Dengue and Zika Virus Infections are enhanced by Live Attenuated Dengue Vaccine but not by Recombinant DSV4 Vaccine Candidate in Mouse Models

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Supplementary File

1. Methods S1: Animal Experiments

1.1 Housing & husbandry

BALB/c, CD11c-*Ifnar1*$^{-/-}$ and AG129 mice were housed in individually ventilated cages (IVCs; dimensions: 425 mm x 266 mm x 155 mm) provided with corn bedding along with enrichment materials such as cardboard shelters, autoclaved tissue paper and/or wooden cubes. The IVCs were maintained in SPF animal facility under the following conditions: temperature of 19-25^{o}C, relative humidity of 30-70%, light intensity of 130-325 Lux, noise level <60 dB and a photoperiod of 12 hours light and 12 hours dark phase, with air changes of 10-15/hour. Gamma-irradiated, non-contaminated and nutritionally adequate, sterile (Gamma-irradiated) and non-contaminated food pellets and sterile (autoclaved) potable fresh drinking water were provided *ad libitum*.

Studies using C57BL/6 *Stat2*$^{-/-}$ mice were carried out in an animal biosafety level 2 plus facility under a protocol approved by the Icahn School of Medicine at Mount Sinai Animal Care and Use Committee. C57BL/6 *Stat2*$^{-/-}$ mice were housed in Allentown IVC polycarbonate plastic cages that house up to 5 mice. Each shoebox cage is supplied ventilation *via* a wall-mounted HEPA-filtered supply and water filtered using reverse osmosis. Mice were provided irradiated food (Purina Pico #5053) and sterile water *via* bottles. Mice were provided with ¼ inch corn cob bedding along with 4 grams of nesting material (Enviro dri) and igloos for enrichment. Mice were housed in conventional holding rooms at a temperature between 20-24°C, relative humidity of 55 ± 10% and a circulation of 10-16 air changes per hour. The holding room was maintained on a 12h light: 12h dark cycle turned on at 7:00am. After infection, mice were monitored daily and weight measurements taken.

1.2 Baseline data, sample size and allocation to groups

Health monitoring of animals for pathogen (fungal, bacterial and viral) infection status was performed regularly and genetic status confirmed prior to use in experiments. Before commencing experiments, animals were weighed daily (morning and evening) and healthy animals, showing no significant weight loss (<15% weight loss) over a week, were selected. Age and gender-matched animals were randomly assigned to control and test groups. All animals were treated and accessed equally for various interventional steps (such as immunisation, challenge or bleeding) entailed in a given experiment. The number of animals to be used was estimated by pair-wise *t*-test with confidence level of 90%, error margin of 5% and effect size of 80% (using statistical software 'G*power 3.1').

1.3 Study design

The animal experimentation work entailed the following five arms. **Arm-1** was designed to generate polyclonal immune sera to three immunogens, DSV4, Dengvaxia and TV DENV, in BALB/c mice (*please see Figure 1 in the main article*). Arm-1 consisted of four groups (*n*=20 per group) of BALB/c mice, which received the following i.m. injections: group-1, 1x PBS (mock immunisation); group-2, DSV4 (20 µg/dose, formulated in alum); group-3, Dengvaxia (0.1 human dose corresponding to 3.5-5.0 $\log_{10}$ $CCID_{50}$ of the four serotypes of chimeric vaccine viruses) and group-4, TV DENV [tetravalent mixture containing 10^5 FIUs each of DENV-1 (West Pac 74), DENV-2 (S-16803), DENV-3 (CH53489) and DENV-4 (TVP360)]. Arm-1 also included a fifth group in which CD11c-*Ifnar1*$^{-/-}$ mice were immunised with Dengvaxia, using the same dose and schedule as for BALB/c mice. **Arm-2** was designed to evaluate the effect of passively transferred BALB/c immune sera into AG129 mice, on their susceptibility to subsequent sub-lethal challenge with DENV-2 S221 (*please see Figure 2 in the main article*). Arm-2 consisted of the following seven groups (*n*=5/group),

