Japanese encephalitis virus–primed CD8+ T cells prevent antibody-dependent enhancement of Zika virus pathogenesis

Dong Chen1,3*, Zhiiliang Duan1,3*, Wenhua Zhou1, Weiwei Zou1, Shengwei Jin5, Dezhou Li3, Xinyu Chen3, Yongchao Zhou4, Lan Yang3, Yanjun Zhang6,7, Sujan Shresta7, and Jinsheng Wen1,4*,8

Cross-reactive anti-flaviviral immunity can influence the outcome of infections with heterologous flaviviruses. However, it is unclear how the interplay between cross-reactive antibodies and T cells tilts the balance toward pathogenesis versus protection during secondary Zika virus (ZIKV) and Japanese encephalitis virus (JEV) infections. We show that sera and IgG from JEV-vaccinated humans and JEV-inoculated mice cross-reacted with ZIKV, exacerbated lethal ZIKV infection upon transfer to mice, and promoted viral replication and mortality upon ZIKV infection of the neonates born to immune mothers. In contrast, transfer of CD8+ T cells from JEV-exposed mice was protective, reducing the viral burden and mortality of ZIKV-infected mice and abrogating the lethal effects of antibody-mediated enhancement of ZIKV infection in mice. Conversely, cross-reactive anti-JEV antibodies or CD8+ T cells displayed the same pathogenic or protective effects upon JEV infection, with the exception that maternally acquired anti-ZIKV antibodies had no effect on JEV infection of the neonates. These results provide clues for developing safe anti-JEV/ZIKV vaccines.

Introduction

Zika virus (ZIKV), a member of the Flaviviridae family, Flavivirus genus, shares a high degree of amino acid similarity with other flaviviruses, including yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). ZIKV was initially isolated from a rhesus monkey in Uganda in 1947, and subsequently caused large outbreaks in French Polynesia (2013–2014) and South America (2015–2016). By early 2017, ZIKV had been reported in 84 countries or territories worldwide (World Health Organization, 2017). Most ZIKV infections cause mild symptoms of fever and headache but can also induce the neurological autoimmune disease Guillain–Barré syndrome (Monsalve et al., 2017). Moreover, infection of pregnant women has been linked to severe fetal defects, including microcephaly (Li et al., 2016a; Mlakar et al., 2016). JEV circulates mainly in Western Pacific, East Asian, Southeast Asian, and South Asian countries (Centers for Disease Control and Prevention [CDC], 2013). Like ZIKV infection, JEV predominantly causes mild or no symptoms, but ~67,900 cases annually progress to Japanese encephalitis, which has a case fatality rate of 20 to 30% (Campbell et al., 2011; Centers for Disease Control and Prevention [CDC], 2013).

Current evidence suggests that exposure to one flavivirus can either protect against or exacerbate secondary infections with a heterotypic serotype or flavivirus (Bardina et al., 2017; Dejnirattisai et al., 2010, 2016; Fowler et al., 2018; George et al., 2017; Ngono and Shresta, 2018; Tesh et al., 2002; Vázquez-Calvo et al., 2017). The mechanisms by which flavivirus cross-reactive immune responses contribute to protection or pathogenesis are not fully understood but may be influenced by the degree of sequence homology, the sequence of infections, and the interval between infections (Elong Ngono and Shresta, 2019; Ngono and Shresta, 2018). Given that many countries routinely vaccinate against JEV (Campbell et al., 2011) and that ZIKV is rapidly spreading to JEV-endemic regions, including heavily populated countries such as China and India (Khaiboullina et al., 2018; Kutsuna et al., 2014; Quyen et al., 2017; Ruchusatsawat et al., 2019; World Health Organization, 2017;...
Antibody (Ab)-dependent enhancement (ADE) of infection can influence the severity of illness following flavivirus infections (Ngono and Shresta, 2018). ADE describes a phenomenon whereby cross-reactive, sub-neutralizing Abs induce death during infection with one flavivirus promote infection of Fcγ receptor-bearing cells upon secondary infection by a heterotypic virus, thereby exacerbating the disease (Katzelnick et al., 2017; Salje et al., 2018). ADE was first experimentally characterized for DENV in studies showing that passive transfer of DENV-immune sera can enhance subsequent DENV infection and disease severity in naive mice (Balsitis et al., 2010; Zellweger et al., 2010). A growing body of evidence suggests that prior infection with DENV may have both positive and negative implications for the clinical consequences of ZIKV infection, depending on the context and balance of humoral and cellular immunity (Elong Ngono and Shresta, 2019; Wen and Shresta, 2019). For instance, recent studies using mice and human placental explants have demonstrated that DENV-specific Abs can mediate ADE of ZIKV infection and pathogenesis (Bardina et al., 2017; Brown et al., 2019; Rathore et al., 2019; Zimmerman et al., 2018). Although preexisting anti-DENV Abs may exacerbate ZIKV infection via ADE, cross-reactive anti-DENV cellular immunity appears to play a protective role during ZIKV infection. Mouse models of sequential DENV-ZIKV infection have revealed that DENV-elicited CD8+ T cells mediate short-term cross-protection against subsequent ZIKV infection in both nonpregnant and pregnant mice (Regla-Nava et al., 2018; Wen et al., 2017a, b). Consistent with these findings in mice, recent epidemiological studies indicate that prior DENV immunity confers cross-protection against ZIKV infection in humans (Gordon et al., 2019; Pedroso et al., 2019; Rodriguez-Barraquer et al., 2019). Thus, interplay between preexisting cross-reactive Ab and T cell responses likely determines the outcome of a subsequent ZIKV infection.

In contrast to sequential DENV-ZIKV and ZIKV-DENV infections, no studies have yet examined the impact of interplay between prior JEV humoral and cellular immunity on ZIKV infection, or vice versa. In hamsters and mice, immunization with a live-attenuated vaccine strain of JEV confers protection againstencephalitis and/or death upon WNV and DENV challenge (Li et al., 2016c; Tesh et al., 2002). In addition, sera from JEV-vaccinated individuals can mediate ADE of DENV infection in Fcγ receptor-positive baby hamster kidney (BHK)-21 cells (Saito et al., 2016), and preexisting JEV Abs have been shown to increase the severity of symptomatic dengue illness in children and adults (Anderson et al., 2011; Sato et al., 2015). Pre-existing JEV Abs also enhance subsequent infection with live-attenuated YFV, thereby improving its immunogenicity (Chan et al., 2016). Thus, while the prevailing evidence suggests that coexisting JEV-elicited cross-reactive Abs and T cells are likely to affect ZIKV infection, this has not yet been investigated.

In the present study, we sought to address these knowledge gaps by modeling sequential infections with JEV and ZIKV or vice versa. We determined how transfer of serum, serum-derived IgG, and CD8+ T cells from mice or humans exposed to one flavivirus affects the development and outcome of a subsequent infection with the heterologous flavivirus in mice. We also assessed the effect of maternally acquired anti-JEV Abs on ZIKV infection in neonatal mice, and vice versa. We show that (i) JEV-immune human and mouse sera mediate ADE of ZIKV infection in vitro and in vivo and decrease the survival of recipient mice following ZIKV infection; (ii) maternally acquired anti-JEV Abs increase the mortality of neonatal mice challenged with ZIKV; (iii) CD8+ T cells from JEV-primed mice cross-react with immunodominant ZIKV epitopes, and transfer of such T cells protects against lethal ZIKV infection; and (iv) cotransfer of JEV-primed CD8+ T cells with JEV-immune serum to naive mice abrogates ADE of ZIKV infection. Importantly, most, but not all, of these results are also observed in the context of preexisting ZIKV immunity and subsequent JEV infection. Collectively, these results suggest that cross-reactive CD8+ T cells can protect against ADE of heterologous flaviviral infections, and thus imply that a precise balance between cross-reactive Ab and T cell responses determines the outcome of subsequent infection with a heterologous flavivirus.

**Results**

Sera from JEV- and ZIKV-immune mice and humans cross-react with heterologous flaviviral EDIII proteins

The amino acid homology between ZIKV and JEV ranges from ~38 to 55% in the structural proteins (capsid [C], premembrane/membrane [prM/M], and envelope [E]) and ~36 to 68% in the nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; BLAST search; data not shown). Flaviviral protein E contains three extracellular domains, I, II, and III, and is the main target for Ab responses to flaviviruses (Luca et al., 2012; Sirohi et al., 2016). E protein domain III (EDIII) encodes epitopes that bind to host cellular receptors and can generate potently neutralizing Abs (Luca et al., 2012). Notably, JEV and ZIKV EDIIIs are ~54% identical at the amino acid level (BLAST search; data not shown).

