Supporting Information

Rumyantsev et al. 10.1073/pnas.1306245110

SI Materials and Methods

Biosafety. Central European strain Hypr of tick-borne encephalitis (TBE) virus, and all vaccine candidates were handled in biosafety level (BSL) 3+ conditions by personnel vaccinated against TBE in accordance with government regulations. Subsequently, selected RepliVax-TBE variants were downgraded to BSL2 based on accumulated biological data.

Cells and Viruses. To generate helper cells for propagation of RepliVax-TBE variants, BHK (1) or Vero (American Type Culture Collection, ATCC) cells were transfected with a Venezuelan equine encephalitis virus replicon (rVEE, based on the TC-83 vaccine strain) expressing the West Nile (WN) virus C protein and puromycin *N*-acetyl-transferase selective marker (1), or an additionally constructed rVEE helper expressing the C protein of TBE Hypr. The cells were maintained in FBS-containing media supplemented with 10 μg/mL of puromycin. RepliVax-TBE titers were determined by immunofocus assay in Vero cells (2) using anti-TBE mouse hyperimmune ascitic fluid (ATCC) or polyclonal rabbit antibodies raised against an inactivated human vaccine against TBE (FSME; Baxter AG) as primary antibodies. The FSME vaccine was used as inactivated vaccine (INV) control in animal studies. Live viruses and chimeras were propagated in regular Vero cells in media without puromycin. Yellow fever (YF) 17D virus (YF-VAX, Sanofi Pasteur) used in animal experiments was prepared by amplification in Vero cells. YF/Japanese encephalitis (JE) chimera was described (3). Langat (LGT) virus strains TP21 (isolated in Malaysia from *Ixodes granulatus* ticks in 1956) and T1674-73 (isolated in Thailand from *Haemaphysalis papuana* ticks in 1973), and wild-type TBE strain Hypr were from R. Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston). Infectious titers/doses of RepliVax constructs and live viruses are expressed in focus forming units (FFU; equivalent to PFU for live viruses).

Construction of RepliVax-TBE Variants and Live Chimeras. RepliVax-TBE based on a WN backbone (RV-WN/TBE) was constructed by replacing the WN-specific prM-E genes in the RepliVax-WN prototype (1) with those from TBE virus strain Hypr isolated in 1953 from a patient in Czechoslovakia (GenBank accession no. U39292; the nucleotide sequence was optimized to have preferential human-genome codon use and to eliminate repeats >8 nt). The TBE genes were synthesized by DNA2.0. A variant with the TBE-specific signal for prM was used in all experiments. (Another variant, with WN-specific prM signal, was also constructed but not used because it replicated to several fold lower titers in helper cells.) RV-YF/TBE was constructed by replacing the YF 17D prM-E genes in RepliVax-YF prototype (1) with the TBE prM-E genes. RV-LGT/TBE was constructed using a synthetic infectious clone of Langat E5 virus assembled from three DNA fragments synthesized based on published LGT E5 sequence (GenBank accession no. AF253420.1). RV-TBE/TBE based entirely on the TBE Hypr sequence was assembled from three DNA fragments synthesized based on published TBE Hypr sequence (GenBank accession No. U39292).

YF/TBE and YF/LGT chimeras containing the TBE Hypr and LGT E5 prM-E genes, respectively, in the YF 17D backbone were made by replacing the JE-specific prM-E genes with the corresponding TBE Hypr/LGT E5 genes in ChimeriVax-JE. Dengue type 2 (DEN2)/TBE chimera was constructed using a synthetic infectious clone of DEN2 virus strain PDK-53 assembled from two DNA fragments synthesized based on published sequence (GenBank accession no. NC_001474). LGT/TBE chimera was made by inserting the complete C protein gene of LGT E5 into the RV-LGT/TBE construct.

Resulting plasmids were transcribed in vitro and appropriate cells were transfected with RNA transcripts to generate infectious RepliVax and chimeric viruses. For DEN2/TBE chimera (the genome split in two plasmids), the full-length cDNA template was prepared by two-fragment in vitro ligation followed by transcription and transfection of Vero cells.

Mouse Studies. All procedures were performed under approved Institutional Animal Care and Use Committee (Sanofi Pasteur, Acambis site) protocols in accordance with the National Institutes of Health requirements for humane treatment of laboratory animals. Outbred Institute for Cancer Research (ICR) mice were from Taconic. Inoculation routes/doses, and bleeding/challenge days were as described in Results. Challenge was done by the i.p. route with 500 LD_{50} (500 FFU) of TBE Hypr. In neurovirulence/neuroinvasiveness tests, and after challenge, mice were monitored for 21 d for survival. Doses causing 50% mortality (LD_{50}) were calculated using the Reed and Muench method. Plaque reduction neutralization ($PRNT_{50}$) titers were determined in heat-inactivated serum samples collected by mandibular bleeding against wild-type Hypr or YF/TBE viruses. Titers of IgG isotypes were determined by isotype-specific ELISA as described (2) using YF/TBE virus as a coating agent.