which received passive transfer of either mAb 4G2 (group-1) or pooled immune sera (20 µl/mouse, i.p.) from mock- (group-2), DSV4- (group-3), Dengvaxia- (group-4) and TV DENV- (group-5) immunised BALB/c mice, from Arm-1. One group (group-6) received immune sera from Dengvaxia-vaccinated CD11c-*Ifnar1*$^{-/-}$ mice, from Arm-1. A reference group (group-7) in Arm-2 was comprised of AG129 mice which received neither passive transfer of mAb/immune serum nor DENV-2 S221. **Arm-3** was designed to evaluate the effect of injecting either partially (30%) or fully (100%) neutralised ICs, generated *in vitro*, by mixing the sub-lethal dose of DENV-2 S221 with anti-DSV4 (α-DSV4), anti-Dengvaxia (α-Dengvaxia) and anti-TV DENV (α-TV DENV) immune sera from Arm-1), into AG129 mice (*please see Figure 3 in the main article*). This arm included the following six test groups (*n*=6/group): α-DSV4 30% nICs, group-1, DSV4 100% nICs, group-2, α- Dengvaxia 30% nICs, group-3, Dengvaxia 100% nICs, group-4, α-TV DENV 30% nICs, group-5 and α-TV DENV 100% nICs, group-6. Arm-3 also included three control groups (*n*=6/group): 4G2 ICs, group-7 (fully neutralised with mAb 4G2); Virus Control, group-8 (only sub-lethal virus dose injected) and 'Uninfected', group-9 (no nICs injected). **Arm-3a** was designed to determine DENV-2 viremia as well as investigate evidence of intestinal haemorrhage in AG129 mice receiving different nICs. This arm consisted of the same 9 groups described in Arm-3, except that the group size was smaller (*n*=3/group). **Arm-4** was designed to test the efficacy of passively transferred immune sera, from Arm-1, into AG129 mice, to assess their efficacy in conferring protection against lethal challenge with DENV-2 S221. This arm included the following groups: α-DSV4-100 µl group (group-1); α-DSV4-300 µl group (group-2); α-Dengvaxia-100 µl group (group-3); Dengvaxia-300 µl group (group-4); Dengvaxia-CD11c-300 µl group (group-5); 3H5 group (group-6); 4G2 group (group-7); Virus Control group (only lethal virus dose injected, group-8); and Uninfected group (group-9). **Arm-5** was designed to investigate the effect of passively transferred BALB/c immune sera (from Arm-1) into C57BL/6 *Stat2*$^{-/-}$ mice, on their susceptibility to subsequent challenge with ZIKV PRVABC59 (*please see Figure 7 in the main article*). This arm consisted of groups (*n*=6-8/group) of C57BL/6 *Stat2*$^{-/-}$ mice. There were four test groups which received passive transfer of mock-immune serum (group-1), α-DSV4 (group-2), α-Dengvaxia (group-3) or α-TV DENV (group-4) immune sera from Arm-1, prior to ZIKV PRVABC59 infection. In addition, there were two control groups in this arm, with one receiving passive transfer of DENV-seropositive human plasma (group-5), before ZIKV infection and the other receiving neither passive serum/plasma transfer nor ZIKV infection (group-6).

1.4 Experimental procedures

All experiments were conducted during the active hours of animals. For immunisation, mice were anaesthetised in an anesthesia chamber (Table Top Research Anesthesia Machine w/O_2 Flush, Parkland Scientific, Inc. Coral Springs, USA) using 2% isofluorane and 2 L min^{-1} oxygen flow. BALB/c and CD11c-*Ifnar1*$^{-/-}$ mice in Arm-1 were immunised on days 0, 21 and 42 by i.m. injections and bled on day 56. AG129 mice in Arm-2 were given passive transfer of mAb 4G2 or immune sera by i.p. injections. These were then administered a sub-lethal dose of DENV-2 S221 by i.v. injection (2x10^4 FIUs/mouse). The AG129 mice were followed for 15 days post-infection. During this time their survival, body weight and clinical signs of illness were monitored. Clinical scores were based on a 5-point system, with the maximum score denoting death: 0.5, mild ruffled fur; 1.0, ruffled fur; 1.5, compromised eyes; 2, compromised eyes with hunched back; 2.5, loose stools; 3.0, limited movement; 3.5, no movement/hind leg paralysis; 4.0, euthanised if cumulative score was 5. AG129 mice in Arm-3 received i.v. injections of different nICs, following which they were monitored for 15 days, exactly as described for the AG129 mice in Arm-2. AG129 mice in Arm-3a were euthanised on day-4 post nIC-inoculation to collect blood (for viremia determination) and small intestinal tissue (for assessing vascular leakage and estimating cytokine production) to evaluate the extent of ADE-induced pathology. In Arm-4, AG129 mice received passive transfer of mAb or polyclonal murine sera and bled 2 hours later (for circulating nAb titre determination) and challenged with a lethal dose (10^5 FIU/mouse) of DENV-2 S221. These were then monitored for survival, clinical signs and body weight change for 15 days as above. In Arm-5, C57BL/6 *Stat2*$^{-/-}$ mice were given passive transfer of different immune sera (i.p.) and challenged 2 hours later with ZIKV PRVABC59 (5x10^3 PFUs, i.d.). The C57BL/6 *Stat2*$^{-/-}$ mice were followed for 15 days post-infection. During this time their survival, body weight and clinical signs of illness were monitored. Clinical scores were based on a 4-point system to grade signs of illness: 0, no symptom; 0.5, mild ruffling of fur; 1.0, ruffled fur; 1.5, compromised eyes; 2.0, hunched back position; 2.5, very limited movement/one lag paralysis; 3.0, no movement and both hind leg paralysis; 4.0, dead or euthanised.