We first tested sera from JEV-naïve infants or JEV-naïve, JEV-vaccinated children and young adults (aged 7–20 yr, with no history of DENV, ZIKV, or YFV infection or vaccination) for their ability to bind recombinant ZIKV-EDIII or JEV-EDIII using indirect ELISAs. As shown in Fig. 1, sera from JEV-vaccinated individuals contained comparably high titers of IgG to both JEV-EDIII and ZIKV-EDIII, suggesting strong antigenic cross-reactivity between the two proteins (Fig. 1, A–C). Moreover, the anti-JEV-EDIII and anti-ZIKV-EDIII titers did not differ markedly between the sera from JEV-immune individuals of different ages (Fig. 1, A and B), suggesting that JEV vaccination induced long-lasting Ab responses to both viruses. A cross-reactive IgG response to JEV-EDIII and ZIKV-EDIII was also observed in sera from WT C57BL/6 mice inoculated with JEV (Fig. 1, D and E). Analysis of sera from ZIKV-inoculated WT mice gave similar results, with high IgG titers to both ZIKV-EDIII and JEV-EDIII (Fig. 2, A and B). These data indicate that exposure of humans or mice to JEV or ZIKV elicits an Ab response that strongly cross-reacts with ZIKV or JEV, respectively.
Sera from JEV-immune mice and humans contain modest neutralizing activity and can enhance ZIKV infection in vitro

The ADE paradigm of viral infection proposes that failure to elicit a fully neutralizing Ab response during a primary viral infection can render the host susceptible to enhancement of subsequent infections. Therefore, we determined the ability of JEV-elicited ZIKV-reactive Abs to mediate protection vs. enhancement of ZIKV infection. We first performed a micro-neutralization assay to assess the ability of JEV-immune sera to block ZIKV infection of Vero cells in vitro. Naive mouse and human sera failed to neutralize ZIKV infection, as expected; however, mouse and human JEV-immune sera had only modest neutralizing activity (Fig. S1, A and B). The 50% neutralizing titers for mouse and human JEV-immune sera were 1:112 and 1:25–1:79, respectively. To determine whether JEV-immune sera could mediate ADE of ZIKV infection in vitro, we performed infection enhancement assays with the human macrophage cell line U937, which is relatively resistant to infection by ZIKV without ADE (Dejnirattisai et al., 2016). Interestingly, sera from JEV-immune humans or mice caused significant enhancement of ZIKV infection of cells (12–31-fold and 17-fold, respectively) compared with JEV-naive sera (Fig. S1, E and F). Thus, JEV-elicited human and mouse sera with modest ZIKV-neutralizing activity are able to enhance ZIKV infection of cells in vitro.

Sera from JEV-immune mice and humans promote lethal infection with the heterologous virus

Next, we examined whether JEV-immune sera could promote ADE of ZIKV infection in vivo as well as in vitro. For these experiments, we selected 1-d-old WT mice because ZIKV infection is lethal in neonates but not in adults (Li et al., 2018; Manangeeswaran et al., 2016). Although the precise reasons for this differential...
susceptibility are unclear, it may be due to the immaturity of the neonatal immune system, including the antiviral type I IFN response, as shown for herpes simplex virus and respiratory syncytial virus infections (Remot et al., 2016; Wilcox et al., 2015), and the antiviral CD8+ T cell response, as shown for Hantaan virus (Araki et al., 2004). In the present study, 1-d-old mice were injected s.c. with 3, 15, or 75 µl of pooled JEV-naive or JEV-immune human sera, and then challenged with ZIKV (10^2 focus-forming units [FFU] s.c.) 6 h later. Mice that received 3 or 15 µl of JEV-immune serum lost more weight (Fig. 3, A and C) and succumbed more rapidly to ZIKV infection than mice receiving JEV-naive serum (100% vs. 50–60% death, respectively; Fig. 3, B and D). A higher volume of sera (75 µl) was not markedly more pathogenic than 15 µl (Fig. 3, E and F). Similar results were observed when mice were injected with JEV-immune or mock-immune mouse serum before ZIKV infection; in this case, mortality was 100%,
100%, and 60% for mice receiving 3, 15, and 75 µl JEV-immune mouse serum and 70%, 75%, and 64% for mice receiving 3, 15, and 75 µl mock-immune mouse serum, respectively (Fig. 4). Taken together, these results suggest that preexisting JEV Abs may confer an increased risk of severe disease upon infection with ZIKV. To confirm that the pathogenic effects of JEV-immune sera were mediated by IgG, we injected 1-d-old mice s.c. with either 12 µg mouse IgG (purified from naive or JEV-immune mouse serum; equivalent to 3 µl of serum) or 75 µg of human IgG (purified from naive or JEV-immune human serum; equivalent to 15 µl of serum), and infected the mice 6 h later with ZIKV. As shown in Fig. S2, purified mouse and human IgG from JEV-immune sera had the same effect as the originating sera, accelerating both weight loss and mortality of ZIKV-infected mice. These data indicate that IgG mediates the lethal ADE of ZIKV pathogenesis induced by JEV-immune human and mouse sera.

We then determined whether ZIKV-immune sera could accelerate the pathogenesis of JEV infection in vivo using the

Figure 3. Transfer of JEV-immune human sera increases the survival of JEV-infected mice but decreases that of ZIKV-infected mice. (A–J) 1-d-old naive C57BL/6 mice were injected s.c. with the indicated volumes of JEV-naive or JEV-immune human sera. 6 h later, the mice were injected s.c. with (A–F) ZIKV (10² FFU) or (G–J) JEV (12 PFU). Mouse weights and survival were recorded daily for up to 28 d. Data are presented as the mean ± SD and are pooled from two experiments, each with 5–10 mice per group (A–F) or 3–6 mice/group (G–J). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by two-tailed Mann–Whitney U test (A, C, E, G, and I) or log-rank test (B, D, F, H, and J).
same experimental model. 1-d-old naive C57BL/6 mice were injected s.c. with the indicated volumes of mock- or JEV-immune mouse sera, and 6 h later, the mice were injected s.c. with (A–F) ZIKV (10^2 FFU) or (G–J) JEV (12 PFU). Mouse weights and survival were recorded daily for up to 28 d. Data are presented as the mean ± SD and are pooled from two experiments, each with five to eight mice per group (A–F) or two or three mice per group (G–J). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by two-tailed Mann–Whitney U test (A, C, E, G, and I) or log-rank test (B, D, F, H, and J).

Sera from JEV- or ZIKV-immune mice and humans protect against lethal infection with the homologous virus

Having shown that JEV- and ZIKV-immune sera enhanced the lethality of ZIKV and JEV infection, respectively, we next performed similar experiments to determine whether the sera
could also mediate ADE of infection with the same virus. Mice receiving serum from JEV-naïve humans (Fig. 3, G–j) or mock-immune mice (Fig. 4, G–j) died within 8 d of JEV infection, as expected. However, mice receiving JEV-immune human sera showed a dose-dependent and significant reduction in mortality, with ∼33% and ∼86% survival of mice receiving 3 and 15 µl sera, respectively (Fig. 3, G–j). Similarly, ∼60% and 100% of mice receiving 3 and 15 µl, respectively, of JEV-immune mouse sera survived a lethal JEV infection (Fig. 4, G–j). We observed the same pattern of protection by ZIKV-immune mouse sera against ZIKV infection, namely, reduced weight loss and protection against ZIKV-induced death in mice receiving ZIKV-immune compared with mock-immune sera (54–92% vs. 8–30% survival, respectively; Fig. 2, G–j). These results indicate that JEV- or ZIKV-elicited Abs protect against subsequent infection with the homologous virus, in striking contrast to the detrimental effect of the Abs on subsequent infection with the heterologous virus.

