ZIKA VIRUS

Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection

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Zika virus (ZIKV), a mosquito-borne flavivirus with homology to Dengue virus (DENV), has become a public health emergency. By characterizing memory lymphocytes from ZIKV-infected patients, we dissected ZIKV-specific and DENV–cross-reactive immune responses. Antibodies to nonstructural protein 1 (NS1) were largely ZIKV-specific and were used to develop a serological diagnostic tool. In contrast, antibodies against E protein domain I/II (EDI/II) were cross-reactive and, although poorly neutralizing, potently enhanced ZIKV and DENV infection in vitro and lethally enhanced DENV disease in mice. Memory T cells against NS1 or E proteins were poorly cross-reactive, even in donors preexposed to DENV. The most potent neutralizing antibodies were ZIKV-specific and targeted EDIII or quaternary epitopes on infectious virus. An EDIII-specific antibody protected mice from lethal ZIKV infection, illustrating the potential for antibody-based therapy.

A fter its introduction into Brazil in 2015, Zika virus (ZIKV) has spread rapidly, and in February 2016, the World Health Organization (WHO) declared it a Public Health Emergency of International Concern (1–3). The main route of ZIKV infection is through bites by *Aedes* mosquitos, but the virus may also be sexually (4) and vertically transmitted (5). Although most of the ZIKV infections are asymptomatic or cause only mild symptoms, there is evidence that ZIKV infection can lead to neurological complications, such as Guillain-Barré syndrome in adults (6) and congenital birth defects, including microcephaly in the developing fetus (5, 7, 8), likely through its ability to infect human neural progenitor cells (9).

Whereas flavivirus envelope (E) proteins mediate fusion and are the main target of neutralizing antibodies, the nonstructural protein 1 (NS1) is secreted by infected cells and is involved in immune evasion and pathogenesis (10). Two recent studies showed a high level of structural similarity between the E protein of ZIKV and that of other flaviviruses—such as dengue virus (DENV), yellow fever virus (YFV), and West Nile virus (WNV)—but also revealed distinct features that may be related to the ZIKV neurotropism (11, 12). Similarly, the structural analysis of ZIKV NS1 revealed conserved features with NS1 of other flaviviruses, although with different electrostatic characteristics (13).

A phenomenon that is characteristic of certain flaviviruses is the disease-enhancing activity of cross-reactive antibodies elicited by previous infections by heterologous viruses, termed antibody-dependent enhancement (ADE). In the case of DENV, for which four serotypes are known, there is epidemiological evidence that a primary infection protects from reinfection with the same serotype but represents a risk factor for the development of severe disease upon reinfection with a different serotype (14). The exacerbated disease is triggered by E- and prM-specific antibodies that fail to neutralize the incoming virus but instead enhance its capture by Fc receptor–expressing (FcR⁺) cells, leading to enhanced viral replication and activation of cross-reactive memory T cells. The resulting cytokine storm is thought to be the basis of the most severe form of disease, known as dengue hemorrhagic fever/dengue shock syndrome (15, 16). The role of antibodies in severe dengue is supported by studies showing that waning levels of maternal antibodies in infants represent a higher risk for

development of severe dengue disease (16–19). Whether individuals with antibodies induced by previous DENV infections can develop a more severe ZIKV infection or have higher risk of fetal transmission is unknown. Similarly, it is unclear whether ZIKV antibodies may affect subsequent DENV infection. Therefore, it is important to dissect the level of cross-reactive immunity at the B cell and T cell level in response to ZIKV infection.

To understand the role of antibodies in ZIKV neutralization and heterologous enhancement of flavivirus infection, we isolated a panel of 119 monoclonal antibodies (mAbs) from four ZIKV-infected donors from the current epidemic, of which two were DENV-naïve (ZA and ZD) and two had serological records of DENV infection (ZB and ZC) (table S1) (20). These mAbs were selected from Epstein-Barr virus–immortalized memory B cells on the basis of their binding to ZIKV NS1 or E proteins and for their ability to neutralize ZIKV infection, and were compared, side by side, with a panel of mAbs previously isolated from DENV-infected donors (21). The mAbs were primarily immunoglobulin G (IgG), were highly polyclonal, and carried a lower level of somatic mutations as compared with that of other acute or recurrent infections (fig. S1 and table S2).