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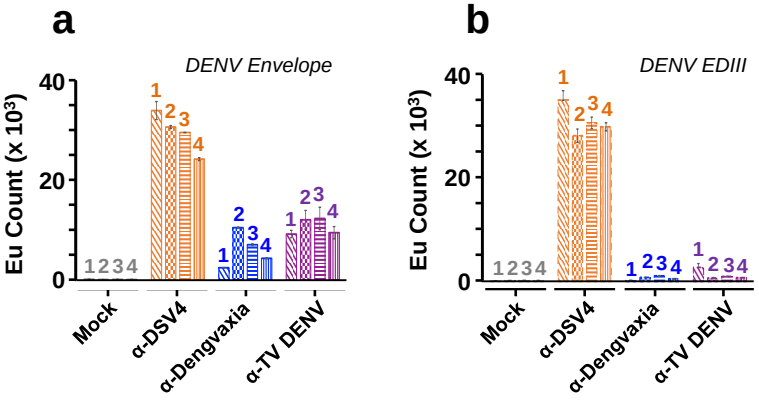


Figure S1: Total anti-DENV antibody titres in sera of DSV4-, Dengvaxia-, and TV DENV-immunised BALB/c mice. *(a) TRF-IA of pooled (n=20) BALB/c mock-immune sera (mock, grey), anti-DSV4 (α-DSV4, orange), anti-Dengvaxia (α-Dengvaxia, blue) and anti-TV DENV (α-TV DENV, purple) antisera using insect cell-expressed recombinant DENV E proteins corresponding to DENV-1, DENV-2, DENV-3 and DENV-4, as the capture antigens. The four DENV E serotypes are denoted by the numbers 1-4 above the bars. Bound total anti-E antibodies were detected using anti-mouse IgG-Eu^{3+} conjugate by TRF. (b) Similar TRF-IA as in panel 'a', except that the coating antigens were purified yeast-expressed recombinant EDIII proteins corresponding to the four DENVs. The colours to distinguish the different immune sera and numbers to denote serotypes are the same as in panel 'a'.*