Maternally acquired anti-JEV Abs, but not anti-ZIKV Abs, promote lethal infection with the heterologous virus in neonates

Because ADE of ZIKV infection in pregnant women could have devastating effects on the developing offspring, we next investigated whether maternally acquired anti-JEV Abs could enhance the severity of ZIKV infection in newborn mice. Sera from 1-d-old naive pups born to JEV-immune mothers contained moderate-to-high titers of IgG reactive to JEV-EDIII and cross-reactive with ZIKV-EDIII (Fig. 5, A and B). The titer of ZIKV-reactive IgG in 1-d-old pups born to JEV-immune mothers was similar to that in the serum of mice receiving 3 µl of mouse JEV-immune serum, and lower than that in mice receiving 75 µl of mouse JEV-immune serum (Fig. S3 and Fig. 5, A and B). ZIKV infection of 1-d-old mice was more lethal for pups born to JEV-immune mothers than pups born to naïve mothers (65% vs. 30% mortality; Fig. 5, C and D), suggesting that the maternally acquired anti-JEV Abs could mediate ADE of ZIKV infection. Consistent with this, ZIKV levels at day 3 after infection were significantly higher in the serum (25-fold, P < 0.01) and slightly higher in the brain (threefold, P = 0.1667) of pups born to JEV-immune mothers compared with those born to naïve mothers (Fig. 5, E and F). These data indicate that maternally acquired anti-JEV Abs mediate ADE of ZIKV infection and increase the severity of disease in 1-d-old mouse pups. We performed similar experiments to determine whether the maternally acquired anti-JEV Abs similarly increased the lethality of JEV infection in neonates. However, in this case, pups born to JEV-immune mothers were protected against JEV infection (Fig. 5, G and H). Thus, whether passively transferred or maternally acquired, anti-JEV Abs protect against JEV infection but exacerbate ZIKV infection.

Intriguingly, we did not find the same results when we examined the effects of maternally acquired anti-ZIKV Abs on the severity of JEV infection in newborn mice. Thus, although sera from 1-d-old naïve pups born to ZIKV-immune mothers contained low-to-moderate titers of ZIKV/JEV-EDIII cross-reactive Abs (Fig. S4, A and B), they did not mediate ADE of JEV infection in neonates (Fig. S4, C and D). These data indicate that maternally acquired anti-JEV Abs, but not anti-ZIKV Abs, mediate ADE of heterologous virus infection in mouse pups. This suggests that ADE occurs in a context-dependent manner and varies based on the heterologous virus infection and the maternal vs. nonmaternal origin of the preexisting Abs.

CD8+ T cells from JEV- or ZIKV-primed mice cross-react with immunogenic ZIKV or JEV epitopes in vitro

Our previous work suggested that the balance between preexisting Ab and T cell immunity to DENV, particularly CD8+ T cell immunity, determines the outcome of subsequent infections with heterotypic DENV serotype or ZIKV (Regla-Nava et al., 2018; Wen et al., 2017a; Zellweger et al., 2013, 2014, 2015). Moreover, we previously identified numerous H-2b-restricted CD8+ T cell epitopes in the ZIKV proteome (Elong Ngono et al., 2017) and demonstrated that at least five of these ZIKV-derived epitopes elicit cross-reactive CD8+ T cell responses to DENV (Wen et al., 2017a). Therefore, we next assessed whether JEV or ZIKV immunization may elicit cross-reactive CD8+ T cells, as observed for the Ab response. To this end, we tested the in vitro response to eight immunodominant H-2b-restricted ZIKV epitopes (prM44-52, E4-12, E57-65, E420-428, NS3276-284, NS3347-355, NS568-779, and NS5222-230) and the corresponding JEV variants (prM47-55, E4-12, E57-65, E416-424, NS3293-301, NS3364-370, NS5320-329, and NS5474-485; Table I) using an IFN-γ ELISpot assay (Wen et al., 2017b). CD8+ T cells were purified from the spleens of 6-wk-old WT mice infected with ZIKV (105 FFU) or JEV (105 FFU) 7 or 28 d earlier, and then incubated with antigen-presenting cells pre pulsed with peptides in vitro. After 24-h incubation, IFNγ-secreting CD8+ T cells were enumerated. CD8+ T cells from JEV-primed mice (7-d infection) mounted significant responses to seven of the eight JEV peptides tested (the exception being NS5320-329) and to five of the corresponding ZIKV peptides (except NS3276-284, NS568-779, and NS5222-230; Fig. 6 A and B). The number of CD8+ T cells responding to the ZIKV and JEV peptide analogues was similar for most peptides (Fig. 6 A). Conversely, CD8+ T cells from ZIKV-primed mice were reactive against seven of the eight ZIKV peptides (except E420-428) and six of the corresponding JEV peptides (except E416-424 and NS3364-370; Fig. 6 B). Although the magnitude of the CD8+ T cell responses to analogous ZIKV and JEV peptides was more variable in mice primed with ZIKV compared with JEV, these data demonstrate that many of the immunodominant epitopes in ZIKV and JEV elicit cross-reactive CD8+ T cells. Of note, the frequencies of peptide-specific CD8+ T cells decreased between 7 and 28 d after infection (Fig. 6, A–D), indicating that 28 d was sufficient time for the virus-elicted CD8+ T cell response to contract and develop memory.

JEV-elicited CD8+ T cells protect against ADE of infection with ZIKV

Recent studies from our group and others have demonstrated that preexisting anti-DENV CD8+ T cells contribute to cross-protection against subsequent infection with ZIKV or heterotypic DENV (Elong Ngono et al., 2016; Regla-Nava et al., 2018; Wen et al., 2017a, b; Zellweger et al., 2018), and similarly, ZIKV-elicited CD8+ T cells can mediate cross-protection against DENV infection (Huang et al., 2017). We therefore determined whether
JEV-elicited CD8+ T cells protect against ZIKV infection. CD8+ T cells were purified from the spleens of 6-wk-old WT mice 28 d after infection with JEV and transferred to 1-d-old mice, which were infected 2 h later with a lethal dose of ZIKV. In sharp contrast to the effects of JEV-immune Abs, JEV-elicited CD8+ T cells significantly increased the survival of ZIKV-infected mice compared with CD8+ T cells from control enterovirus 71 VP1 protein (EV71-VP1)–immunized mice (78% vs. 28% survival; Fig. 7, A and B). Moreover, we found that CD8+ T cells purified from ZIKV-infected mice and transferred to 1-d-old mice could also significantly protect against a subsequent lethal infection with JEV, compared with EV71-VP1-elicited CD8+ T cells (33% vs. 89% death; Fig. 8, A and B). These data indicate that cross-reactive CD8+ T cells elicited by either JEV or ZIKV could protect against infection with the heterologous virus.

We next investigated whether JEV-elicited CD8+ T cells could rescue or attenuate the lethal effects of JEV-immune Abs on ZIKV infection. We cotransferred serum and CD8+ T cells from EV71-VP1-immune or JEV-immune mice at the same time and infected the mice with ZIKV 6 h later. Remarkably, JEV-elicited CD8+ T cells significantly protected the mice against ADE of infection mediated by JEV-immune Abs (47% vs. 11% survival; Fig. 7, C and D). Thus,

Figure 5. Pups born to JEV-immune mothers have decreased survival and increased viral burden upon ZIKV challenge compared with pups born to naive mothers. 6-wk-old female C57BL/6 mice were i.p. injected with 1 mg anti-Ifnar1 mAb (MAR1-5A3) 1 d before r.o. injection with JEV (10^2 PFU). 4 wk later, naive or JEV-immune females were mated with 10-wk-old naive male mice. (A and B) 1-d-old C57BL/6 pups were sacrificed, and sera were prepared. JEV-EDIII and ZIKV-EDIII–reactive IgG were measured by indirect ELISA. Mouse anti-His mAb was used as positive control. Data are presented as the mean ± SEM. (C–H) 1-d-old pups were injected s.c. with (C–F) ZIKV (10^2 FFU) or (G and H) JEV (12 PFU). (C, D, G, and H) Mouse weights and survival were recorded daily for up to 28 d. Data are presented as the mean ± SD and are pooled from three experiments totaling 20 infected mice born to 3 naive mothers and 17 infected mice born to 3 JEV-immune mothers (G and H). (E and F) Mice were sacrificed 3 d after ZIKV infection, and blood and brains were collected for measurement of viral burden using the FFA. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by two-tailed Mann–Whitney U test (B, C, E, and G) or log-rank test (D and H).
A convergence of factors has revitalized interest in developing new flaviviral vaccines; they include the emergence of ZIKV in DENV-endemic regions; the ease of global travel, which increases not only the spread of disease but also the likelihood that individuals may be exposed to multiple flaviviruses; and the finding that flaviviral proteins share significant amino acid homology. These observations have stimulated the exploration of humoral and cellular immunity to primary flaviviral infections and their beneficial and detrimental effects on subsequent infections with heterologous flaviviruses. In particular, the spread of ZIKV into JEV-endemic countries underscores the need to understand how interplay between humoral and cellular immunity to JEV modulates the severity of disease caused by ZIKV infection and vice versa. In this study, our major goals were to examine whether JEV-elicited Abs enhance ZIKV infection via ADE, and, if so, to determine whether anti-JEV CD8+ T cell immunity transfer could abrogate JEV Ab-mediated ADE of ZIKV infection. Indeed, the results of this study have uncovered critical roles for JEV-elicited cross-reactive CD8+ T cells in protecting against ADE of infection with ZIKV. Our investigation of the reverse infection scenario revealed that ZIKV-elicited Abs and CD8+ T cells affected the outcome of JEV infection by mediating ADE and contributing to protection, respectively. These data have important implications for the design of safe and effective anti-JEV/ZIKV vaccines.