Nonhuman Primate Studies. The in-life steps of the two nonhuman primate (NHP) studies were performed at BIOQUAL in accordance with Animal Welfare Regulations (US Department of Agriculture), Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

The first NHP study (schedule is shown in Fig. S2) was done in 34 experimentally naïve Rhesus monkeys of Chinese origin prescreened to be seronegative for JE, WN, TBE and YF by $PRNT_{50}$ (titers <10). To establish a surrogate challenge model, three groups (three to four animals per group) were inoculated by the s.c. route with 6 $\log_{10}$ FFU per dose of two strains of LGT virus (TP21 and a plaque purified variant of T1674-73) and YF/TBE chimera, followed by measuring viremia on days 1–8. To evaluate immunogenicity, four groups (four animals per group) were inoculated by the intradermal (i.d.), i.m., and s.c. routes (one or two doses for s.c., with the second dose on day 30) with 7 $\log_{10}$ FFU per dose of RV-WN/TBE. Humoral immunity was measured by $PRNT_{50}$ on days 29 and 50 and compared with YF/TBE (6 $\log_{10}$ FFU, s.c.) or three human doses of the FSME INV (administered i.m. on days 0, 14 and 30). i.d. inoculations were performed using a Becton Dickinson i.d. inoculation devise with a 34 gauge 1-mm needle. Efficacy was evaluated by a s.c. challenge on day 59 with the selected LGT T1674-73 challenge virus followed by measuring postchallenge viremia using immunofocus assay.

The second NHP study consisted of two parts, short-term (Fig. S3) and long-term (Fig. S4). Rhesus monkeys of Chinese origin seronegative for JE, WN, YF, TBE, and DEN 2 and 4 were used, except for two animals that were found to be seropositive for WN. The latter were assigned to a group assessing the effect of anti-vector preimmunity (subgroup 3a in Fig. S3); additional two naïve animals (subgroup 3b) were first preimmunized with RV-WN (7 $\log_{10}$ FFU, i.d.) before vaccination with RV-WN/TBE (7 $\log_{10}$

FFU, i.d.). Two other groups received 6 and 5 $\log_{10}$ FFU per dose of RV-WN/TBE by the i.d. route to evaluate dose–responses using a schedule of steps similar to the first NHP study. In the long-term part (Fig. S4), the dynamics of TBE neutralizing antibody responses were monitored for 6 mo following immunization with 7 $\log_{10}$ FFU per dose of RV-WN/TBE by the i.d. and i.m. routes compared with YF/TBE (5 $\log_{10}$ FFU, s.c.) and three human doses of FSME. $PRNT_{50}$ titers in sera were measured on days 29 and 50 and then at 3, 4, 5, and 6 mo. The immunized animals and a control unimmunized group were challenged with LGT T1674-73 following the final bleeding. In addition to focus assay, postchallenge viremia was measured by real time, quantitative RT-PCR (RT-qPCR) performed on RNA isolated from monkey sera using LGT T1674 E-specific primers. RT-qPCR was performed by using the TaqMan One-Step RT-PCR kit and the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). LGT T1674 strain was used to generate a standard curve and the amount of infectious RNA transcripts per reaction corresponded to the known FFU per reaction expressed as equivalent FFU (Eq FFU). The detection limit was 0.32 $\log_{10}$ Eq FFU/mL LGT E5 RNA concentration curves were also generated, and the conversion factor was ~ 0.0038 Eq FFU per copy (GE).

Statistical Analyses. Calculations of end point titers and analyses of statistical significance were performed using GraphPad Prism 5 (GraphPad Prism Software).

1. Mason PW, Shustov AV, Frolov I (2006) Production and characterization of vaccines based on flaviviruses defective in replication. *Virology* 351(2):432–443.
2. Rumyantsev AA, et al. (2011) Characterization of the RepliVax platform for replication-defective flavivirus vaccines. *Vaccine* 29(32):5184–5194.
3. Guirakhoo F, et al. (1999) Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. *Virology* 257(2):363–372.