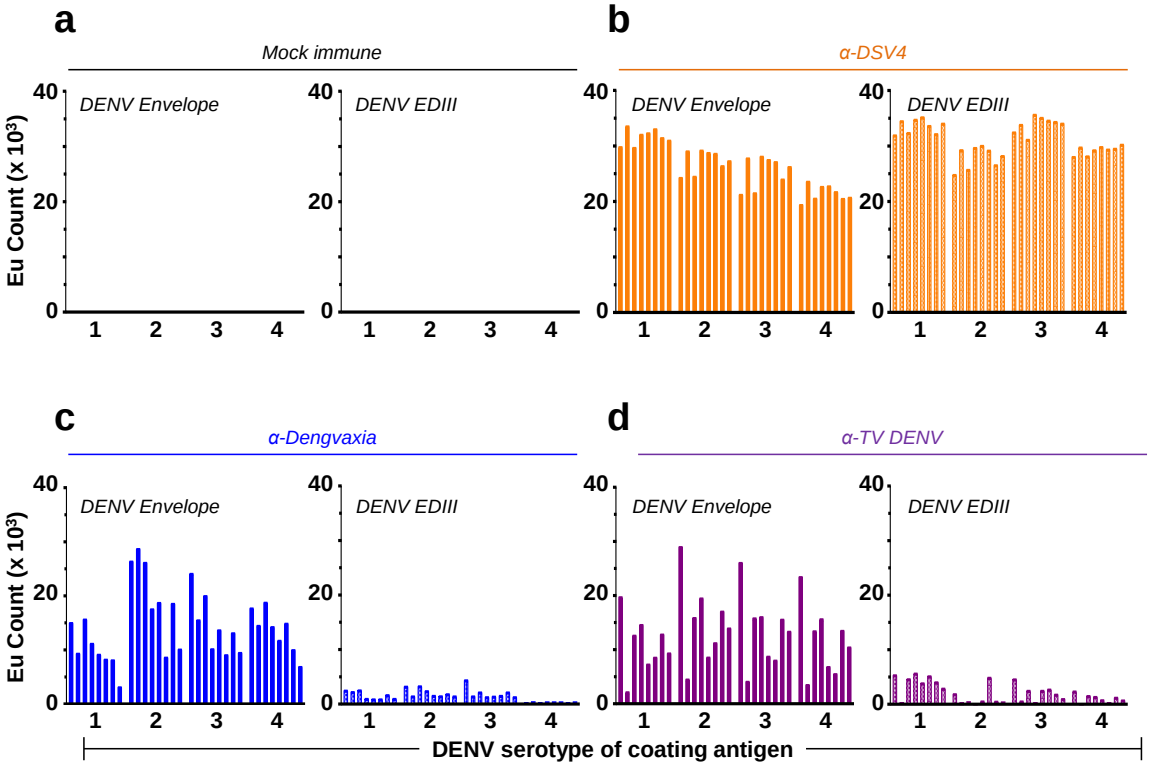
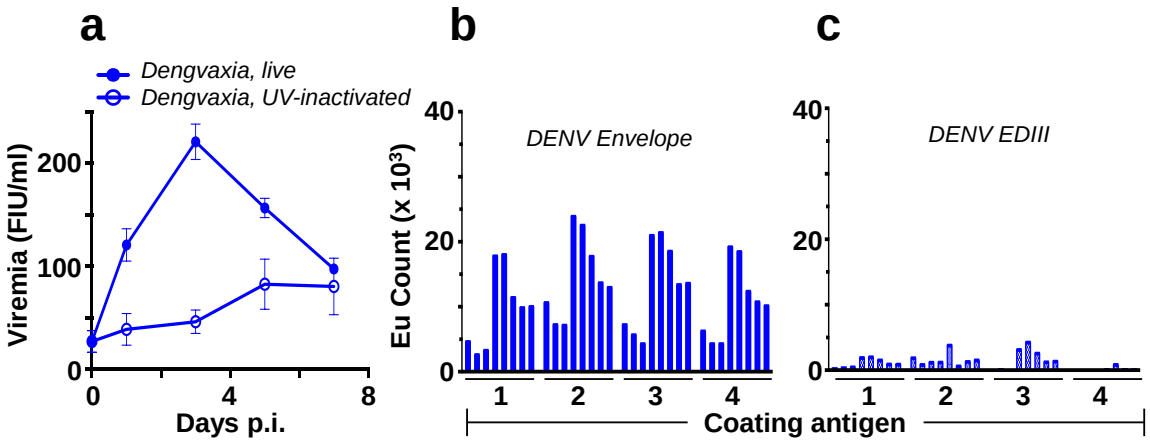


Figure S2: TRF-IA analysis of sera from individual BALB/c mice immunised with DSV4, Dengvaxia, and TV DENV. *Mock-immune (panel 'a', mock, grey), anti-DSV4 (panel 'b', α-DSV4, orange), anti-Dengvaxia (panel 'c', α-Dengvaxia, blue) and anti-TV DENV (panel 'd', α-TV DENV, purple) antisera, from 6 individual mice (out of the 20 mice in each immunisation group in Figure S1), were analysed by TRF-IA using recombinant DENV envelope (DENV E) and EDIII (DENV-EDIII) proteins described in Figure S1 legend. Apart from the 6 individual sera (indicated by the first 6 bars for each coating antigen), two sera pools were also tested in parallel. One was a pool of the 6 individual sera (the 7th bar) and the other, a pool of all 20 sera of each immunisation group (the 8th bar). None of the sera from the mock-immunised mice (the 6 individual sera as well as the two sera pools) showed any discernible reactivity in the TRF-IA.*



