune mothers infected for 2 months (n = 3).

(E and F) Anti-ZIKV IgG (E) and anti-DENV2 IgG (F) were detected via ELISA.

Data are pooled from three independent experiments and are expressed as mean ± SEM.

mothers is likely due to a lack of ZIKV binding by maternally acquired DENV2 Abs.

Increased Viral Burden in DENV2-Infected Mice with Passively Transferred IgG from Pups Born to ZIKV-Immune Mothers

To confirm that maternal ZIKV Abs were responsible for enhanced DENV2 infection, we isolated serum immunoglobulin G (IgG) from 4- to 5-week-old pups born to ZIKV-immune or naive mothers and then passively transferred it into age-matched naive 4- to 5-week old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ recipient mice immediately prior to infection. Two different preparations of IgG that were isolated from pooled sera collected from pups born to ZIKV-immune or naive mothers showed binding to both ZIKV and DENV but no neutralizing activity against DENV (Figures S4A–S4D). Mice that received 145 μg (amount that was determined from IgG isolated from a single mouse) of IgG from pups born to ZIKV-immune mothers had higher DENV2 RNA levels in the serum (7-fold, $^{**}p < 0.01$) and spleen (4-fold, $^{*}p < 0.05$) than mice receiving IgG from pups born to naive mothers at day 3 after challenge (Figure 3A). These results imply that maternally acquired IgG obtained from ZIKV-immune mothers contributes to the enhanced DENV2 infection and pathogenesis phenotypes observed after DENV2 challenge of pups at 4–5 weeks of age. As expected, when 10-fold less IgG was transferred to naive 4- to 5-week old recipient mice, no difference in DENV2 RNA levels in tissues was observed between mice that received IgG from ZIKV-immune versus naive mothers (Figure 3B), revealing the Ab concentration-dependent nature of the DENV2 enhancement phenotype.

TNF Levels Are Increased in Mice with Passively Transferred ZIKV IgG upon DENV2 Challenge

We next determined whether tumor necrosis factor (TNF) levels were increased in mice that received IgG from pups born to ZIKV-immune pups relative to mice that received IgG from naive pups. Human studies have shown that patients with severe dengue have higher levels of several pro-inflammatory cytokines, including TNF, compared with individuals with mild dengue (Green et al., 1999; Hober et al., 1993; Kittigul et al., 2000; Wang et al., 2007), and in a small observational study individuals on anti-TNF Ab therapy did not develop DHF/DSS (Deligny et al., 2014). Consistent with several studies demonstrating that the lethal ADE-mediated dengue disease in mice is TNF dependent (Ng et al., 2014; Phanthanawiboon et al., 2016; Shresta et al., 2006; Watanabe et al., 2015; Zellweger et al., 2010), TNF levels were increased in ZIKV IgG recipient mice compared with naive IgG recipient mice (Figure S4E), implying that ZIKV IgG treatment potentiates TNF induction.

TNF Blockade Decreases DENV2-Induced Lethality in Mice Born to ZIKV-Immune Mothers

To assess the significance of TNF induction in our mouse model, we determined whether DENV2-induced lethal disease in pups born to ZIKV-immune mice was mediated by TNF by performing blocking experiments with a neutralizing anti-TNF Ab. Mice born to ZIKV-immune mothers were inoculated with DENV2 and administered 100 μg of an anti-TNF or an isotype control Ab on days 1, 2, and 3 p.i. The anti-TNF Ab-treated group exhibited improved clinical scores, weight gain, and increased survival

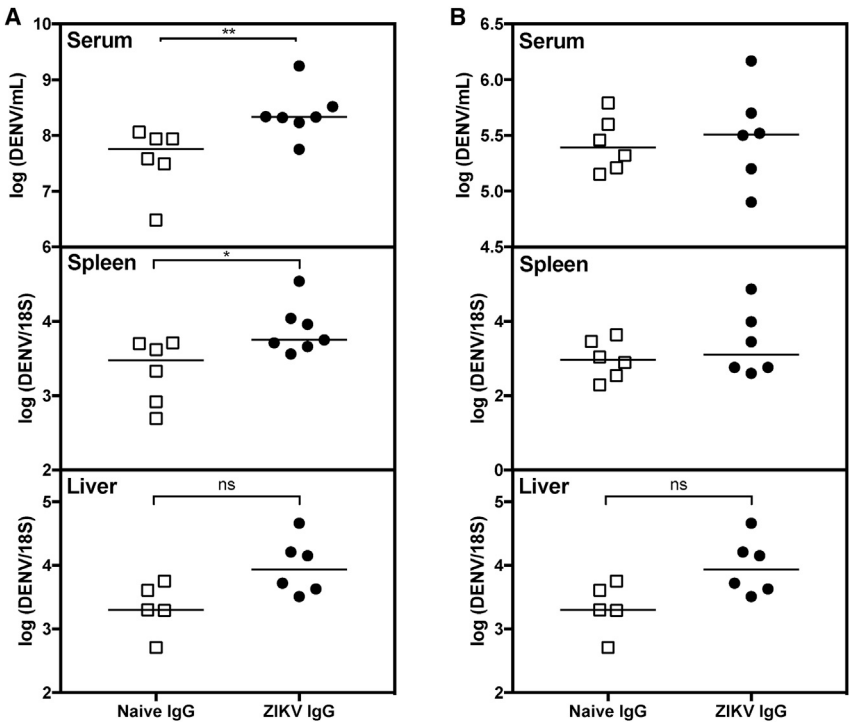

Figure 3. Passive Transfer of IgG Isolated from Mice Born to ZIKV-Immune Mothers into Naive Mice Increases Viral Burden upon DENV2 Challenge