Out of the 119 ZIKV-reactive mAbs isolated, 41 bound to NS1. ZIKV and DENV NS1 share 51 to 53% of amino acid identity (Fig. 1, A and B, and fig. S2). The mAbs isolated from ZIKV-infected, DENV-naïve donors were to a large extent ZIKV-specific and did not cross-react to DENV NS1, whereas those isolated from ZIKV-infected, DENV-immune donors showed, as expected, a higher degree of cross-reactivity (Fig. 1C, fig. S3, and table S3). The limited cross-reactivity of NS1-reactive mAbs from ZIKV-infected, DENV-naïve donors was corroborated by the lack of reactivity to DENV1-4 NS1 proteins of plasma from ZIKV-infected, DENV-naïve donors (fig. S4). Conversely, the plasma of the ZIKV-infected, DENV-immune donors reacted strongly to DENV1-4 NS1 proteins, which is consistent with a recall response to NS1 dominated by cross-reactive antibodies. Reciprocally, NS1-reactive mAbs isolated from DENV-immune, ZIKV-naïve donors showed variable patterns of cross-reactivity between DENV serotypes but, with the exception of a single mAb, did not cross-react with ZIKV NS1 (Fig. 1D, fig. S5, and table S4).

Given the interest in developing a diagnostic tool that would discriminate ZIKV-specific from DENV-specific antibodies, we identified antigenic sites (figs. S1 and S2) on NS1 targeted by ZIKV-specific but not by cross-reactive mAbs (fig. S6, A to D). MAbs reacting to two distinct sites on ZIKV NS1 were used in a two-determinant immunoassay so as to detect circulating NS1 protein in body fluids during acute infection (fig. S6E). In addition, mAb ZKA35 binding to the antigenic site S2 was used as a probe in a blockade-of-binding assay (22) so as to detect ZIKV-specific, but not DENV-specific, serum antibodies to NS1 (Fig. 1E) and has a potential to be developed in a serological assay in order to detect clinical and subclinical ZIKV infections at the population level.

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Given the role of T cells in antibody production and in immunopathology, we also investigated the specificity and cross-reactivity of CD4+ memory T cells from the same donors using the T cell library method (Fig. 1, F and G) (23). NS1-specific memory CD4+ T cells were present in the CXCR3+ T helper 1 (T_H1) compartment and, with variable frequencies, in the CXCR3− T_H compartment. With a few exceptions, these T_H cells were specific for either ZIKV or DENV NS1 (Fig. 1, F and G, and fig. S7A), which is consistent with a low level of T cell cross-reactivity, even in subjects who were already exposed to DENV.

The E protein is formed by three domains: EDI, which is involved in the conformational changes required for viral entry; EDII, which contains the fusion loop; and EDIII, which may be involved in binding to cellular receptors (24). DI, DII, and DIII of ZIKV and DENV share 35, 51, and 29% amino acid identity, respectively (Fig. 2A). According to the structure of the ZIKV E protein dimer (11, 12), the majority of the ZIKV- and DENV-conserved solvent-accessible residues are located in EDII, particularly in the fusion loop and the neighboring region (Fig. 2B and fig. S8). Strikingly, the majority of ZIKV EDI/II–specific mAbs isolated from the four ZIKV donors (65%; 24 of 37)

cross-reacted with the E protein of all four DENV serotypes (Fig. 2C, fig. S9, and table S5). Similarly, a large proportion (67%; 31 of 46) of DENV EDI/II–reactive mAbs isolated from DENV donors were also cross-reactive with ZIKV E protein (Fig. 2D, fig. S10, and table S6). These data indicate that the human antibody response to the E protein and in particular to EDI/II is cross-reactive between ZIKV and DENV, which is consistent with previous reports on mAbs from mice immunized with flavivirus antigens (11, 25). ZIKV-immune plasma was also found to cross-react with ZIKV and DENV1-4 E proteins (fig. S11), possibly because of the abundant EDI/II cross-reactive antibody response (21, 26–28).