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Serum	nAb Titre (FNT_{50})			
	DENV-1	DENV-2	DENV-3	DENV-4
Mock immune	<10	<10	<10	<10
α-Dengvaxia	601	1001	758	315

Figure S3: Analysis of the immunogenicity of Dengvaxia in CD11-Ifnar1$^{-/-}$ mice. *Immunisation schedule, route and the number of mice were the same as done for BALB/c mice, but the Dengvaxia dose per mouse was one-twentieth the human dose. (a) Mice (n=3) were bled on day 4 after the first immunisation dose for determination of serum CYD-2 viremia by RT-qPCR. For comparison, serum viremia was evaluated in parallel in sera of mice which received the same dose of Dengvaxia which had been UV-inactivated (as described in the Materials & Methods section of the main article). Serum viremia, expressed as FIUs/ml was calculated with reference to genomic RNA isolated from a dilution series of titred DENV-2 stock. (b) Mice were bled 2 weeks after the last dose for TRF-IA. Sera from 6 individual mice (denoted by the first 6 bars of the histogram) and two sera pools (the 7th and 8th bars, as described in Figure S2) were titrated against recombinant DENV Envelope proteins of the four serotypes (1, 2, 3 and 4). (c) The same experiment as in panel 'b', except that the coating antigens were recombinant DENV EDIIIs. (d) Determination of DENV nAb titers in pooled (n=20) sera from mock-immunised (Mock immune) and Dengvaxia-immunised (α-Dengvaxia) CD11c-Ifnar1$^{-/-}$mice, determined using the FACS-based virus neutralisation assay. The nAb titres depicted as <10, denote that there was no discernible DENV neutralisation, even at the lowest serum dilution tested, which was 1:10.*

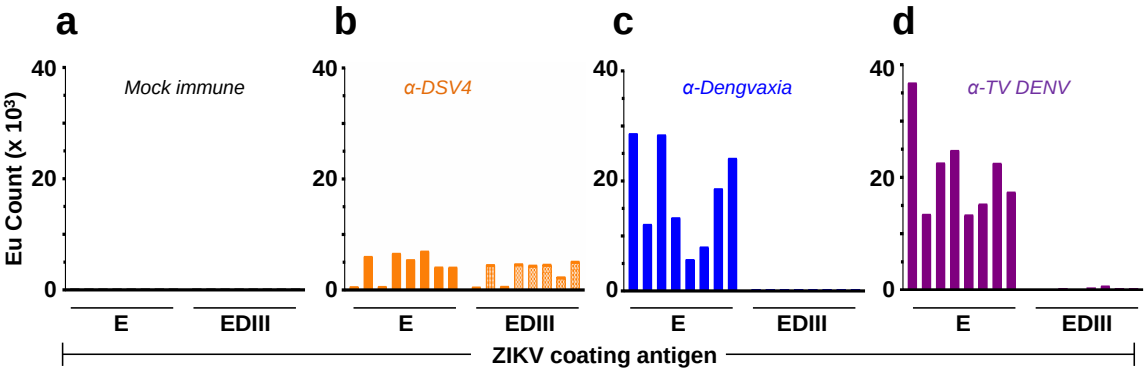

Figure S4: TRF-IA analysis for anti-ZIKV antibodies in sera from individual BALB/c mice immunised with DSV4, Dengvaxia, and TV DENV. *Mock-immune (panel 'a', mock immune, grey), anti-DSV4 (panel 'b', α-DSV4, orange), anti-Dengvaxia (panel 'c', α-Dengvaxia, blue) and anti-TV DENV (panel 'd', α-TV DENV, purple) antisera, from 6 individual mice (out of the 20 mice in each immunisation group described in Figure S1), were analysed by TRF-IA using insect cell-expressed recombinant ZIKV E (E) and yeast-expressed recombinant ZIKV EDIII (EDIII) proteins. Apart from the 6 individual sera (indicated by the first 6 bars for each coating antigen), two sera pools were also tested in parallel (as in Figure S2). One was a pool of the 6 individual sera (the 7th bar) and the other, a pool of all 20 sera of each immunisation group (the 8th bar). None of the sera from the mock-immunised mice (the 6 individual sera as well as the two sera pools, in panel 'a') showed any discernible reactivity in the TRF-IA.*