Many adult mouse models are now available for investigating ZIKV infection and pathogenesis, including mice deficient in IFN-α/β receptor 1 and/or IFN-γ receptor 1 (Ifnar1−/−, Ifngr1−/−), IFN regulatory factors (IRfβ−/−, IRf5−/−, IRf7−/−), and IFN signaling proteins (Stat2−/−); as well as WT SJL mice and anti-Ifnar1 mAb-treated WT C57BL/6 mice (Li et al., 2016b, 2018). Two recent studies demonstrated that ZIKV can replicate in the brain and spinal cord and cause death in 1- to 3-d-old neonatal C57BL/6 mice in a viral dose- and host age-dependent manner (Li et al., 2018; Manangeeswaran et al., 2016). In the present study, we used a model in which 1-d-old C57BL/6 mice are infected with 10^2 FFU of ZIKV, a dose that causes ∼40% of animals to die within 28 d. The neonatal WT mouse model was selected for this study because ADE of ZIKV pathogenesis cannot be modeled in adult WT mice, which rapidly clear ZIKV infection, likely due to heightened Ifnar1 signaling in adult compared with neonatal WT mice. Additionally, the interplay between Ab and CD8+ T cell responses to ZIKV may be more faithfully recapitulated in mice with an intact antiviral IFN response because the Ifnar1 deficiency could limit the adaptive immune response (Kolumam et al., 2005; Wang et al., 2012). We also strived to ensure that our findings were as relevant as possible to humans by using both mouse and human JEV-immune sera. However, in some experiments involving the transfer of human JEV-naïve serum, 60–80% (instead of the expected 40%) of animals succumbed to ZIKV infection, suggesting that this mouse model may be better suited for studies with mouse than human sera.

We found that mice infected with live JEV produced Abs that strongly cross-reacted with purified ZIKV-EDIII protein but had modest ZIKV-neutralizing activity. Cross-recognition of JEV-EDIII by ZIKV-induced Abs was also detected. Transfer of JEV-immune serum protected mice against lethal infection with JEV but enhanced disease severity and promoted mortality following ZIKV infection, and vice versa with ZIKV-immune sera and JEV infection. These data are in line with recent studies showing ADE of DENV and YFV infections by anti-JEV Abs (Anderson et al., 2011; Chan et al., 2016; Saito et al., 2015, 2016) and ADE

### Discussion

A convergence of factors has revitalized interest in developing new flaviviral vaccines; they include the emergence of ZIKV in DENV-endemic regions; the ease of global travel, which increases...
of DENV infection by anti-ZIKV Abs (Fowler et al., 2018; George et al., 2017). We also observed that maternally acquired anti-JEV Abs accelerated ZIKV infection and increased the mortality of 1-d-old mice after ZIKV challenge. These results are in accordance with studies showing that anti-DENV Abs and anti-WNV Abs enhance ZIKV infection in cells and exacerbate ZIKV pathogenesis in mouse models (Bardina et al., 2017; Brown et al., 2019; Rathore et al., 2019). Thus, preexisting humoral immunity to JEV or ZIKV may increase the risk of developing severe disease upon infection with ZIKV or JEV, respectively. At present, the evidence to support ADE of ZIKV or JEV in humans is weak, although ZIKV-immune human sera have been shown to mediate ADE of ZIKV infection in Ifnar1−/− mice (Shim et al., 2019), and maternal anti-ZIKV Abs are associated with ADE of ZIKV infection and fetal microcephaly in humans (Robbiani et al., 2019). Future epidemiological studies may provide evidence to support ADE of both viruses.

In contrast to our finding that anti-JEV Abs enhanced ZIKV pathogenesis, a recent report showed that passive transfer of anti-JEV Abs from mice vaccinated with the live-attenuated JEV vaccine SA14-14-2 had no effect in adult Ifnar1−/− mice infected with ZIKV (Zhang et al., 2020). This discrepancy between studies is likely due to differences in the quality of anti-JEV Abs used for passive transfer, namely, sera from JEV SA14-14-2–vaccinated mice in the study by Zhang et al. (2020) vs. Abs from JEV-vaccinated humans or JEV-immune mice in our study. We also confirmed the results of our passive transfer experiments using a model in which maternal anti-JEV Abs were transferred to mouse pups before infection with ZIKV. However, based on our finding that passive transfer of nonmaternal anti-ZIKV Abs mediated ADE in mice with JEV infection, whereas maternal Abs had no effect, we hypothesize that the influence of preexisting humoral immunity may be highly context-dependent and subject to modulation by multiple factors, including Ab quality, mouse strain, and virus. In fact, two studies have found that Abs have no effect on infection with heterologous flavivirus. One study showed that sera from individuals inoculated with tick-borne encephalitis virus vaccine did not affect the mortality of ZIKV-infected Stat2−/− mice (Duehr et al., 2018), and the second study found that sera from macaques inoculated with a YFV vaccine did not enhance ZIKV infection in vitro (Bardina et al., 2017). Phylogenetic analysis of the E protein amino acid
sequences shows that JEV, WNV, and DENV are more closely related to ZIKV than are YFV and tick-borne encephalitis virus (Duehr et al., 2018); therefore, the phylogenetic relationship between flaviviruses may determine how the Ab response to one flavivirus affects the pathogenesis of a secondary heterologous flavivirus infection. It seems likely that the capacity to elicit ADE is attributable to the close phylogenetic distance between flaviviruses.

We observed that JEV-elicited CD8+ T cells protected against lethal ZIKV infection, and vice versa. This is consistent with the results of studies examining the protective effects of CD8+ T cells cross-reactive with various flaviviruses. In several mouse models, DENV-reactive CD8+ T cells have been found to cross-protect against heterotypic DENV infection (Elong Ngono et al., 2016; Zellweger et al., 2015), reduce tissue viral loads and mortality of ZIKV-infected mice (Wen et al., 2017a, b), and protect fetuses upon ZIKV infection of pregnant mice (Regla-Nava et al., 2018). ZIKV-elicited CD8+ T cells were also reported to protect against DENV infection (Huang et al., 2017), and JEV SA14-14-2-primed CD8+ T cells were recently shown to
Figure 8. **Transfer of ZIKV-elicited CD8+ T cells increases the survival of JEV-infected mice.** (A and B) 1-d-old naive C57BL/6 mice were r.o. injected with 2 × 10⁶ CD8+ T cells from EV71-VP1–immune mice that had been s.c. immunized with EV71-VP1 (50 µg/mouse/each; twice, 2 wk apart) 28 d earlier (n = 9) or ZIKV-exposed mice injected r.o. with ZIKV (10² PFU) 28 d earlier (n = 9). 2 h later, the neonates were injected s.c. with JEV (12 PFU). Mouse weights (A) and survival (B) were recorded daily for 14 d. Data are presented as the mean ± SD and are pooled from two experiments, each with four or five mice/group. *, P < 0.05; ***, P < 0.001 by two-tailed Mann–Whitney U test (A) or log-rank test (B).

Figure 9. **Transfer of JEV-elicited CD8+ T cells modulates anti-JEV Ab-mediated ADE of ZIKV infection.** (A and D) 1-d-old C57BL/6 naive mice were s.c. injected with 3 µl of mock-immune or JEV-immune mouse serum. 6 h later, mice were injected s.c. with ZIKV (10² FFU). (B and E) 1-d-old C57BL/6 naive mice were r.o. injected with 2 × 10⁶ CD8+ T cells from EV71-VP1–immune or JEV-exposed mice and injected s.c. with ZIKV (10² FFU) 2 h later. (C and F) Mice were treated as described for B and E except that the CD8+ T cells were from EV71-VP1–immune or JEV-exposed mice injected s.c. with 3 µl of JEV-immune mouse sera before s.c. injection of ZIKV (10² FFU). ZIKV viral loads in serum and brain were measured using the FFA at 3 or 8 d after infection. Data are presented as the mean ± SEM and are pooled from two experiments, each with three to six mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by two-tailed Mann–Whitney U test.
CD8+ T cells resulting from either inoculation with JEV vaccines or... gai nst... China, Cambodia, India, Nepal, and Sri Lanka). The inactivated Vero vaccine has been available for more than 50 yr and is used in six countries or territories. The live-attenuated SA14-14-2 vaccine be-

Materials and methods

Peptide synthesis

Eight previously identified H-2b-restricted CD8+ T cell epitopes in ZIKV (ZIKV-prM44-52, ZIKV-E4-12, ZIKV-E57-65, ZIKV-E420-428, ZIKV-NS3276-284, ZIKV-NS3287-295, ZIKV-NS568-77, and ZIKV-NS5222-229; Elong Ngono et al., 2017) and their variants in JEV (JEV-prM58-66, JEV-E4-12, JEV-E57-65, JEV-E416-424, JEV-NS3287-295, JEV-NS3364-372, JEV-NS5320-329, and JEV-NS574-243) were synthesized by ChinaPeptides with a purity >95% (Table 1). All peptides were dissolved in dimethyl sulfoxide at 20 mg/ml.