In contrast, most of the EDIII-reactive mAbs (90%; 27 of 30) isolated from ZIKV or DENV donors were specific for either ZIKV or DENV E protein (Fig. 2, C and D; figs. S9 and S10; and tables S5 and S6). In addition, whereas the EDI/II mAbs showed only modest or even no neutralizing activity against ZIKV infection, the EDIII-reactive antibodies were overall more highly neutralizing than EDI/II–reactive antibodies (table S5). By screening for ZIKV neutralization, we also isolated several highly potent neutralizing antibodies that failed to bind to ZIKV E and EDIII

proteins (Fig. 2C). These mAbs, that we define as neutralizing non-E-binding (NNB), could recognize quaternary epitopes that are displayed on the infectious virions but not on soluble proteins, as recently demonstrated for DENV (29–31). E-specific CD4+ T cells were primarily detected in the CXCR3+ T_H cell subset and, with only a few exceptions, were specific for ZIKV E protein (Fig. 2, E and F, and fig. S7B). Furthermore, E-reactive T cells directly isolated from ex vivo cultures of memory CD4+ T cells of ZIKV- or DENV-infected donors (fig. S12) did not cross-react upon secondary stimulation with heterologous E proteins (Fig. 2G). These findings indicate an overall low level of T cell cross-reactivity between ZIKV and DENV E proteins.

To address the biological properties of ZIKV and DENV antibodies, we used a selected mAb panel and measured in parallel their binding (Fig. 3A), neutralizing, and ADE activity (Fig. 3, B and C). This panel also contained Fc mutant versions of mAbs that do not bind to FcγR and complement [LALA mutants; leucine (L) to alanine (A) substitution at the position 234 and 235] (21, 32). The EDIII-specific mAbs ZKA64 and ZKA190 and the NNB mAb ZKA230 were highly potent in ZIKV neutralization, with median inhibitory concentration (IC_{50}) values of 93, 9, and 10 ng/ml, respectively. Furthermore, all these mAbs enhanced infection of ZIKV in the non-permissive K562 human erythroleukemic cell line at a broad range of concentrations, including those that fully neutralized ZIKV infection on Vero cell line. Whereas EDIII mAbs ZKA64 and ZKA190 did not enhance ZIKV infections of K562 cells above 1 μg/ml, the NNB mAb ZKA230 failed to do so, a result that might be due to the different mechanisms of neutralization of free viruses versus FcγR-internalized viruses. In contrast, the cross-reactive EDI/II–specific mAbs ZKA3 and ZKA78 only partially neutralized ZIKV infectivity, while effectively enhancing infection of K562 cells. Consistent with their cross-reactivity, these EDI/II–specific mAbs also neutralized and enhanced DENV1 infection.

The above results suggest that cross-reactive antibodies elicited by either ZIKV or DENV infection and primarily directed to EDI/II can mediate heterologous ADE. We therefore asked whether ADE could be also induced with immune sera and whether this could be blocked by neutralizing mAbs delivered in a LALA format (Fig. 3, D and E). In a homologous setting, four ZIKV-immune plasma collected from convalescent patients showed similar capacity to enhance ZIKV infection of K562 cells, and this ADE effect was completely blocked by the EDIII-specific ZKA64-LALA mAb, but only partially inhibited by the cross-reactive EDI/II DV82-LALA mAb (Fig. 3D). In a heterologous setting, the four ZIKV-immune plasma strongly enhanced infection by DENV1 to levels comparable with those observed with DENV3 plasma. The enhancement of DENV1 infection by both ZIKV and DENV3 plasma was completely inhibited by DV82-LALA, but not by ZKA64-LALA because of its lack of cross-reactivity to DENV1 (Fig. 3E).
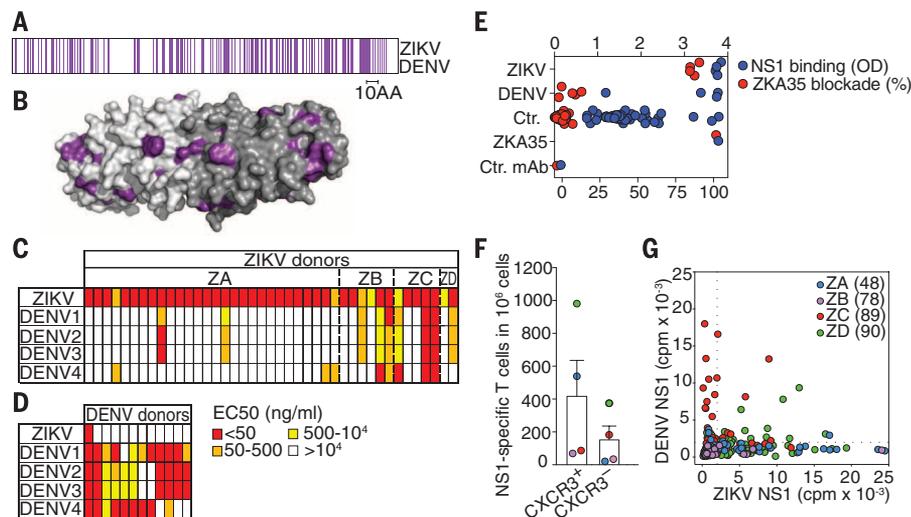