(A and B) IgG was isolated from serum of 36 pups born to ZIKV-immune mothers infected for 6–12 months (n = 8) or from 32 age-matched naive mice from naive *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mothers (n = 7). 145 μg (A) or 14.5 μg (B) of purified IgG isolated from pups born to ZIKV-immune mothers (ZIKV IgG) or naive mothers (naive IgG) was passively transferred into naive 4- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ recipient mice, followed by challenge of the passively transferred recipient mice with 2 × 10^{5} FFU of DENV2 strain S221 via tail vein injection. Tissues were harvested 3 days p.i. and DENV2 RNA levels in the serum, spleen, and liver were quantified by qRT-PCR. n = 6–7 ZIKV IgG recipient mice and n = 6 naive IgG recipient mice. Mann-Whitney test (**$p < 0.01$, *$p < 0.05$).

compared with isotype control Ab-treated mice (Figures 4A–4D). This result is consistent with the hypothesis that DENV2-infected pups born to ZIKV-immune mothers have increased disease severity through ADE and overexuberant production of pro-inflammatory cytokines.

Monoclonal Ab Treatment Decreases DENV2-Induced Lethality in Mice Born to ZIKV-Immune Mothers

The original studies demonstrating ADE-mediated lethal dengue disease in mice showed that neutralizing DENV monoclonal Abs (mAbs) that blocked fusion could abrogate ADE (Balsitis et al., 2010; Zellweger et al., 2010). Given these data, we assessed whether the DENV2-induced lethal disease in pups born to ZIKV-immune mothers could also be prevented via treatment with neutralizing mAbs. We tested two human EDE1 mAbs (C8 and C10) that cross-neutralize different DENV serotypes and ZIKV (Dejnirattisai et al., 2015; Fernandez et al., 2017; Swanstrom et al., 2016) for their ability to reduce disease severity in DENV2-infected pups born to ZIKV-immune *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mothers. Administration of 100 μg of EDE1 C8 or EDE1 C10 Ab on days 1, 2, and 3 p.i. decreased dengue disease compared with treatment with PBS alone, based on clinical scores (Figures 4E–4G), weight loss (Figure 4H), and survival (Figure 4I). Thus, administration of EDE1 mAbs can prevent severe dengue disease in mice born to ZIKV-immune mothers.

DISCUSSION

Currently, little is known about how prior ZIKV immunity affects DENV pathogenesis and infection *in vivo*. One study showed that previous ZIKV exposure increased peak DENV viremia in macaques (George et al., 2017), and another reported enhanced DENV pathogenesis in mice administered a ZIKV mAb (Stettler et al., 2016). Consistent with these findings, our data show increased viral burden, worsened clinical signs, and decreased survival in mice born to ZIKV-immune mothers relative to those born to naive mothers. Our study demonstrates maternal Ab-mediated infant DHF/DSS in the context of pre-existing anti-flavivirus immune sera other than anti-DENV immune sera (Martinez Gomez et al., 2016; Ng et al., 2014). In comparison, maternal DENV Abs exerted mainly neutral effects against ZIKV in our mouse model, in agreement with the observation that prior DENV immunity (1–3 years long) did not influence subsequent ZIKV infection in macaques (McCracken et al., 2017; Pantoja et al., 2017) and consistent with reports that the anti-DENV Ab response in humans becomes less cross-neutralizing against ZIKV over time (Collins et al., 2017; Montoya et al., 2018). However, our result contrasts with a published study demonstrating increased ZIKV infection and pathogenesis in *Stat2*$^{-/-}$mice that were passively transferred with DENV-immune human plasma (Bardina et al., 2017). The disparity in results may be related to the magnitude and quality of Ab responses (e.g., binding, neutralization, isotype, specificity, and avidity) and perhaps the use of *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ versus *Stat2*$^{-/-}$mice.