Cell lines, viruses, and reagents

Vero, U937, and BHK-21 cells were purchased from the American Type Culture Collection and were maintained in RPMI-1640 (Vero and U937 cells) or MEM-α (BHK-21 cells) medium ( Gibco). JEV strain ZJ14-52 (GenBank accession no. MK558811) was originally isolated from the serum of a patient with Japanese encephalitis in July 2014. ZIKV strain Zhejiang04 (GenBank accession no. KX117076.1) was originally isolated from the serum of a ZIKV-infected patient who lived in Wenzhou, China, and traveled to the Republic of Suriname in February 2016 during the ZIKV South American epidemic. ZIKV and JEV viral stocks were produced by propagating in Vero cells for two passages followed by titering in a BHK-21 cell-based focus-forming assay (FFA) in the case of ZIKV (Elong Ngono et al., 2017) or plaque-forming assay (PFA) in the case of JEV. Complete Freund’s ad-
v

Human sera and mice

This study was approved by the ethical committee of Zhejiang Provincial Center for Disease Control and Prevention, and written informed consent was obtained from all subjects. Blood was collected from healthy JEV-vaccinated donors (7-, 8-, 9-, 10-, 13 of 18

Chen et al.

JEV-primed CD8+ T cells prevent ADE of Zika virus
and EV71-VP1 protein were synthesized by Genewiz and cloned. Gene sequences encoding the viral (JEV and ZIKV) EDIII proteins were expressed in bacteria using the pET21a plasmid. The plasmids were transfected into Escherichia coli BL21, and the recombinant proteins were purified using a nickel-nitrilotriacetic acid column according to the manufacturer’s instructions.

Expression of JEV and ZIKV EDIII proteins

Gene sequences encoding the viral (JEV and ZIKV) EDIII proteins and EV71-VP1 protein were synthesized by Genewiz and cloned into plasmid pET21a downstream of a 6× His-tag. The plasmids were transfected into Escherichia coli BL21, and the recombinant proteins were purified using a nickel-nitrilotriacetic acid column according to the manufacturer’s instructions.

Viral infection of adult mice

Three protocols were employed. In the first, 6-wk-old male WT mice were injected i.p. with 1 mg of the anti-Ifnar1 Ab MAR1-5A3 1 d before retro-orbital (r.o.) injection with 10^3 PFU of JEV or 10^3 FFU of ZIKV in 200 µl 10% FBS/PBS. 7 or 28 d later, naive and virus-infected mice were sacrificed, and the spleens were collected. CD8+ T cells were isolated from splenocytes by positive selection with magnetic beads and used for the IFN-γ ELISPOT assays.

In the second protocol, 6-wk-old female WT mice were injected i.p. with 1 mg of the anti-Ifnar1 Ab MARI-SA3 1 d before r.o. injection of 10^3 PFU of JEV or 10^3 FFU of ZIKV in 200 µl 10% FBS/PBS. Mock-immune mice were injected r.o. with 200 µl 10% FBS/PBS. As additional controls, EV71-VP1 was emulsified in complete Freund’s adjuvant and injected s.c. into the backs of 6-wk-old female mice (50 µg/mouse). After 2 wk, mice were boosted with the same amount of EV71-VP1 in incomplete Freund’s adjuvant. 28 d later, the mock-immune mice, EV71-VP1-immune mice, JEV-immune mice, and ZIKV-immune mice were sacrificed, blood samples were collected and processed, and sera were used for ELISA, microneutralization assays, and passive transfer experiments. IgG was purified from mock-immune and JEV-immune sera using protein-G affinity columns and used for passive transfer experiments. CD8+ T cells were purified from splenocytes by negative selection with magnetic beads and used for adoptive transfer experiments.

In the third protocol, 6-wk-old female WT mice were injected i.p. with 1 mg of the anti-Ifnar1 Ab MARI-SA3 1 d before r.o. injection of 10^3 PFU of JEV or 10^3 FFU of ZIKV in 200 µl 10% FBS/PBS. 28 d later, the naive, JEV-immune, and ZIKV-immune mice were mated with 10-wk-old naive male mice. 1-d-old pups born to naive, JEV-immune, or ZIKV-immune mice were sacrificed, blood was collected and prepared, and sera were used for ELISA.

Anti-EDIII IgG capture ELISAs

Anti-JEV-EDIII- and anti-ZIKV-EDIII-reactive IgG in human and mouse sera were detected by indirect capture ELISAs. In brief, 96-well plates were coated with 1 µg/ml recombinant JEV-EDIII or ZIKV-EDIII proteins overnight at 4°C, and the wells were then blocked with 10% FBS/PBS for 1 h at 25°C. Human sera and mouse sera (mock-, JEV-, and ZIKV-immune) were serially diluted threefold starting from 1:9 dilutions. Sera from 1-d-old pups born to naive, JEV-immune, or ZIKV-immune mice were serially diluted threefold starting with 1:15 dilutions. Threefold serially diluted mouse anti-His mAb (starting with 6 µg/ml) was used as a positive control. Diluted serum samples (100 µl) were added to the wells of JEV-EDIII or ZIKV-EDIII protein-coated ELISA plates and incubated for 1.5 h at 25°C. After washing the wells, bound IgG was detected by incubation with HRP-conjugated goat anti-human or mouse IgG mAb (1:5,000) for 1 h at 25°C. The plates were then developed with TMB for 10 min.
at 25°C, and the absorbance at 450 nm was measured. Endpoint titers were defined as the reciprocal of the serum dilution that gave twice the average OD value obtained with naive serum.

**Microneutralization assay**

ZIKV- and JEV-neutralizing activities of human and mouse sera were determined using a microneutralization assay as described previously (Abbink et al., 2016) with slight modifications. In brief, 50 µl of twofold serially diluted (from 1:4 to 1:256) serum samples was incubated with 50 µl of ZIKV (10^2 FFU) or JEV (10^2 FFU) at 37°C for 1 h. The mixture was then added to Vero cell monolayers that had been cultured overnight in 96-well plates, and the cells were incubated for 1 h. Samples containing virus but not serum were included as controls. The viral supernatants were removed, and the wells were overlaid with 1% CMC medium and incubated for 2 d at 37°C. Cells were then fixed with 4% paraformaldehyde/PBS, permeabilized with 1% Triton X-100, and incubated with mouse anti-ZIKV E protein mAb (1 µg/ml) or mouse anti-JEV E protein mAb (1 µg/ml) followed by HRP-conjugated goat anti-mouse IgG (1:5,000). Color development was achieved by the addition of TMB substrate, and the absorbance at 450 nm (OD450) was recorded. Percent neutralization of infection was calculated as (OD450 in control wells - OD450 in serum-containing wells)/OD450 in control wells. The 50% neutralizing titer was calculated as the reciprocal of the average serum dilution yielding 50% inhibition of the OD450 compared with naive serum values.

**In vitro ADE assay**

To assess ADE in vitro, we developed an assay similar in principle to the previously described microneutralization assay (Abbink et al., 2016). Human and mouse sera were serially diluted twofold from 1:4 to 1:256 in PBS and added to 96-well plates at 50 µl/well. Aliquots of ZIKV (10^4 FFU/50 µl/well) were added, and the plates were incubated for 1 h at 37°C. The well contents were transferred to wells containing 10^4 U937 cells that had been pretreated with 0.5 µg/ml PMA for 2 d, and the plates were incubated for 2 h at 37°C. Viral supernatants were removed, and the cells were overlaid with 1% CMC medium and incubated for 2 d at 37°C. Cells treated in the same manner but without serum served as controls. After incubation, the U937 cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 1% Triton X-100, and incubated with mouse anti-ZIKV E protein mAb (1 µg/ml) followed by HRP-conjugated goat anti-mouse IgG (1:1,000). Color development was achieved by addition of TMB, and the absorbance at 450 nm was measured. ADE of infection was expressed as the fold increase compared with control wells: (OD450 in serum-containing wells - OD450 in control wells)/OD450 in control wells.