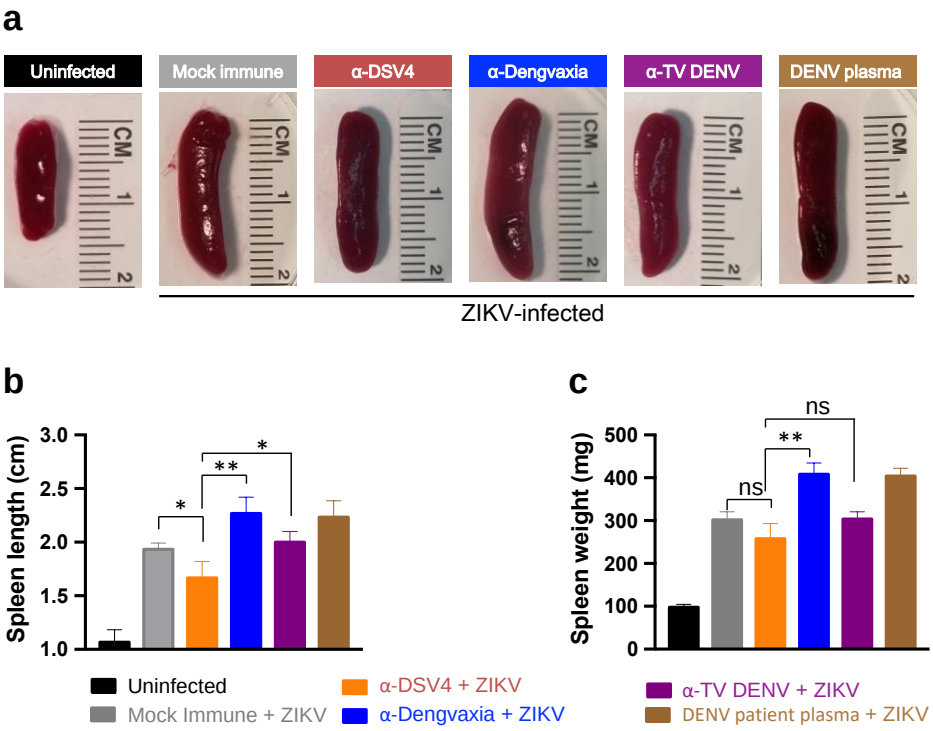

Figure S5: Splenomegaly in ZIKV-challenged mice. *(a) Comparison of spleens from the different groups of mice described in Figure 7, main article. (b) Lengths of the spleens shown in 'a'. (c) Wet weights of the spleens shown in 'a'. In panels 'b' and 'c', significant and very significant (double asterisks) differences (unpaired two-tailed 't' test) are denoted by single and double asterisks, respectively; ns: not significant. Spleens from ZIKV-infected mice which had received prior transfer of α-DSV4 immune serum were significantly smaller (both in terms of length and wet weight) than those which had received prior transfer of α-Dengvaxia immune serum.*