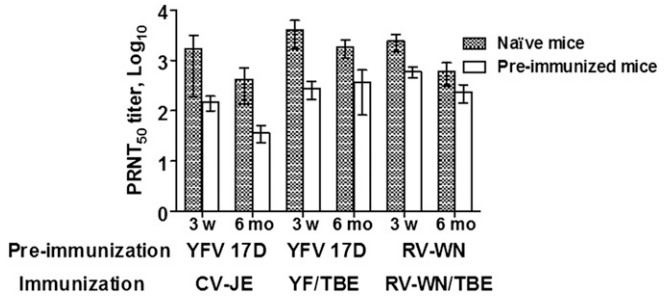
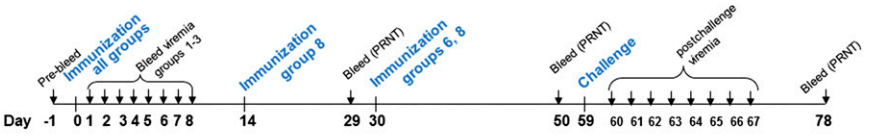
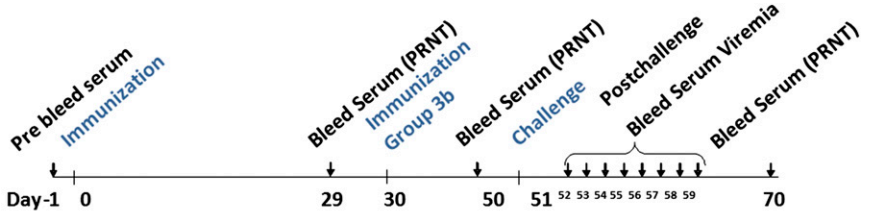


Fig. S1. Effect of anti-vector preimmunity on immunogenicity of chimeric viruses and RepliVax-TBE. Mice (eight per group) were preimmunized with YF 17D and then immunized 3 wk or 6 mo later with ChimeriVax-JE or YF/TBE viruses. Mice were similarly preimmunized with RV-WN and then immunized with RV-WN/TBE. All doses were 5 $\log_{10}$ by the i.p. route. Vaccine-specific N Ab titers were measured 21 d after immunization; geometric mean titers are shown.



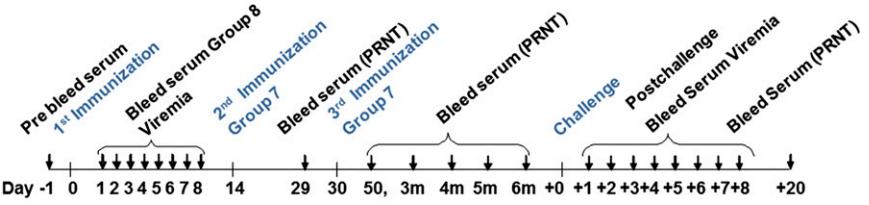
Group	Animals/ Route	Immunization Day 0	Bleed-viremia Days 1-8	Immunization Day 14	Bleed-PRNTs Day 29	Immunization Day 30	Bleed-PRNTs Day 50	Challenge Day 59	Bleed-viremia Days60-67	Bleed-PRNTs Day 78
1. YF/TBE	4 (2M/2F) 1x SC	10^6 SC in 1 ml	X		X		X	X	X	X
2. LGT 1674	3 (1M/2F) 1x SC	10^6 SC in 1 ml	X		X		X	X	X	X
3. LGT TP21	3 (1M/2F) 1x SC	10^6 SC in 1 ml	X		X		X	X	X	X
4. RV-WN/TBE	4 (2M/2F) 1x SC	10^7 SC in 1 ml			X		X	X	X	X
5. RV-WN/TBE	4 (2M/2F) 1x IM	10^7 IM in 1 ml			X		X	X	X	X
6. RV-WN/TBE	4 (2M/2F) 2x SC	10^7 SC in 1 ml			X	10^7 SC in 1 ml	X	X	X	X
7. RV-WN/TBE	4 (2M/2F) 1x ID	10^7 ID 0.2 ml			X		X	X	X	X
8. INV	4 (2M/2F) 3x IM	IM, human dose; 0.5 ml		IM, human dose; 0.5 ml	X	IM, human dose; 0.5 ml	X	X	X	X
9. Mock	4 (2M/2F) 1x SC	Diluent SC in 1 ml			X		X	X	X	X

Fig. S2. Design of NHP study 1.