By modeling a potential epidemiologic scenario in which infants born to ZIKV-experienced women are infected with DENV, our study has revealed a pathogenic potential of ZIKV maternal Abs during DENV infection. Mouse versus human differences related to the quantity and potentially quality of maternal IgG transferred into infants may affect the timing or duration of enhanced DENV pathogenesis in human infants versus mice. In our mouse model, 4- to 5-week-old pups born to flavivirus-naive mothers with either short-term (2 months) or long-term (>6 months) exposure to ZIKV exhibited increased dengue disease severity. In humans, the magnitude and quality of the Ab response to ZIKV may vary depending on the length of exposure (acute versus early convalescence versus late convalescence) and

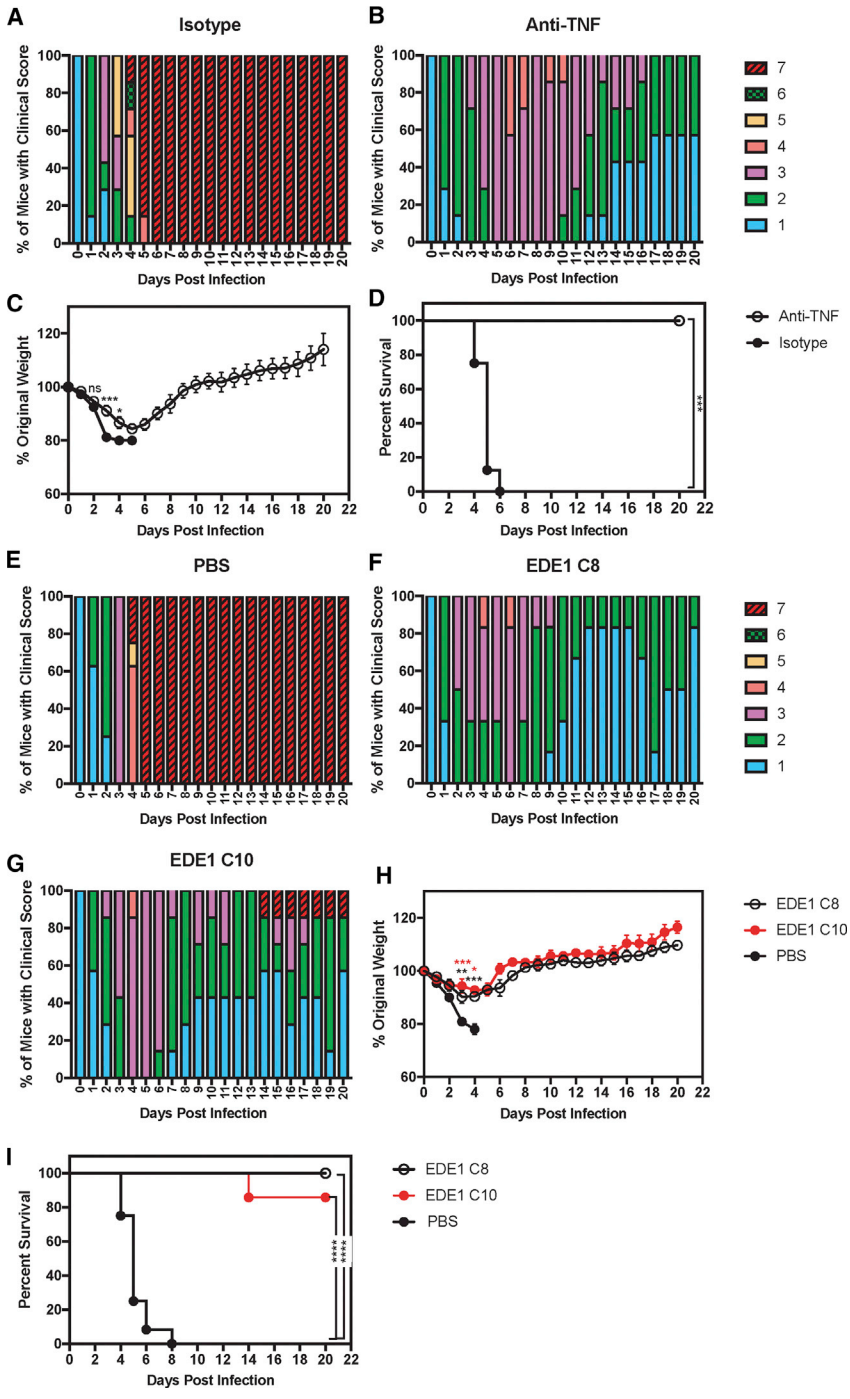

Figure 4. Administration of Anti-TNF Ab or EDE1 C8 and C10 Abs, which Recognize EDE1 Epitopes, Prevents Lethal Dengue Disease in Mice Born to ZIKV-Immune Mothers

Four- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice born to mothers infected with ZIKV strain SD001 (10^6 FFU via retro-orbital route) for 7–9 months (n = 3) were treated via an intraperitoneal injection with 100 μg of isotype control Ab (clone HPRN) or anti-TNF Ab (clone XT3.11) on days 1, 2, and 3 following inoculation with 10^6 FFU of DENV2 strain S221 via tail vein injection.

(A–D) Clinical scores of isotype control Ab-treated mice (n = 7) (A), clinical scores of anti-TNF Ab-treated mice (n = 8) (B), and weight loss (C) and survival rates (D) of isotype control Ab-treated mice (black circles) and anti-TNF Ab-treated mice (open circles). Administration of EDE1 C8 or EDE1 C10 Abs was performed in 4- to 5-week-old *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice born to mothers infected with ZIKV strain SD001 (10^6 FFU via retro-orbital route) for 12–13 months (n = 4). These pups were injected via an intraperitoneal route with PBS, EDE1 C8 Ab (100 μg), or EDE1 C10 Ab (100 μg) on days 1, 2, and 3 following challenge with DENV2 strain S221 (10^6 FFU via tail vein).