Fig. 1. Specificity and cross-reactivity of NS1-reactive mAbs and T cells derived from ZIKV- and DENV-infected donors. (**A**) Sequence conservation of NS1 proteins as determined by the alignments shown in fig. S2. Conserved residues in NS1 are in purple. (**B**) Structure of the ZIKV NS1 dimer [Protein Data Bank (PDB) 5IY3]. The two monomers are shown with different shades of gray. The surface residues of each protomer that are conserved between ZIKV and DENV are colored as in (A). (**C** and **D**) Heat map of the reactivity of (C) 41 mAbs derived from four ZIKV-infected donors (ZA-ZD) and (D) 12 mAbs derived from two DENV-infected donors. The mAbs were tested for binding to NS1 proteins of ZIKV and DENV1-4 [median effective concentration (EC_{50}), nanograms per milliliter]. A description of the immune status of the donors and the mAbs codes is provided in table S1. The binding curves of NS1-reactive mAbs are shown in figs. S3 and S5, and EC_{50} values are reported in tables S3 and S4. Data are representative of at least two independent experiments. (**E**) Plasma from ZIKV-infected (n = 4 donors), DENV-infected (n = 5 donors), and control donors (n = 48 donors) (1/10 dilution) were tested for their capacity to bind NS1 (blue dots) and to inhibit the binding of the biotinylated mAb ZKA35 to NS1 (red dots). (**F** and **G**) T cell libraries were generated from CXCR3+ and CXCR3− memory CD4+ T cells of the four ZIKV-infected donors and screened for reactivity against ZIKV or DENV1-4 NS1 proteins by use of ^{3}H-thymidine incorporation. Shown is the estimated number of ZIKV NS1–specific T cells per 10^6 cells [mean + SEM and color-coded values for individual donors (F)]. Shown is also the proliferation [counts per minute (cpm)] of individual cultures to either ZIKV or DENV1-4 NS1 proteins (G). The cultures from different donors are color-coded, and their numbers are shown in parentheses. Dotted lines represent the cut-off value.

The ADE-blocking ability of a single EDIII-specific LALA mAb could be related not only to its capacity to out-compete serum-enhancing antibodies but also to neutralize virus once internalized into endosomes, similarly to what has been reported for the WNV-neutralizing antibody E16 (33). Conversely, the lack of ADE-blocking ability of DV82-LALA might be explained by the inability of antibodies to EDI/II to effectively neutralize endocytosed virus.

The above results suggest that previous ZIKV and DENV immunity may pose a risk of a more severe disease upon exposure to heterologous virus through ADE. To address this possibility in vivo, we selected two EDI/II cross-reactive mAbs elicited by ZIKV (ZKA78) or DENV (DV82) and tested them for their capacity to enhance infection by DENV or ZIKV in animal models. Pre-administration of ZKA78 or DV82, but not their LALA versions, to DENV2-infected AG129 mice led to severe symptoms and lethality by day 5 (Fig. 4A), suggesting that ZIKV immunity could predispose to enhanced DENV pathology in vivo. Given the high lethality of ZIKV infection in immunodeficient mice (34–36), we investigated a possible ZIKV disease–enhancing activity of the DENV cross-reactive mAb DV82 in 129Sv/ev immunocompetent mice that are not permissive for productive ZIKV infection. However, in this mouse model we did not observe signs of enhanced disease or infection (fig. S13).

To develop a therapeutic approach to ZIKV infection, we tested the LALA form of the potently neutralizing EDIII-specific mAb ZKA64 in prophylactic and therapeutic settings. ZKA64-LALA completely protected A129 mice challenged with a lethal dose of ZIKV from body weight loss and lethality when given 1 day before or 1 day after ZIKV challenge (Fig. 4B).