Group	Virus	Monkeys per group	Day 0 Inoculation route (FFU)	Day 29 Bleed-PRNT	Day 30	Day 50 Bleed-PRNT	Day 51 Challenge
1	RV-WN/TBE	4	ID (1 x 10^6)	X		X	X
2	RV WN/TBE	4	ID (1 x 10^5)	X		X	X
3a	RV WN/TBE (anti-vector immunity)	2 WNV positive	ID (1 x 10^7)	X		X	X
3b	RV WN/TBE (anti-vector immunity)	2 WN naive	RV WNV ID (1 x 10^7)	X	RV WN/TBE ID (1 x 10^7)	X	X
4	Mock-1	4	ID 200ul diluent	X		X	X

Fig. S3. Design of NHP study 2, short segment.



Group	Virus	Monkeys per group (M/F)	Day 0 Inoculation route (FFU)	Days 14 Inoculation	Days 30 Inoculation	6 months + 1 day Challenge
5	RV WN/TBE	2/2	IM (1 x 10^7)			+
6	RV WN/TBE	2/2	ID (1 x 10^7)			+
7	INV	2/2	IM 0.5 ml	IM, 0.5 ml	IM, 0.5 ml	+
8	YF/TBE	2/2	SC (1x10^5)			+
9	Mock	2/2				+

Bleed days for PRNT: d29, d50, and then monthly at 3, 4, 5 and 6 months

Fig. S4. Design of NHP study 2, long segment.

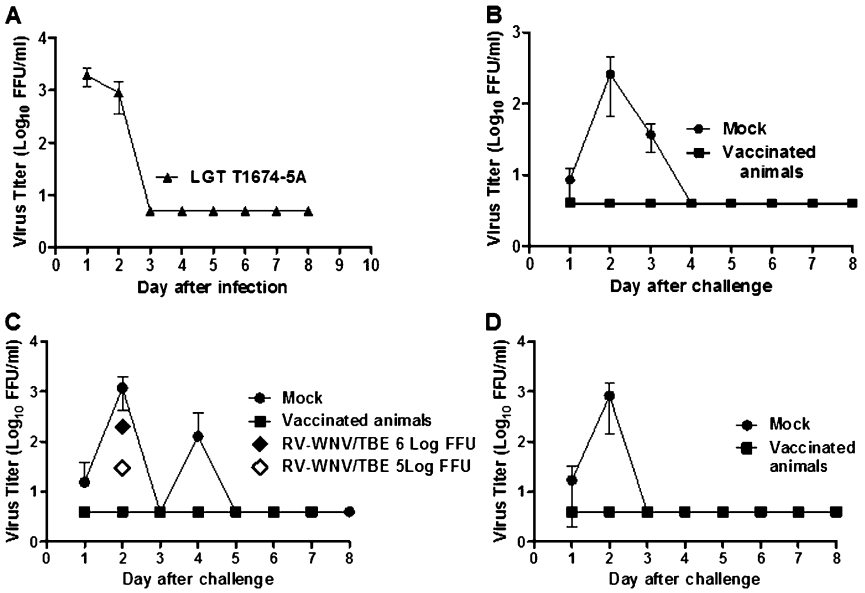


Fig. S5. Postchallenge LGT 1674 virus viremia in NHP determined by plaque assay. (*A*) Establishing the model using 6 $\log_{10}$ FFU dose. To achieve better resolution of viremia, the dose was reduced to 5 $\log_{10}$ FFU to test efficacy of vaccines. (*B*) Challenge in NHP study 1. Only Mock animals showed viremia, whereas monkeys in all immunized groups showed no detectabale viremia. (*C*) Postchallenge viremia in the short segment of NHP study 2. (*D*) Postchallenge viremia in the long-term segment of NHP study 2, at 6 mo.

Table S1. Evaluation of prime-boost vaccination regimens with RV-WN/TBE and INV in mice

Prime day 0	Day 21 $PRNT_{50}$ GMT± SD	Boost day 22	Day 42 $PRNT_{50}$ GMT± SD	Postchallenge mortality, %
INV	48 ± 31	INV	1,019 ± 831	0
INV	57 ± 238	Diluent	115 ± 45	40
INV	59 ± 46	RV-WN/TBE	3,287 ± 1,059	0
RV-WN/TBE	341 ± 342	RV-WN/TBE	6,291 ± 3,871	0
RV-WN/TBE	558 ± 666	INV	14,205 ± 1,433	0
RV-WN/TBE	565 ± 371	Diluent	2,535 ± 561	0
Diluent	<20	Diluent	<10	100

All immunizations were by the i.p. route. RV-WN/TBE was administered at 5 $\log_{10}$ FFU per dose, and INV at 1/20th of a human dose. i.p. challenge was on day 43 with 500 LD_{50} of TBE Hypr.