(E–H) Clinical scores of PBS control mice (n = 9) (E), clinical scores of EDE1 C8 Ab-administered mice (n = 10) (F), clinical scores of EDE1 C10 Ab-administered mice (n = 8) (G), and weight loss (H) and survival rates (I) of PBS-treated (black circles), EDE1 C8-treated (open circles), and EDE1 C10-treated (red circles) mice.

Data are pooled from two independent experiments and are expressed as mean ± SEM. Unpaired Student's t test of groups for each day was used in (C) and (H). Log-rank test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) was used in (D) and (I).

flavivirus-naive versus immune status of mothers (Collins et al., 2017; Montoya et al., 2018; Yu et al., 2017), thereby affecting the window of both potentially protective and pathogenic periods in infants.

In summary, our findings have implications for understanding DENV infections in countries with co-circulation of ZIKV and DENV or women with prior ZIKV immunity through natural infection or, possibly, vaccination. Mounting evidence supports a key role for ADE in pathogenesis of DENV infection in children and adults with secondary heterotypic DENV infection and infants born to DENV-immune women (Balsitis et al., 2010; Halstead, 2007; Katzelnick et al., 2017; Ng et al., 2014; Zellweger et al., 2010). Our results suggest that if women are infected with ZIKV or potentially immunized with a ZIKV vaccine that elicits a cross-reactive Ab response, their infants might have an increased risk of developing DHF/DSS. A ZIKV vaccine with fusion loop mutations in the E protein has been reported to reduce cross-reactive Ab responses and minimize enhancement of DENV infection and pathogenesis in mice (Richner et al., 2017), suggesting one potential avenue for designing ZIKV vaccines that avoid ZIKV maternal Ab-mediated severe DENV disease in infants. Thus, the emergence of ZIKV immunity in DENV-endemic regions may create an added risk for ADE-mediated severe dengue disease. Our mouse model may be useful for testing the effects of ZIKV and DENV (including different serotypes) on infant DENV and ZIKV infections and for evaluating the effects of ZIKV and DENV vaccine candidates that are designed for deployment in DENV-endemic regions. As T, B, and most dendritic cell responses are normal in

LysMCre^{+}*Ifnar1*$^{fl/fl}$ mice, this mouse model may also be useful for investigating mechanisms of DENV pathogenesis and immunity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.chom.2018.09.015.

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AUTHOR CONTRIBUTIONS

W.W.T. and S.S. conceived the project. A.M.F., W.W.T., M.P.Y., A.M., K.M.V., and J.G. performed and analyzed experiments. M.D.M., A.F.C., and R.T.S. obtained the ZIKV clinical isolate SD001. J.S. and R.S.B. were responsible for generating and testing EDE1-C8 and -C10 neutralizing antibodies. A.M.F. and S.S. wrote the manuscript. M.S.D., R.S.B., and S.S. conceived experiments and edited the manuscript.