This study reports the first characterization of the human immune response to ZIKV infection. The highly cross-reactive antibodies to EDI/II elicited by ZIKV or DENV infection that are poorly neutralizing but potently enhancing may pose a risk for heterologous ADE. We have shown in vitro and in a relevant animal model that an EDI/II cross-reactive mAb raised by ZIKV can induce lethal DENV infection. However, enhancement of ZIKV infection by DENV-elicited cross-reactive antibodies could be observed in vitro but not in vivo, a finding that may be due to the tropism of the virus or to the limitation of the mouse model. It will be important to address this issue in clinical and epidemiological studies, which may be facilitated by the development of serological diagnostics, such as the blockade-of-binding assay described in this study.

T cells in flavivirus infections play a complex role in both protection and pathogenesis (15). The low degree of CD4+ T cell cross-reactivity between DENV and ZIKV, which we observed even in individuals who are immune to both viruses, suggests that in ZIKV infection original antigenic sin may not play a pathogenic role. Our findings also suggest that the risk of cytokine storm and consequent severe disease after enhanced heterologous infection of DENV by antibodies to ZIKV may be mitigated by the poor T cell cross-reactivity.

Our study describes two classes of potent neutralizing antibodies that are specific for ZIKV and directed either against EDIII or quaternary epitopes present on infectious virus, the latter being particularly frequent, similar to what has been described in DENV (30). Given the high potency and in vivo efficacy shown in this study, these antibodies, developed in wild type or their LALA versions produced in wild-type version or in LALA version so as to avoid possible enhancement, could be used in prophylactic or therapeutic settings to prevent congenital ZIKV infection in pregnant women living in high-risk areas (37).
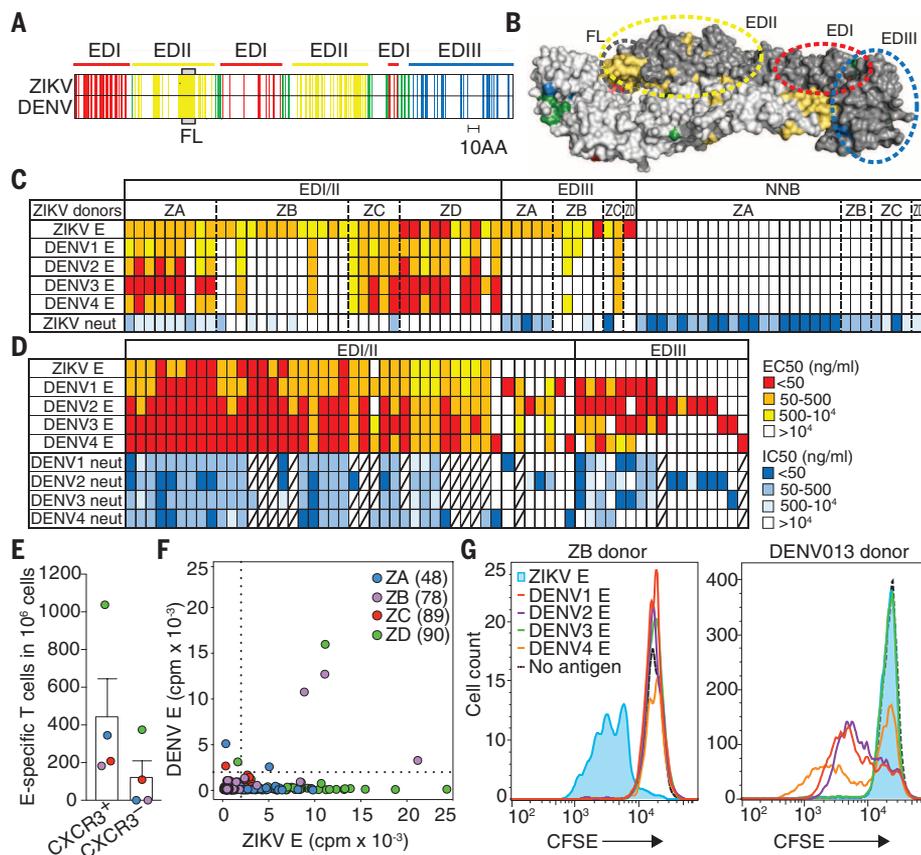