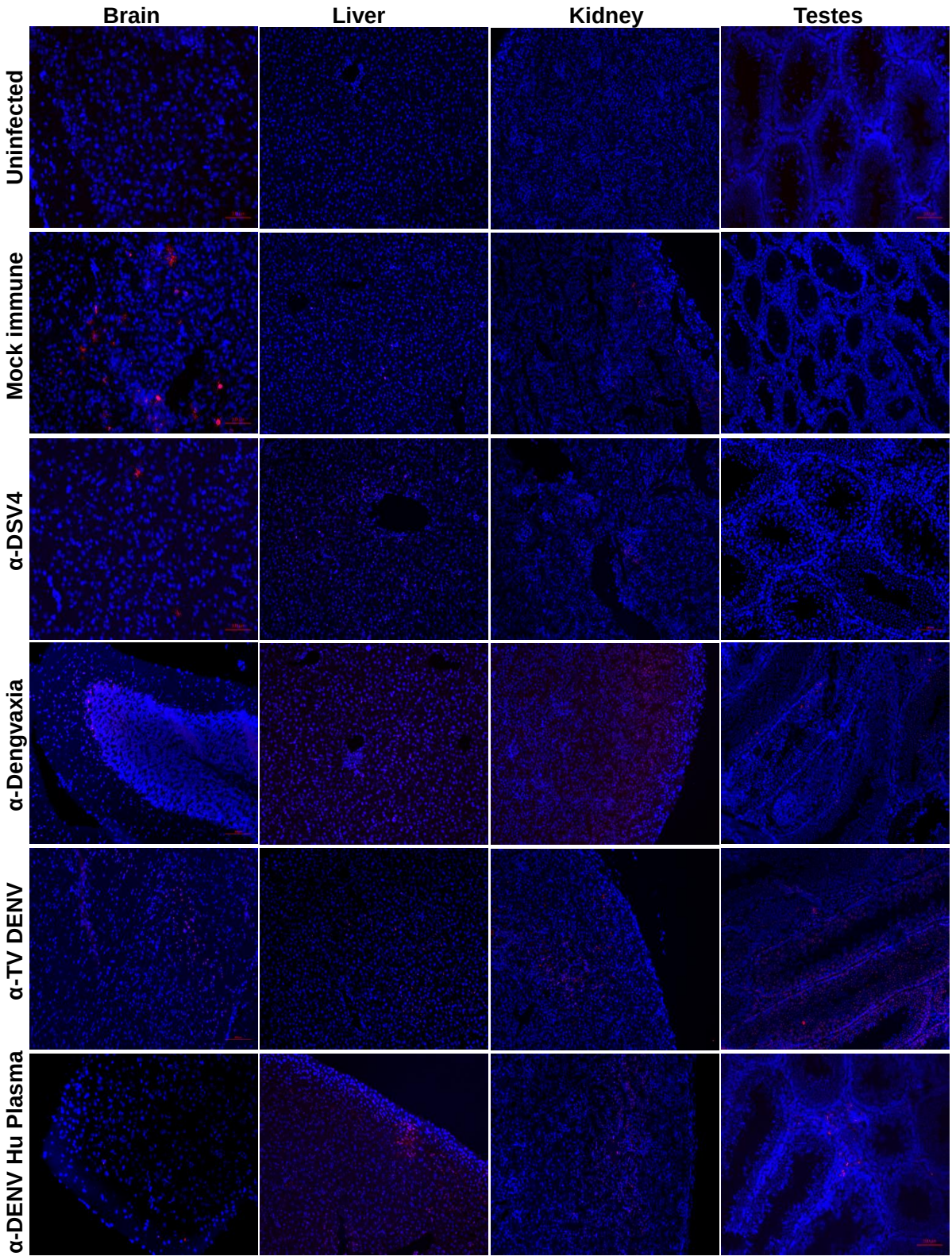

Figure S6: FISH images of tissue slices from ZIKV-challenged mice (related to Figure 7 of the main article). *Tissue slices from various groups of ZIKV-infected mice in Figure 7 were probed for ZIKV RNA using V-ZIKVsph2015 (ACD catalogue# 467871) and visualised by fluorescence microscopy. The bottom row is a positive control performed on tissues from ZIKV-infected mice which had received human DENV seropositive plasma, as described before.*[47] *Red=ZIKV RNA; blue=DAPI.*