DECLARATION OF INTERESTS

M.S.D. is a consultant for Inbios and is on the Scientific Advisory Board of Moderna. R.S.B. has consulted with Takeda and Sanofi Pasteur. All other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
4G2	ATCC	Cat. #HB-112 RRID: CVCL_J890
PerCP/Cy5.5 anti-mouse CD3e (clone: 145-2C11)	TONBO Biosciences	Cat. #65-0031-U100 RRID: AB_394599
Brilliant Violet 510 anti-mouse CD8a (clone: 53-6.7)	BioLegend	Cat. #100751 RRID: AB_2561389
CD4 Monoclonal Antibody APC-eFluor 780 (clone: GK1.5), eBioscience	Thermo Fisher Scientific	Cat. #47-0041-82 RRID: AB_11218896
CD19 Monoclonal Antibody PE (eBio1D3 (1D3)), eBioscience	Thermo Fisher Scientific	Cat. #12-0193-83 RRID: AB_657660
PerCp/Cy5.5 anti-mouse CD138 (Syndecan-1) (clone: 281-2)	BioLegend	Cat. #142510 RRID: AB_2561601
BD Pharmingen FITC rat anti-mouse IgD (clone: 11-26c.2a)	BD Biosciences	Cat. #553439 RRID: AB_394859
EDE1 C8	Ralph Baric (Swanstrom et al., 2016)	N/A
EDE1 C10	Ralph Baric (Swanstrom et al., 2016)	N/A
In vivo MAb rat IgG1 isotype control, anti-horseradish peroxidase (clone: HPRN)	BioXCell	Cat. #BE0088 RRID: AB_1107775
BD Pharmingen PE-labeled anti-human CD209 (clone: DCN46)	BD Biosciences	Cat. #551265 RRID: AB_394123
In vivo Mab anti-mouse TNFα (clone: XT3.11)	BioXCell	Cat. #BE0058 RRID: AB_1107764
Peroxidase conjugated affini-pure goat anti-mouse IgG $F(ab')_2$ fragment	Jackson ImmunoResearch	Cat. #115-035-072 RRID: AB_2338507
Peroxidase conjugated affini-pure goat anti-mouse IgG Fcγ	Jackson ImmunoResearch	Cat. #115-035-008 RRID: AB_2313585
Bacterial and Virus Strains		
ZIKV (SD001)	A.F.C. et al., unpublished data	N/A
DENV2 (S221)	Yauch et al., 2009	N/A
Chemicals, Peptides, and Recombinant Proteins		
Cytofix/Cytoperm	BD Biosciences	Cat. #554714
Qiamp Viral Mini Kit	Qiagen	Cat. #52904
RNAlater	Thermo Fisher Scientific	Cat. #AM7021
QuantaBio qScript one-step qRT-PCR kit	VWR	Cat. #101414-172
eBioscience TMB solution	Thermo Fisher Scientific	Cat. #00-4201-56
Experimental Models: Cell Lines		
Aedes albopicticus: C6/36	ATCC	Cat. #ATCC: CRL-1660 RRID: CVCL_Z230
Baby Hamster Kidney (BHK)-21	ATCC	Cat. #ATCC: CCL-10 RRID: CVCL_1915
U937-DC SIGN cells	ATCC	Cat. #CRL-3253 RRID: CVCL_2Z95
Experimental Models: Organisms/Strains		
Mouse: *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ C57BL/6	Michael S. Diamond (Clausen et al., 1999)	N/A
Oligonucleotides		
DENV2 forward primer: CATATTGACGCTGGGAAAGA	Prestwood et al., 2008	N/A
DENV2 reverse primer: AGAACCTGTTGATTCAAC	Prestwood et al., 2008	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
18S forward primer: CGGCTACCACATCCAAGGAA	Prestwood et al., 2008	N/A
18S reverse primer: GCTGGAATTACCGCGGCT	Prestwood et al., 2008	N/A
DENV2 probe – [Fam]-TGCTGGCCTC – [TamraQ]	Eurofins	N/A
18S probe – [Fam] – CTGTCTGGCA – [TamraQ]	Eurofins	N/A
Software and Algorithms		
FlowJo version 10	FlowJo	https://www.flowjo.com/
Graphpad Prism 7	Graphpad Prism Software	https://www.graphpad.com/
Other		
Pierce FITC antibody labelling kit	Thermo Fisher Scientific	Cat. #53027
Nab Protein G Spin Kit	Thermo Fisher Scientific	Cat. #89979
Slide-A-Lyzer	Thermo Fisher Scientific	Cat. #66212
Mouse TNF-α Quantikine ELISA kit	R&D Systems	Cat. #MTA00B
ZIKV E protein Suriname strain	Native Antigen Company	Cat. #ZIKVSU-ENV
KPL True Blue	SeraCare	Cat. #5510-0030

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sujan Shresta (sujan@lji.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Viruses

SD001 is a ZIKV clinical isolate obtained from an adult female traveler infected in Caracas, Venezuela in 2016 (A.F.C. et al., unpublished data). Infectious virus was isolated from a filtered urine sample from patient SD001 and propagated in C6/36 *Aedes albopictus* cells (ATCC, cat. # ATCC: CRL 1660). Mouse-adapted DENV2 strain S221 was propagated in C6/36 cells. ZIKV-SD001 and DENV2-S221 were titrated by focus-forming assay using baby hamster kidney (BHK-21) (ATCC, cat. #ATCC: CCL 10) cells as described in the virus quantification section.

Cell Lines

C6/36 mosquito cells were propagated in Leibovitz's L-15 (Thermo Fisher Scientific, cat. #11415064) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, cat. #16000044), 1% penicillin/streptomycin (Thermo Fisher Scientific cat. #15140-122), and 1% HEPES (Thermo Fisher Scientific, cat. #15630080) at 28°C. BHK-21 cells were grown in MEM α (Fisher, cat. #12-561-072) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES at 37°C in a 5% CO_2 atmosphere. U937-DC-SIGN cells were propagated in RPMI 1640 (Thermo Fisher Scientific, cat. #11875093) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES at 37°C in a 5% CO_2 atmosphere.

Mice

LysMCre^{+}*Ifnar1*$^{fl/fl}$ C57BL/6 mice lack type I interferon (IFN) receptors in a subset of myeloid cells (Clausen et al., 1999; Diamond et al., 2011) and were characterized previously as a model for DENV (Pinto et al., 2015; Zust et al., 2014) and ZIKV (Elong Ngono et al., 2017; Tang et al., 2016) infection. Female mice were inoculated via retro-orbital route with 10^6 focus-forming units (FFU) of ZIKV-SD001 diluted in 10% FBS/PBS (100 μL total volume) or with 5 x 10^5 FFU of DENV2-S221 via tail vein injection. At four weeks post-ZIKV or DENV infection, females were bred with naive 6- to 8-week old male *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice. Male and female pups born to ZIKV- or DENV-infected *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ females were challenged at 4- to 5-weeks of age. Analyses were not performed on whether or not the sex of the mouse influenced the overall outcome as the outcome did not differ based on the sex of the mouse. Four- to 5-week-old age-matched pups (both males and females) born to naive *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mothers were used as controls. The mothers' ZIKV or DENV infection lengths are stated in each figure legend. All experiments were performed following the La Jolla Institute Animal Care and Use Committee-approved animal protocol #AP 00001029. Mice were housed at a maximum of 5 per cage with the same sex of mouse. Water, food, and housing with bedding and enrichment were autoclaved prior to being utilized. Cages were changed every 2 weeks under a laminar flow hood. Proper personal protective equipment was worn when in the vivarium and handling the mice. All mice were bred and maintained under specific pathogen free (SPF) conditions, and infected mice were housed in a BSL2 SPF room.