Fig. 2. Specificity and cross-reactivity of E-reactive mAbs and T cells derived from ZIKV- and DENV-infected donors. (A) Sequence conservation of E proteins as determined by the alignments of ZIKV isolates and DENV1-4 reference strains, as shown in fig. S8. Conserved residues in EDI, EDII, EDIII, and the hinge regions of the E protein are colored in red, yellow, blue, and green, respectively. FL, fusion loop. (B) Structure of the ZIKV E protein dimer (PDB 5JHM). The two monomers are shown with different shades of gray, and the residues conserved between ZIKV and DENV are colored as in (A). (C and D) Heat map of the reactivity of (C) 78 mAbs derived from four ZIKV donors and (D) 61 mAbs derived from nine DENV donors. NNB, neutralizing non-E-binding mAbs. The mAbs were tested for binding to E-recombinant proteins of ZIKV and DENV1-4 (EC_{50}, nanograms per milliliter). Also shown is the virus-neutralizing activity (IC_{50}, nanograms per milliliter). Strikethrough cells indicate not tested. Binding curves of E-reactive mAbs are shown in figs. S9 and S10. The EC_{50} and IC_{50} values of E-reactive and NNB mAbs are shown in tables S5 and S6. Data are representative of at least two independent experiments. (E and F) T cell libraries were generated from CXCR3+ and CXCR3− memory CD4+ T cells of the four ZIKV-infected donors and screened for reactivity against ZIKV or DENV1-4 E proteins by using ^{3}H-thymidine incorporation. Shown is the estimated number of ZIKV E–specific T cells per 10^{6} cells [mean + SEM and color-coded values for individual donors (E)]. Also shown is the proliferation (cpm) of individual cultures to either ZIKV E or DENV1-4 E proteins (F). The cultures from different donors are color-coded, and their numbers are shown in parentheses. Dotted lines represent the cut-off value. (G) CD4+ CXCR3+ T cells from peripheral blood mononuclear cells of a ZIKV-infected (ZB) or DENV-infected (DENV013) donor were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with ZIKV or DENV1-4 E proteins, respectively. Proliferating T cells were isolated by means of cell sorting, relabeled with CFSE, and stimulated with the indicated proteins. Shown are the CFSE profiles on day 5.