**IFN-γ ELISPOT assay**

IFN-γ-producing CD8+ T cells were enumerated using an IFN-γ ELISPOT assay as previously described (Wen et al., 2017b). In brief, CD8+ T cells were isolated from splenocytes from JEV- or ZIKV-infected mice by positive selection using magnetic beads. LPS blasts (antigen-presenting cells) were prepared by stimulation of naive C57BL/6 splenocytes with LPS and dextran sulfate as described previously (Wen et al., 2017b). CD8+ T cells (2 × 10^5), LPS blasts (10^5), and 10 µg of JEV or ZIKV peptides were added to each well of ELISPOT plates precoated with anti–IFN-γ mAb (CT655-10, U-CyTech Biosciences). After 24 h incubation, the wells were incubated sequentially with biotinylated anti-mouse IFN-γ mAb (CT655-10, U-CyTech Biosciences), HRP-conjugated streptavidin, and 3-amino-9-ethylcarbazole color development solution. The results are expressed as the number of peptide-reactive spot-forming cells (SFCs) per 10^6 CD8+ T cells. The presence of >20 SFCs/well with a stimulation index of >2 (index = ratio of test SFCs to control SFCs) was considered a positive response.

**Viral challenge of mice transferred with serum and/or CD8+ T cells**

For the serum transfer experiments, two protocols were used. In the first protocol, 1-d-old C56BL/6 mice were injected s.c. with 3 µl, 15 µl, or 75 µl of pooled human sera (from JEV-naive or 8-yr-old JEV-vaccinated donors) or mouse sera (mock- or JEV-immune); or with 75 µg IgG purified from human JEV-naive or JEV-immune sera (8-yr-old JEV-vaccinated donors); or with 12 µg IgG purified from mouse mock-immune or JEV-immune sera. Transfer of 75 µl of sera was accomplished with two injections of 37.5 µl each, administered 3 h apart. 6 h later, mice were injected s.c. with 12 PFU of JEV or 10^3 FFU of ZIKV in 30 µl 10% FBS/PBS. In the second protocol, 1-d-old C56BL/6 mice were injected s.c. with 0.05 µl or 10 µl of pooled sera from mock- or ZIKV-immune mice. 6 h later, mice were injected s.c. with 12 PFU of JEV or 10^3 FFU of ZIKV in 30 µl 10% FBS/PBS.

For the cell transfer experiments, three protocols were used. CD8+ T cells were isolated from the spleens of EV71-VP1-, JEV-, or ZIKV-immune C57BL/6 mice, as described above. For the first protocol, aliquots of 2 × 10^6 CD8+ T cells in 10 µl of 10% FBS/PBS were injected r.o. into 1-d-old naive mice, and 2 h later, the mice were injected s.c. with 10^2 FFU of ZIKV in 30 µl 10% FBS/PBS. For the second protocol, 1-d-old naive mice were injected r.o. with 2 × 10^6 CD8+ T cells in 10 µl of 10% FBS/PBS and s.c. with 3 µl mouse JEV-immune serum. 6 h later, the mice were injected s.c. with 10^2 FFU of ZIKV in 30 µl 10% FBS/PBS. For the third protocol, 1-d-old naive mice were injected r.o. with 2 × 10^6 CD8+ T cells in 10 µl of 10% FBS/PBS and injected s.c. with 12 PFU of JEV in 30 µl 10% FBS/PBS 6 h later. For all experiments, mouse weights and deaths were recorded daily for up to 28 d. In selected experiments, ZIKV viral burden in serum and brain was measured on days 3 and 8 after ZIKV challenge.

**ZIKV/JEV challenge of pups born to JEV/ZIKV-immune mice**

Three protocols were used. In the first, 1-d-old pups born to naive and JEV-immune mice were injected s.c. with 12 PFU of JEV or 10^5 FFU of ZIKV in 30 µl of 10% FBS/PBS. Mouse weights and deaths were recorded daily for up to 28 d. In the second protocol, 1-d-old pups born to naive and JEV-immune mice were injected s.c. with 10^2 FFU of ZIKV in 30 µl of 10% FBS/PBS. 3 d after infection, the mice were sacrificed, and blood and brain were collected for measurement of viral burden. In the third protocol, 1-d-old pups born to naive and ZIKV-immune mice were injected s.c. with 12 PFU of JEV in 30 µl of 10% FBS/PBS. Mouse weights and deaths were recorded daily for up to 14 d.
Anderson, K.B., R.V. Gibbons, S.J. Thomas, A.L. Rothman, A. Nisalak, R.L. Thenary.

Internally acquired anti-ZIKV Ab on the outcome of JEV-infected

Accepted: 4 May 2020

Revised: 31 March 2020

Submitted: 14 November 2019

October 3, 2019, application no. 62/910,337 pending “La Jolla In-

stitutional funds from the La Jolla Institute for Immunology.

Shresta was supported by in-

stitutional funds from the La Jolla Institute for Immunology.

Author contributions: J. Wen and S. Shresta designed the study. D. Chen, Z. Duan, W. Zhou, W. Zou, S. Jin, D. Li, X. Chen, Y. Zhou, L. Yang, and Y. Zhang performed the experiments. D. Chen, Y. Zhang, J. Wen, and S. Shresta interpreted the data. J. Wen and S. Shresta wrote the manuscript. S. Shresta edited the manuscript.

Disclosures: S. Shresta reported a patent to “Flavivirus peptide

sequences, epitopes, and methods and uses thereof,” filed Oc-

cember 13, 2011, application no. 62/910,337 pending “La Jolla In-

stitute.” No other disclosures were reported.

Submitted: 14 November 2019

Revised: 31 March 2020

Accepted: 4 May 2020

References

Abbkink, P., R.A. Larocca, R.A. De La Barrera, C.A. Bricault, E.T. Moseley, M. Boyd, M. Kirilova, Z. Li, D. Ng’ang’a, O. Nanayakkara, et al. 2016. Protective efficacy of multiple vaccine platforms against Zika virus challenge in rhesus monkeys. Science. 353:1129–1132. https://doi.org/10.1126/science.aab6157

Anderson, K.B., R.V. Gibbons, S.J. Thomas, A.L. Rothman, A. Nisalak, R.L. Berkelman, D.H. Libraty, and T.P. Endly. 2011. Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. PLoS Negl. Trop. Dis. 5. e1381. https://doi.org/10.1371/journal.pntd.0001311

Araki, K., K. Yoshimatsu, B.H. Lee, M. Okumura, H. Karaiwa, I. Takashima, and J. Arikawa. 2004. Age-dependent hantavirus-specific CD8(+) T cell responses in mice infected with Hantaan virus. Arch. Virol. 149: 1373–1382. https://doi.org/10.1007/s00705-003-0285-4

Balsitis, S.J., K.L. Williams, R. Lachica, D. Flores, J.L. Kyle, M. Mielshop, S. Johnson, M.S. Diamond, P.R. Beatty, and E. Harris. 2010. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog. 6. e1000790. https://doi.org/10.1371/journal.ppat.1000790

Bardina, S.V., P. Bunduc, S. Tripathi, J. Duehr, J.A. Frere, J.A. Brown, R. Nachbagauer, G.A. Foster, D. Krysztof, D. Tortorella, et al. 2017. Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. Science. 356:175–180. https://doi.org/10.1126/science.aal4365

Brown, J.A., G. Singh, J.A. Acklin, S. Lee, J.E. Duehr, A.N. Chokola, J.J. Frere, K.W. Hoffman, G.A. Foster, D. Krysztof, et al. 2019. Dengue Virus Immunity Increases Zika Virus-Induced Damage during Pregnancy. Immunity. 50:751–762.e5. https://doi.org/10.1016/j.immuni.2019.01.005

Campbell, G.L., S.L. Hills, M. Fischer, J.A. Jacobson, C.H. Hoke, J.M. Hombach, A.A. Marfin, T. Solomon, T.F. Tsai, V.D. Tsu, et al. 2011. Estimated global incidence of Japanese encephalitis: a systematic review. Bull. World Health Organ. 89:766–774. https://doi.org/10.1016/j.ameic.2011.03.012

Centers for Disease Control and Prevention (CDC). 2013. Japanese encephalitis surveillance and immunization--Asia and the Western Pacific. 2012. MMWR Morb. Mortal. Wkly. Rep. 62:658–662.