METHOD DETAILS

Disease Scoring

Pups born to ZIKV-immune or naive mothers at 4- to 5-weeks of age were inoculated with 1 x 10^6 FFU of DENV2 S221 diluted in 10% FBS/PBS (200 μL total volume) per mouse via tail vein injection. Mice were monitored daily for weight and clinical scores from day 0 to day 20 p.i. Clinical scores ranged from 1 - 7: 1, heathy mice with a smooth coat and bright, alert eyes; 2, mice are slightly ruffled around the head and neck, but active and alert; 3, mice have a ruffled coat throughout the body, but still active and alert; 4, mice have a very ruffled coat and slightly closed eyes, they walk slowly, and they have mild lethargy; 5, mice have a very ruffled coat and closed inset eyes, slow to no movement but will return to the upright position if put on the side; 6, mice have a very ruffled coat and closed inset eyes, are moribund, they have no movement or uncontrollable spastic movements, will not return to upright position if put on its side, and completely unaware or in noticeable distress and require humane euthanasia; 7, mice are deceased. Mice were humanely euthanized if weight loss was greater than or equal to 20% of their body mass or if their clinical score was a 6.

Flow Cytometric Analysis of Immune Cell Populations

Spleens were harvested from pups born to ZIKV-immune or naive mothers at 4-5 weeks of age after being humanely euthanized with CO_2. Splenocytes were plated into 96-well round-bottom plates at 1 x 10^6 cells/well in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES. Splenocytes were washed with PBS and stained with anti-CD3 PerCP-Cy5.5 (Tonbo Biosciences, cat. #65-0031-U100), anti-CD4 APC eflour780 (Thermo Fisher Scientific, cat. #47-0041-82), and anti-CD8 BV510 (BioLegend, cat. #100751) or with anti-CD19 PE (Thermo Fisher Scientific, cat. #12-0193-83), anti-CD138 PerCP-Cy5.5 (BioLegend, cat. #142510), and anti-mouse IgD FITC (BD Biosciences, cat. #553439). Cells were incubated with these Abs (each at 1:200 dilution) for 30 min, followed by washing for 3 times with FACs buffer. The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, cat. #554714), washed and resuspended in FACs buffer.

Infectious Virus Quantification

Four- to 5-week old *LysMCre*$^+$*Ifnar1*$^{fl/fl}$ mice born to ZIKV-immune or naive mothers were inoculated with 2 x 10^5 FFU DENV2 S221 diluted in 10% FBS/PBS via tail vein injection (200 μL total volume) or with 1 x 10^5 FFU ZIKV in 10% FBS/PBS retro-orbitally (100 μL total volume). Mice were humanely euthanized with CO_2 3 days p.i. Blood was obtained via cardiac puncture, centrifuged (16,363 x g for 15 min at 4°C), and serum was stored at -80°C. Mice were perfused with PBS. Spleen and livers were harvested and put in pre-weighed tubes containing complete MEM α containing a metal bead and stored at -80°C. Viral titers were measured using a BHK-21 cell-based focus forming assay (FFA). BHK-21 cells were plated at 2 x 10^5 cells per well in a 24-well plate and incubated overnight in complete MEM α medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES at 37°C in a 5% CO_2 atmosphere. Cells were infected with serial dilutions of virus for 1.5 hr with gentle shaking every 15 min. The medium was then aspirated and replaced with fresh MEM α supplemented with 1% carboxymethyl cellulose (Sigma, cat. #419273), 10% FBS, 1% penicillin/streptomycin, and 1% HEPES. Cells were then cultured for 3 days. At 3 days p.i., cells were fixed with 4% formalin (Fisher Scientific, cat. #SF98-4) for 30 min, washed 3 times with PBS, permeabilized with 1% Triton X-100 (Sigma, cat. #X100-100ML) for 30 min, washed 3 times with PBS, and blocked by 10% FBS in PBS for 30 min. ZIKV or DENV was detected by incubation of cells with 4G2, a pan-flavivirus E protein-specific monoclonal Ab (ATCC, cat. # D1-4G2-4-15 (ATCC HB-112)) (1 μg/mL in 1% FBS/PBS) for 1.5 h at room temperature or overnight at 4°C. Cells were washed 3 times with PBS and incubated for 1.5 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, cat. #115-035-072) (diluted 1:1000 in 1% bovine serum albumin (BSA)/PBS), followed by washing 3 times with PBS. Finally, foci were detected by incubation with KPL True Blue substrate (SeraCare, cat. #5510-0030) for 20 min and rinsed in diH_2O.