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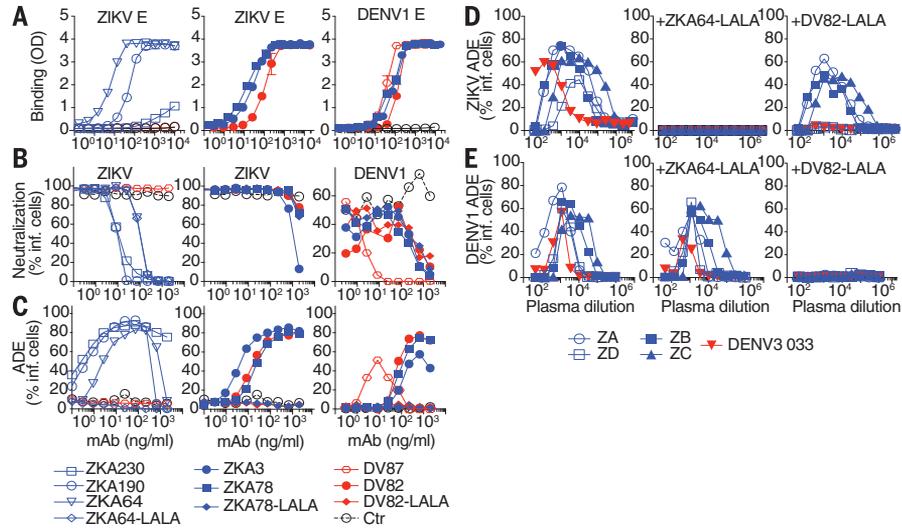12. D. Sirohi et al., Science 352, 467–470 (2016).
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Fig. 3. Neutralization and enhancement of ZIKV and DENV infection by mAbs and immune plasma. (**A**) Binding of mAbs isolated from ZIKV-infected donors (blue) or from DENV-infected donors (red) to (left and middle) recombinant ZIKV E and (right) to DENV1 E protein. Open and solid symbols indicate mAbs specific or cross-reactive for ZIKV and DENV, respectively. (**B**) Neutralization of ZIKV and DENV1 as determined by the percentage of infected Vero cells. (**C**) ADE of ZIKV and DENV1 infection of nonpermissive K562 cells, as determined by the percentage of infected cells on day 5. (**D** and **E**) Inhibition of ADE by LALA mutant mAbs. Serial dilutions of ZIKV- and DENV3-immune plasma were incubated with ZIKV or DENV1 before addition to K562 cells in the absence or presence of a fixed amount (50 µg/ml) of ZKA64-LALA or DV82-LALA mAb. Shown is the percentage of infected K562 cells on day 4. Blue and red symbols indicate plasma derived from ZIKV- or DENV-infected donors, respectively. Data are representative of two independent experiments.
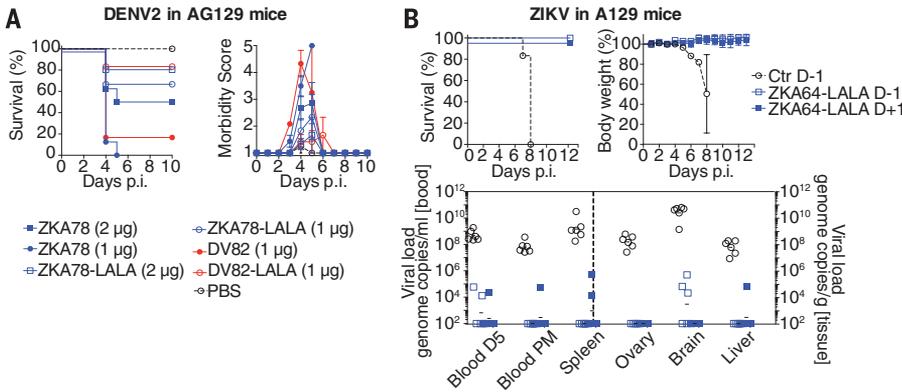
Fig. 4. In vivo enhancement of DENV2 infection by an anti-ZIKV cross-reactive mAb and ZIKV therapeutic efficacy of a potent anti-ZIKV EDIII-specific mAb. (**A**) In vivo ADE. MAbs were administered intraperitoneally to AG129 mice 20 to 24 hours before intravenous inoculation with 2.5×10^5 plaque-forming units (PFU) of DENV2 D2S10. Results are representative of two independent experiments, with $n = 8$ mice for the ZKA78 1 and 2-µg groups; $n = 6$ for the DV82 and all LALA mAb groups; and $n = 4$ for phosphate-buffered saline (PBS) group. (Left) A Kaplan-Meier survival curve is shown; significance was determined by using the Mantel-Cox log-rank test. ZKA78 versus PBS, $P = 0.0008$; ZKA78 versus ZKA78-LALA, $P = 0.0081$; DV82 versus PBS, $P = 0.0187$; and DV82 versus DV82-LALA, $P = 0.0255$. (Right) Mice were monitored over a 10-day period and scored for morbidity and mortality by using a standardized 5-point system (38). (**B**) Prophylaxis and therapy of ZIKV infection. MAbs (15 mg/kg) were administered intraperitoneally to A129 mice ($n = 6$ mice) 24 hours before or after subcutaneous inoculation with 10^2 PFU of ZIKV MP1751. (Top left) A Kaplan-Meier survival curve is shown. (Top right) Mice were monitored over a 13-day period for body weight loss. (Bottom) Viral loads were measured on day 5 in blood of all animals and in blood and indicated tissues when animals were culled at the end of the study or when the humane end points were met. Lines indicate geometric means.

Science

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Characterizing the Zika virus antibody response

Given the public health emergency that Zika virus poses, scientists are seeking to understand the Zika-specific immune response. Stettler *et al.* analyzed 119 monoclonal antibodies isolated from four donors that were infected with Zika virus during the present epidemic, including two individuals that had previously been infected with dengue virus, another member of the flavivirus family. Neutralizing antibodies primarily recognized the envelope protein domain III (EDIII) or quaternary epitopes on the intact virus, and an EDIII-targeted antibody protected mice against lethal infection. Some EDI/II-targeting antibodies cross-reacted with dengue virus in vitro and could enhance disease in dengue-infected mice. Whether dengue and Zika virus antibodies cross-react in humans remains to be tested.

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