Chen, K.R., X. Wang, W.A.A. Saron, E.S. Gan, H.C. Tan, D.Z.L. Mok, S.L. Zhang, Y.H. Lee, C. Liang, L. Wijaya, et al. 2016. Cross-reactive anti-

bodies enhance live attenuated virus infection for increased immuno-

genicity. Nat. Microbiol. 1:16164. https://doi.org/10.1038/nmicrobiol.2016.164

Dejnirattisai, W., A. Jumnainsong, N. Onsirisakul, P. Fitton, S. Vasana-

wanna, W. Limpitikul, C. Puttikhunt, C. Edwards, T. Duangchinda, S. Supasa, et al. 2010. Cross-reacting antibodies enhance dengue virus infection in humans. Science. 328:745–748. https://doi.org/10.1126/science.1185181

Dejnirattisai, W., P. Supasa, W. Wongwiwat, A. Rouvinski, G. Barba-Spaeth, T. Duangchinda, A. Sakuntabhai, V.M. Cao-Lormeau, P. Malasit, F.A. Rey, et al. 2016. Dengue virus sero-cross-reactivity drives antibody-

dependent enhancement of infection with zika virus. Nat. Immunol. 17:1102–1108. https://doi.org/10.1038/ni.3515

Duehr, J., S. Lee, G. Singh, G.A. Foster, D. Krysztof, S.L. Stramer, M.C. Bermúdez González, E. Menichetti, R. Gerevitschläger, C. Gabriel, et al. 2018. Tick-Borne Encephalitis Virus Vaccine-Induced Human Anti-

bodies Mediate Negligible Enhancement of Zika Virus Infection InVitro and in a Mouse Model. mSphere. 3. e00011-18. https://doi.org/10.1128/mSphereDirect.00011-18

Elong Ngono, A., S. Shresta. 2019. Cross- Reactive T Cell Immunity to Dengue and Zika Viruses: New Insights Into Vaccine Development. Front. Immunol. 10. doi:10.3389/fimmu.2019.01316

Elong Ngono, A., H.W. Chen, W.W. Tang, Y. Joo, K. King, D. Weiskopf, J. Sidney, A. Sette, and S. Shresta. 2016. Protective Role of Cross-Reactive CD8 T Cells Against Dengue Virus Infection. EBioMedicine. 13:284–293. https://doi.org/10.1016/j.ebiom.2016.10.006

Elong Ngono, A., E.A. Vizcarra, W.W. Tang, N. Sheets, Y. Joo, K. Kim, M.J. Gorman, M.S. Diamond, and S. Shresta. 2017. Mapping and Role of the CD8(+) T Cell Response During Primary Zika Virus Infection in Mice. Cell Host Microbe. 21:35–46. https://doi.org/10.1016/j.chom.2016.12.010

Fowler, A.M., W.W. Tang, M.P. Young, A. Mamidi, K.M. Viramontes, M.D. McCauley, A.F. Carlin, R.T. Schooley, J. Swanstrom, R.S. Baric, et al. 2018. Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. Cell Host Microbe. 24:743–750.e5. https://doi.org/10.1016/j.chom.2018.09.015

Gao, X., X. Li, M. Li, S. Fu, H. Wang, Z. Lu, Y. Cao, Y. He, W. Zhu, T. Zhang, et al. 2014. Vaccine strategies for the control and prevention of Japanese encephalitis in Mainland China, 1951-2011. PLoS Negl. Trop. Dis. 8. e3015. https://doi.org/10.1371/journal.pntd.0003015

George, J., W.G. Valliant, M.J. Mattapallil, M. Walker, Y.S. Huang, D.L. Van-

landingham, J. Misamore, J. Greenhouse, D.E. Weiss, D. Verthelyi, et al. 2017. Prior Exposure to Zika Virus Significantly Enhances Peak Dengue-2 Viremia in Rhesus Macaques. Sci. Rep. 7:10498. https://doi.org/10.1038/s41598-017-10901-1

Gordon, A., L. Gresh, S. Ojeda, L.C. Katzelnick, N. Sanchez, J.C. Mercado, G. Chowell, B. Lopez, D. Elizondo, J. Coloma, et al. 2019. Prior dengue virus infection and risk of Zika: A pediatric cohort in Nicaragua. PLoS Med. 16. e1002726. https://doi.org/10.1371/journal.pmed.1002726

Chen et al.

JEV-primed CD8+ T cells prevent ADE of Zika virus

https://doi.org/10.1084/jem.20192152
Hadinegoro, S.R., J.L. Arredondo-García, M.R. Capeding, C. Deseda, T. Chotpitayasunondh, R. Dietze, H.I. Muhammad Ismail, H. Reynaudes, K. Linskens, D.M. Rivero-Medina, et al. CYD-TDV Dengue Vaccine Working Group. 2015. Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. N. Engl. J. Med. 373:1195–1206. https://doi.org/10.1056/NEJMoa1506223

Huang, H., S. Li, Y. Zhang, X. Han, B. Jia, H. Liu, D. Liu, S. Tan, Q. Wang, Y. Bi, et al. 2017. CD8+ T Cell Immune Response in Immunocompetent Mice during Zika Virus Infection. J. Virol. 91. e00990-17. https://doi.org/10.1128/JVI.00990-17

Katzelnick, L.C., L. Gresh, M.E. Halloran, J.C. Mercado, G. Kuan, A. Gordon, A. Collier, C. Chen, J. Wan, W. Wang, C. Lam, J.H., Y.L. Chua, P.X. Lee, J.M. Martín-Sanz, et al. 2016c. Zika Virus-induced microcephaly in fetuses. Sci. Adv. 5:v32020. https://doi.org/10.1126/sciadv.aav3202

Regla-Nava, J.A., A. Elong Ngono, K.M. Viramontes, A.T. Huynh, Y.T. Wang, A.T. Nguyen, R. Salgado, A. Mamidi, K. Kim, M.S. Diamond, et al. 2018. Cross-reactive Dengue Virus-specific CD8+ T cells protect against Zika virus during pregnancy. Nat. Commun. 9:3042. https://doi.org/10.1038/s41467-018-05458-0

Remot, A., A. Descamps, L. Jouneau, D. Labroret, C. Dubouyq, S. Bouet, J. Lecopardon, E. Rebour, A. Petit-Camurand, and S. Riffault. 2016. Zika virus induces the immune response to respiratory syncytial virus and limits lung disease upon RSV reexposure in neonate mice. Eur. J. Immunol. 46:874–884. https://doi.org/10.1002/eji.201545929

Robbini, D.F., P.C. Olsen, F. Costa, Q. Wang, T.Y. Oliveira, N. Nery, Jr., A. Aromolaran, M.S. dos Rosário, G.A. Sacramento, J.S. Cruz, et al. 2019. Risk of Zika microcephaly correlates with features of maternal antibodies. J. Exp. Med. 216:2303–2315. https://doi.org/10.1084/jem.201910614

Rodríguez-Barraquer, I., F. Costa, J.E.M. Nascimento, N. Nery, P.M.S. Cas-tanha, G.A. Sacramento, J. Cruz, M. Carvalho, D. De Olivera, J.E. Hagan, et al. 2019. Impact of preexisting dengue immunity on Zika virus emergence in a dengue-endemic region. Science. 363:607–610. https://doi.org/10.1126/science.aav6618

Ruchusatsawat, K., P. Wongjaroen, A. Posanacharoen, I. Rodríguez-Barrera-quer, S. Sangkitporn, D.A.T. Cummings, and H. Salje. 2016. Long-term circulation of Zika virus in Thailand: an observational study. Lancet Infect. Dis. 19:439–446. https://doi.org/10.1016/S1473-3099(18)30718-7

Saito, Y., M.L. Moi, N. Takeshita, C.K. Lim, H. Shiba, K. Hosono, M. Satô, I. Kurane, and T. Takasaki. 2016. Japanese encephalitis vaccine-facilitated dengue virus infection-enhancement antibody in adults. BMC Infect. Dis. 16:578. https://doi.org/10.1186/s12879-016-1873-8

Salje, H., J.A. Cummings, I. Rodríguez-Barracaquer, L.C. Katzelnick, J. Lesser, C. Klungthong, B. Thaisomboonsuk, N. Nisalak, A. Weg, D. Ellison, et al. 2018. Reconstruction of antibody dynamics and infection histories to evaluate dengue risk. Nature. 557:719–723. https://doi.org/10.1038/s41586-018-0157-4