Viral RNA Quantification

Four- to 5-week-old *LysMCre*$^+$*Ifnar1*$^{fl/fl}$ mice born to ZIKV-immune or naive mothers were inoculated with 2 x 10^5 FFU DENV2 S221 diluted in 10% FBS/PBS (200 μL total volume) via tail vein injection. Mice were humanely euthanized 3 days p.i. Blood was obtained via cardiac puncture, centrifuged (16,363 x g for 15 min at 4°C), and serum was stored at -20°C. Mice were perfused with PBS, and their spleens and livers were harvested and stored in RNAlater (Thermo Fisher Scientific, cat. #AM7021) at 4°C. Tissues were homogenized prior to RNA extraction. RNA was isolated via Qiagen QIAmp viral mini kit (Qiagen, cat. #52904) and qRT-PCR was performed as previously described (Prestwood et al., 2008) using the QuantaBio qScript one-step qRT-PCR kit (VWR, cat. #101414-172) and probes and primers described in the Key Resources Table. PCR mixtures were pre-incubated at 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of two-step incubations at 95°C for 15 s and 60°C for 1 min for DENV2. DENV2 samples were compared to a standard curve of 10^3 – 10^7 copies of DENV2 RNA. 18S samples were diluted and compared to a standard of 18S rRNA that was derived from a mouse spleen and diluted to 10^2 – 10^4 18S RNA. 18S RNA was ran for 10 min at 48°C, 5 min at 98°C, and 39 cycles of 15 seconds at 95°C and 1 min at 60°C.

ZIKV and DENV ELISA

Serum samples from mice born to ZIKV- or DENV-immune mothers were tested for ZIKV- and DENV-binding antibodies using a direct ELISA. To detect DENV antibodies, sucrose purified DENV2 S221 virions were used at a concentration of 1 x 10^6 FFU/well as the coating antigen and UV-inactivated. DENV2 was diluted in 50 μL coating buffer (0.1M $NaHCO_3$ in PBS) per well and incubated at

4°C overnight. Wells were washed 3 times with ELISA washing buffer (0.05% Tween20 in PBS) and then blocked with 5% casein in PBS for an hour at room temperature. Serum was first diluted 1:10 in 10% FBS/PBS and then 1:3 for subsequent dilutions and was incubated in wells for 1.5 hr at room temperature. Wells were washed 3 times with ELISA washing buffer. Wells were then incubated with peroxidase conjugated Affini-Pure Goat anti-mouse IgG Fcγ (Jackson ImmunoResearch, cat. #115-035-008) diluted 1:5000 in 1% BSA/PBS at room temperature. Wells were washed 3 times with ELISA washing buffer. 100 μL of TMB substrate solution was added until blue color change, and reaction was stopped with 50 μL of 2N sulfuric acid (Sigma, cat. #339741). To detect ZIKV Abs, ZIKV E protein (Suriname strain, Native Antigen Company, #ZIKVSU-ENV) was adsorbed to 96-well plates at a concentration of 1 μg/mL in coating buffer (0.1M $NaHCO_3$ in PBS) overnight at 4°C and the remaining steps were the same as the DENV ELISA.

Neutralization Assays

Serum samples from naive pups born to ZIKV- or DENV-immune mothers were used in a standard flow cytometry-based neutralization assay using U937-DC-SIGN cells (Wen et al., 2017). Mouse serum was inactivated at 56°C for 30 min . Sera were diluted at 1:10 and then at 1:3 for subsequent dilutions up to 1:7290 and then incubated with 10^5 FFU of DENV2 S221 or with 6 x 10^4 FFU ZIKV-SD001 in RPMI 1640 supplemented with 1% penicillin/streptomycin and 1% HEPES in a 96-well round bottom plate. Virus and sera were incubated together at 37°C with 5% CO_2 for 1 hr . U937-DC-SIGN cells were then seeded into each well (10^5 cells per well in a 96-well round bottom plate), followed by addition of the virus/serum mixture to cells. Plates were incubated at 37°C with 5% CO_2 for 2 hr , rocking every 15 min . Plates were then centrifuged at 300 x *g* for 5 min and media was replaced with RPMI 1640 (supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% HEPES). Sixteen hours later, cells were stained with PE-labeled anti-human CD209 diluted 1:100 (BD Pharmingen, cat. #551265), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, cat. #554714), and then stained with FITC-labeled anti-flavivirus E protein antibody (clone 4G2) diluted 1:100. 4G2 was conjugated to Pierce FITC using an antibody labelling kit (Thermo Fisher Scientific, cat. #53027).

TNF Blockade

At days 1, 2, and 3 post challenge with DENV, pups born to ZIKV-immune mothers (n = 3 mothers infected for 7-9 months) were treated via intraperitoneal injection with 100 μg of anti-TNF (BioXcell, clone XT3.11, Rat IgG1, cat. #BE0058) or an isotype control (BioXcell, clone HRPN, Rat IgG1, cat. #BE0088) Ab that was diluted in PBS (200 μL total volume per mouse). Mice were clinically scored, weighed, and monitored for survival, on a daily basis, until day 20 p.i.