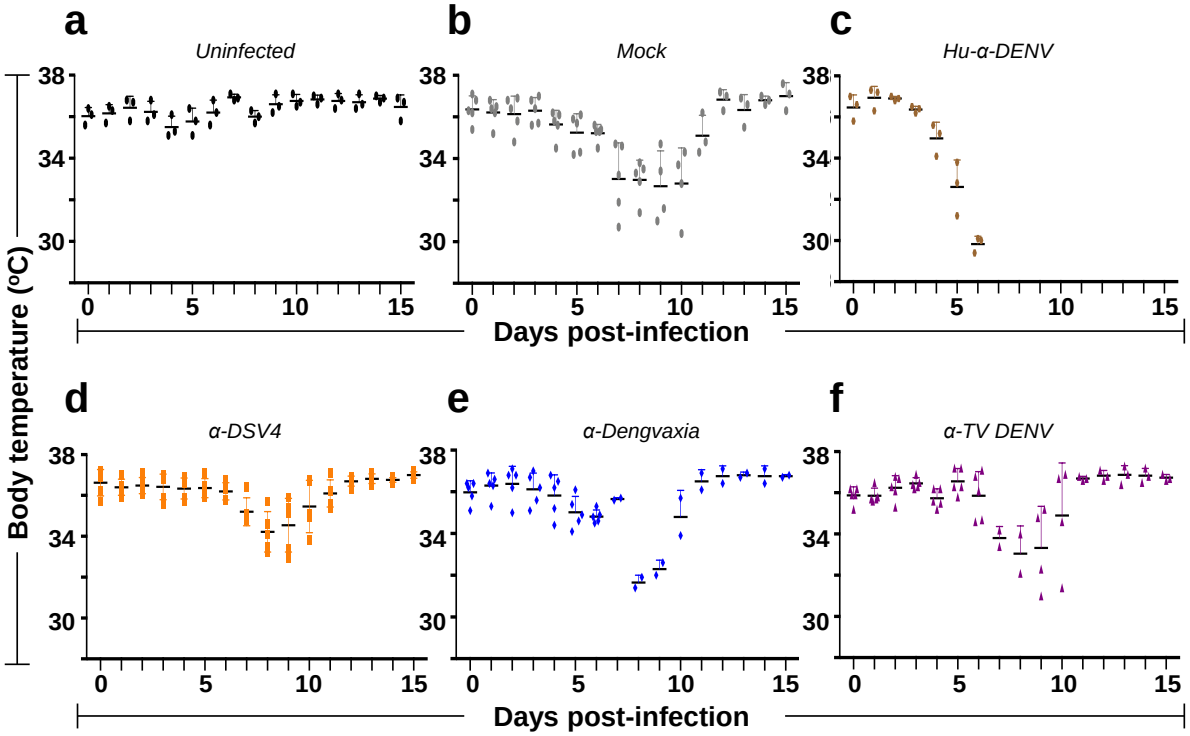

Figure S7: Body temperature changes in ZIKV-challenged C57BL/6 Stat2$^{-/-}$ mice. *The body temperature of each mouse in the different groups (n=6/group), described in Figure 7 of the main article was monitored on a daily basis. The 'Uninfected' panel shows data from mice which neither received passive transfer of any immune serum nor were challenged with ZIKV. Each data point represents the body temperature of a single mouse. The horizontal segment denotes the mean body temperature of the group. Note: The ZIKV-challenged mice which had received prior passive transfer of human DENV seropositive plasma showed a progressive drop in body temperature before succumbing. The elevation in body temperature observed on day 3 post ZIKV challenge earlier[47] was not discernible in the current work for unknown reasons. However, we speculate that differences in the pooled human DENV-seropositive plasma may be one of the underlying causes. The pooled serum in the earlier experiment was generated from about a dozen samples with very high anti-DENV antibody titres, while the pool used in the current work was a mixture of >100 immune sera with much lower and varied anti-DENV antibody titres. The hypothermia seen in the 'Hu-α-DENV' group was, however, transient in the survivors in the other groups of ZIKV-challenged mice.*

Table S1: Enhancement of DENV infection of K562 cells

Immune serum	Peak enhancement titres[a] (% infection)			
	DENV-1	**DENV-2**	**DENV-3**	**DENV-4**
Mock[b]	-	-	-	-
α-DSV4	1080 (16.96)	120 (16.82)	360 (18.6)	40 (5.29)
α-Dengvaxia	40 (16.8)	120 (17.09)	120 (17.36)	120 (14.18)
α-TV DENV	120 (20.48)	120 (18.47)	120 (19.56)	120 (16.41)

[a]*Data shown are related to Figure 2 (panels a-d) of the main article. Peak enhancement titre is the reciprocal of the serum dilution at which maximum number of K562 cells was infected; values shown in brackets represent the percentage of infected cells corresponding to the peak enhancement titre.*
[b]*No discernible infection by any DENV serotype was seen in K562 cells in the presence of mock-immune serum.*

Table S2: Comparison of ZIKV burden in various tissues of *Stat2*$^{-/-}$ mice[a]

Tissue	Immune serum			
	α-DSV4	**α-Dengvaxia**	**α-TV DENV**	**α-Hu-DENV**
Brain	0.0565	>0.9999	<0.0001	<0.0001
Eye	>0.9999	0.8028	0.0011	<0.0001
Spinal cord	>0.9999	>0.9999	>0.9999	0.0830
Testes	>0.9999	<0.0001	<0.0001	<0.0001
Spleen	>0.9999	<0.0001	<0.0001	<0.0001
Lymph node	>0.9999	<0.0001	<0.0001	<0.0001
LIver	>0.9999	<0.0001	<0.0001	<0.0001
KIdney	>0.9999	>0.9999	>0.9999	<0.0001
Large Intestine	>0.9999	0.0003	0.1454	<0.0001
Small intestine	>0.9999	<0.0001	>0.9999	<0.0001

[a]*Comparisons for tissues in mice of each immune serum group with reference to cognate tissue from mice in the mock immune serum group (related to Figure 7 in the main article). Data shown are p values based on two-way ANOVA with Bonferroni's correction.*