Sato, R., N. Hamada, T. Kashiwagi, Y. Imamura, K. Haru, M. Nishimura, T. Kamimura, T. Takasaki, H. Watanabe, and T. Koga. 2015. Dengue Hemorrhagic Fever in a Japanese Traveler with Pre-existing Japanese Encephalitis Virus Antibody. Trop. Med. Health. 43:85–88. https://doi.org/10.2149/tmlh.2014-34

Shim, B.S., Y.C. Kwon, M.J. Ricciardi, M. Stone, Y. Otsuka, F. Berri, J.M. Kwal, D.M. Magnani, C.B. Jackson, A.S. Richard, et al. 2012. Zika Virus-Immunne Plasma from Symptomatic and Asymptomatic Individuals Enhance Zika Pathogenesis in Adult and Pregnant Mice. MBio. 10. e00758-19. https://doi.org/10.1128/mBio.00758-19

Sirohi, D., Z. Chen, L. Sun, T. Klose, T.C. Pierson, M.G. Rossmann, and R.J. Kuhn. 2016. The 3.8 A resolution cryo-EM structure of Zika virus. Science. 352:467–470. https://doi.org/10.1126/science.aaf5316

Solomon, T., P. Soler, and P.M. Winter. 2004. Neurovirulence and host factors in dengue virus infection. Adv. Virus. 11:631–645. https://doi.org/10.1126/science.aan6836

Vázquez-Calvo, Á., A.B. Blázquez, E. Escobedo-Romano, T. Merino-Ramos, J.C. Saiz, M.A. Martin-Acebes, and J.M. Jiménez de Oya. 2017. Zika virus infection confers protection against West Nile encephalitis. Emerg. Infect. Dis. 8:245–251. https://doi.org/10.3201/eid0803.170038

Wikiwand. 2017. Zika virus-induced microcephaly in fetuses. Science. 358:929. https://doi.org/10.1126/science.aaf5316

Zikvik. 2017. Zika Virus Induced Microcephaly in Fetuses. Sci. Adv. 3:32020. https://doi.org/10.1126/sciadv.aav3202

Chen et al. JEV-primed CD8+ T cells prevent ADE of Zika virus
World Health Organization. 2017. Zika situation report: Zika virus, microcephaly and Guillain-Barre syndrome. https://apps.who.int/iris/bitstream/handle/10665/25474/zikasitrep10Mar17-eng.pdf

Wilcox, D.R., N.R. Wadhwani, R. Longnecker, and W.J. Muller. 2015. Differential reliance on autophagy for protection from HSV encephalitis between newborns and adults. *PLoS Pathog.* 11. e1004580. https://doi.org/10.1371/journal.ppat.1004580

Zellweger, R.M., T.R. Prestwood, and S. Shresta. 2010. Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. *Cell Host Microbe.* 7:128–139. https://doi.org/10.1016/j.chom.2010.01.004

Zellweger, R.M., R. Miller, W.E. Eddy, L.J. White, R.E. Johnston, and S. Shresta. 2013. Role of humoral versus cellular responses induced by a protective dengue vaccine candidate. *PLoS Pathog.* 9. e1003723. https://doi.org/10.1371/journal.ppat.1003723

Zellweger, R.M., W.E. Eddy, W.W. Tang, R. Miller, and S. Shresta. 2014. CD8+ T cells prevent antigen-induced antibody-dependent enhancement of dengue disease in mice. *J. Immunol.* 193:4117–4124. https://doi.org/10.4049/jimmunol.1401597

Zellweger, R.M., W.W. Tang, W.E. Eddy, K. King, M.C. Sanchez, and S. Shresta. 2015. CD8+ T Cells Can Mediate Short-Term Protection against Heterotypic Dengue Virus Reinfecion in Mice. *J. Virol.* 89:6494–6505. https://doi.org/10.1128/JVI.00336-15

Zhang, Y., W. Chen, Q. Bi, J. Yan, Y. Sun, E. Chen, H. Yan, X. Lou, H. Mao, et al. 2016. Highly diversified Zika viruses imported to China, 2016. Protein Cell. 7:461–464. https://doi.org/10.1007/s13238-016-0274-5

Zhang, W., Y. Xu, F. Zhao, M. Tarbe, S. Zhou, W. Wang, S. Zhang, W. Zhang, Q. Xu, L. Shi, et al. 2020. The pre-existing cellular immunity to Japanese encephalitis virus heterotypically protects mice from Zika virus infection. *Sci. Bull. (Beijing).* 65:402–409. https://doi.org/10.1016/j.scib.2019.11.006

Zimmerman, M.G., K.M. Quicke, J.T. O’Neal, N. Arora, D. Machia, L. Priyamvada, R.C. Kauffman, E. Register, O. Adekuna, D. Swieboda, et al. 2018. Cross-Reactive Dengue Virus Antibodies Augment Zika Virus Infection of Human Placental Macrophages. *Cell Host Microbe.* 24:731–742.e6. https://doi.org/10.1016/j.chom.2018.10.008

Zhang, Y., W. Chen, G. Wong, Y. Bi, J. Yan, Y. Sun, E. Chen, H. Yan, X. Lou, H. Mao, et al. 2016. Highly diversified Zika viruses imported to China, 2016. Protein Cell. 7:461–464. https://doi.org/10.1007/s13238-016-0274-5

Chen et al. Journal of Experimental Medicine

JEV-primed CD8+ T cells prevent ADE of Zika virus https://doi.org/10.1084/jem.20192152
Figure S1. Modest neutralization but efficient enhancement of ZIKV infection in vitro by JEV-neutralizing, JEV-immune sera from humans and mice. 

(A–D) Human serum samples isolated from JEV-naive infants (6 mo of age, n = 6) or JEV-immune subjects (n = 6 for all age groups), and mouse serum samples (n = 6 for all groups) were tested for neutralization of ZIKV (A and B) or JEV (C and D) infection of Vero cells. Mouse ZIKV-immune serum was used as the positive control. 

(E and F) Enhancement of ZIKV infection by JEV-immune sera from humans and mice was evaluated in U937 cells. Data are expressed as the mean ± SEM.
Figure S2. Transfer of JEV-immune IgG exacerbates lethal ZIKV infection in mice. (A–D) 1-d-old naive C57BL/6 mice were injected s.c. with (A and B) 75 µg of IgG purified from JEV-naive (n = 12) or JEV-immune (n = 8) human sera or (C and D) 12 µg of IgG purified from mock-immune (n = 15) or JEV-immune (n = 13) mice and injected s.c. with ZIKV (10^2 FFU) 6 h later. Weights and survival were recorded daily for up to 28 d. Data are presented as the mean ± SD and are pooled from two experiments, each with four to eight mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by two-tailed Mann–Whitney U test (A and C) or log-rank test (B and D).

Figure S3. JEV- and ZIKV-reactive IgG in the serum of mice 6 h after transfer of mouse JEV-immune serum. (A–F) 1-d-old naive C57BL/6 mice were injected s.c. with 3, 15, or 75 µl of mock- or JEV-immune mouse sera (n = 5 for all groups). 6 h later, the mice were sacrificed, and sera were collected. JEV-EDIII- and ZIKV-EDIII–reactive IgG levels were measured by indirect ELISA. Data are presented as the mean ± SEM. **, P < 0.01 by two-tailed Mann–Whitney U test.
Maternally acquired anti-ZIKV Abs have no effect on the survival of JEV-infected pups born to ZIKV-immune mothers. 6-wk-old female C57BL/6 mice were i.p. injected with 1 mg anti-Ifnar1 mAb (MAR1-5A3) 1 d before r.o. injection with ZIKV (10^2 FFU). 4 wk later, naive or ZIKV-immune females were mated with 10-wk-old naive male mice. (A and B) 1-d-old C57BL/6 pups were sacrificed, and sera were prepared. ZIKV-EDIII and JEV-EDIII–reactive IgG were measured by indirect ELISA. Mouse anti-His mAb was used as positive control. Data are presented as the mean ± SEM. (C and D) 1-d-old C57BL/6 pups were injected s.c. with JEV (12 PFU). Mouse weights and survival were recorded daily for 14 d. Data are presented as the mean ± SD and are pooled from four experiments totaling 23 infected mice born to 4 naive mothers and 25 infected mice born to 4 ZIKV-immune mothers. *, P < 0.05; ****, P < 0.0001 by two-tailed Mann–Whitney U test.