Passive Transfer of IgG

Serum IgG was purified from 4- to 5-week-old mice that were born to naive or ZIKV-immune mothers. For the first IgG preparation, serum was isolated from 23 4- to 5-week old mice from 3 different ZIKV-immune mothers and 20 naive mice from 2 different mothers. For the second IgG preparation, serum was isolated from 13 4- to 5-week-old mice from 2 different ZIKV-immune mothers and 9 naive mice from 2 different mothers. IgG was isolated from the pooled serum using the NAb protein G spin columns, 1 mL (Thermo Fisher Scientific, cat. #89957). Buffer was exchanged with PBS by the Slide-a-lyzer dialysis cassettes, 2K molecular weight cut-off, 12 mL (Thermo Fisher Scientific, cat. #66212) per manufacturer's directions. The isolated IgGs were injected via intraperitoneal route into 4- to 5-week old naive *LysMCre*$^{+}$*Ifnar1*$^{fl/fl}$ mice 30 min prior to inoculation with 2 x 10^5 FFU of DENV2 S221 via the tail vein. Three days p.i., mice were humanely euthanized. Blood was drawn, serum was isolated, and spleens and livers were harvested.

TNF ELISA

Serum samples from mice that received IgG was assessed for TNF levels using a R&D Systems quantikine ELISA kit (R&D Systems, cat. #MTA00B). 52 μL of the serum from each recipient mouse that received 145 μg of IgG was assessed to determine levels of TNF using the kit protocol.

Monoclonal Ab Treatment

At days 1, 2, and 3 post challenge with DENV2, pups born to ZIKV-immune mothers (*n*=4 mothers infected for 12-13 months) were treated via intraperitoneal injection with 100 μg of monoclonal Ab (C8 or C10) recognizing the DENV E-dimer epitope (EDE) (Dejnirattisai et al., 2015) diluted in PBS to a total of 200 μL total volume per mouse. Both EDE1-C8 and EDE1-C10 Abs were produced recombinantly (Swanstrom et al., 2016). Mice were clinically scored, weighed, and monitored for survival on a daily basis for 20 days p.i.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow Cytometric Analysis of Immune Cell Populations

Following Ab staining, splenocytes were resuspended in FACs buffer and analyzed on the LSRII flow cytometer. Data were analyzed using FlowJo software X 10.0.7 (Tree Star).

Infectious Virus Quantification

Infectious viral foci were detected by True Blue peroxidase substrate reaction and counted manually. Viral titers were expressed as log FFU/g tissue or log FFU/mL serum.

Viral RNA Quantification

DENV2 RNA was quantified using the CFX96 Touch real-time PCR detection system (Bio-Rad CFX Manager 3.1) and normalized to volume for serum and to 18S RNA levels for spleens and livers.

ZIKV and DENV ELISA

ZIKV and DENV specific IgG were detected by TMB reaction. Signals were quantified using a Spectramax M2E at 450 nm.

Neutralization Assays

After Ab staining, infected U937-DC-SIGN cells were resuspended in FACS buffer and analyzed on the LSRII flow cytometer. Data were analyzed using FlowJo software X 10.0.7 (Tree Star). Percent inhibition was calculated by taking the value of % infection of the control with no serum - % infection of sample and dividing all by the value of % infection of the control with no serum.

Statistical Analysis

n demonstrates the number of mice used per experiment. For Figures 1A–1D: 11 male and female pups were used from 2 naive mothers and 15 male and female pups were used from 3 ZIKV-immune mothers that were infected for 8-12 months; Figures 1E–1H: 8 male and female mice were used from 2 naive mothers and 8 male and female mice from 2 ZIKV-immune mothers infected for 2 months; Figure 1I: 11 male and female pups from 2 naive mothers and 13 male and female pups from 4 ZIKV-immune mothers infected for 6-13 months; Figure 1J: 7 female and male pups from 2 naive mothers and 7 male and female pups from 2 ZIKV-immune mothers infected for 2 months. For Figures 2A–2D: serum was obtained from 10 male and female pups born to 2 naive mothers and 13 male and female pups born to 4 ZIKV-immune mothers infected for 6-13 months; Figures 2E and 2F: serum from 10 male and female mice from 2 ZIKV-immune mothers infected for 2 months compared to serum obtained in Figures 2A–2D. For Figure 3: IgG was isolated from 32 male and female pups born to 7 naive mothers and 36 male and female pups born to 8 ZIKV-immune mothers infected for 6-12 months and 13 male and female mice from 3 naive mothers were used as recipient mice. For Figures 4A–4D: 15 male and female mice from 3 ZIKV-immune mothers infected for 7-9 months; Figures 4E–4I 27 male and female mice from 4 ZIKV-immune mothers infected for 12-13 months. *n* values can also be found in figure legends.

Data were analyzed using Graphpad Prism, version 7 (Graphpad Software). p values were obtained by using Mann Whitney test, unpaired Student's t test, one-way ANOVA or by using the log-rank test for survival curves. Goodness of fit tests were performed for data analyzed by unpaired Student's t tests to determine normal distribution of data as expected. Statistical details are described in corresponding figure legends along with p values.