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1 A Multi-Agent Alphavirus DNA Vaccine Delivered by Intramuscular Electroporation Elicits
2 Robust and Durable Virus-Specific Immune Responses in Mice and Rabbits and Completely
3 Protects Mice against Lethal Venezuelan, Western, and Eastern Equine Encephalitis Virus
4 Aerosol Challenges

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20 **ABSTRACT**

21 We previously demonstrated that a Venezuelan equine encephalitis virus (VEEV) DNA vaccine
22 that was optimized for increased antigen expression and delivered by intramuscular (IM)
23 electroporation (EP) elicits robust and durable virus-specific antibody responses in multiple
24 animal species and provides complete protection against VEEV aerosol challenge in mice and
25 nonhuman primates. Because our ultimate goal is to develop a single multi-agent vaccine
26 formulation that can elicit protection against VEEV, western equine encephalitis virus (WEEV),
27 and eastern equine encephalitis virus (EEEV), here we performed a comparative evaluation of
28 the immunogenicity and protective efficacy of individual optimized VEEV, WEEV, and EEEV
29 DNA vaccines with that of a 1:1:1 mixture of these vaccines, which we have termed the 3-EEV
30 DNA vaccine, when delivered by IM EP. The individual DNA vaccines and the 3-EEV DNA
31 vaccine elicited robust and durable virus-specific antibody responses in mice and rabbits and
32 completely protected mice from homologous VEEV, WEEV, and EEEV aerosol challenges. In
33 addition, these DNA vaccines provided protection in mice that was similar to that of the
34 respective live-attenuated VEEV vaccine and superior to that of the respective formalin-
35 inactivated WEEV and EEEV vaccines currently used in humans under Investigational New
36 Drug status. Taken together, the results from these studies demonstrate that the individual
37 VEEV, WEEV, and EEEV DNA vaccines and the 3-EEV DNA vaccine delivered by IM EP
38 provide an effective means of eliciting protection against lethal encephalitic alphavirus infections
39 in a murine model and represent viable next-generation vaccine candidates that warrant further
40 development.

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42

43 **IMPORTANCE**

44 Venezuelan equine encephalitis virus (VEEV), western equine encephalitis virus (WEEV), and
45 eastern equine encephalitis virus (EEEV) are recognized as significant biological defense threats.
46 There are currently no licensed human vaccines for these viruses, and existing investigational
47 live-attenuated and inactivated vaccine candidates suffer from issues of high reactogenicity or
48 suboptimal immunogenicity, respectively. In addition, there is evidence of immune inhibition
49 associated with simultaneous or serial administration of the VEEV, WEEV, and EEEV
50 investigational vaccines in humans. Consequently, alternative strategies for developing vaccines
51 that can safely and effectively protect humans against infections caused by these viruses are
52 needed. In this report, we have demonstrated that VEEV, WEEV and EEEV DNA vaccines that
53 were optimized for increased antigen expression elicit robust and durable virus-specific antibody
54 responses in mice and rabbits and completely protect mice from homologous VEEV, EEEV, and
55 WEEV aerosol challenge when delivered individually or in a multi-agent formulation by
56 intramuscular electroporation.

57

58 **INTRODUCTION**

59 Venezuelan equine encephalitis virus (VEEV), western equine encephalitis virus (WEEV), and
60 eastern equine encephalitis virus (EEEV) are non-segmented, positive-sense RNA viruses of the
61 genus *Alphavirus* in the family *Togaviridae* (1). Naturally transmitted by mosquitoes through
62 rodent or bird hosts, VEEV, WEEV, and EEEV are highly pathogenic for equines and humans
63 and have caused periodic epizootics throughout North, Central, and South America (2). Human
64 infection with these New World alphaviruses typically results in an acute, incapacitating disease
65 characterized by fever, headache, nausea, myalgia, and malaise (3). Severe neurological disease,

66 including fatal encephalitis, can also result from VEEV, WEEV, and EEEV infection of humans.
67 Although the human case-fatality rates associated with natural infection are estimated to be low
68 for VEEV (≤1%) and WEEV (3-15%), EEEV is the most severe of the arboviral encephalitides
69 with a human case-fatality rate estimated to be from 33% to as high as 75% (4-7). Moreover,
70 numerous documented laboratory accidents and the results of animal studies have demonstrated
71 that VEEV, WEEV, and EEEV are also highly infectious in aerosols, and infection with
72 aerosolized virus could potentially result in higher human mortality than that observed with
73 natural infection (8-10). In addition to producing incapacitating or lethal infections and being
74 infectious in aerosols, these encephalitic alphaviruses are also easily grown to high titers in
75 inexpensive and unsophisticated cell culture systems and are considerably stable (4).
76 Consequently, VEEV, WEEV, and EEEV represent significant potential biological defense
77 threats and are classified as Category B priority pathogens by both the Centers for Disease
78 Control and Prevention and the National Institute of Allergy and Infectious Diseases.
79 Although there are no licensed human vaccines for the encephalitic alphaviruses, live-
80 attenuated and formalin-inactivated vaccines are currently utilized under U.S. Food and Drug
81 Administration Investigational New Drug (IND) status to protect laboratory workers and other
82 at-risk personnel. The live-attenuated VEEV IND vaccine, TC-83, provides long-lasting
83 immunity and protection from both subcutaneous and aerosol VEEV challenges; however, it
84 causes significant adverse reactions in approximately 25% of recipients, and approximately 20%
85 of recipients fail to develop a detectable neutralizing antibody response (11, 12). The formalin-
86 inactivated VEEV IND vaccine derived from TC-83, C-84, and the formalin-inactivated WEEV
87 and EEEV IND vaccines are well tolerated, but they require frequent boosting to elicit and
88 maintain detectable neutralizing antibody responses in humans and have exhibited suboptimal

89 protection against aerosol viral challenge in animal studies (13-15). In addition, immune
90 interference has been documented when the VEEV, EEEV, and WEEV IND vaccines are
91 administered simultaneously or sequentially in humans (16-18). Due to the significant limitations
92 associated with these existing vaccine candidates, they are not being pursued for licensure. As a
93 result, development of improved vaccines that can safely and effectively protect humans against
94 encephalitic alphavirus infections is needed (19). Toward this goal, next-generation encephalitic
95 alphavirus vaccine candidates, including live-attenuated, inactivated, Sindbis virus-based
96 chimeric, virus replicon particle, virus-like particle, and DNA vaccines, are all currently at
97 various stages of development (20).

98 Vaccination with DNA plasmids that express protein antigens has numerous inherent
99 advantages as a platform for the development of next-generation vaccines. Foremost among the
100 benefits of this approach is that the endogenous expression of target antigens achieved with DNA
101 vaccination can elicit both cellular and humoral immune responses (21-24). Due to the lack of a
102 host immune response to the vector backbone, DNA vaccines also circumvent issues of pre-
103 existing or vaccine-induced vector-based immunity that can deleteriously affect vaccine
104 immunogenicity and safety (25, 26). From a logistical standpoint, DNA vaccines can be rapidly
105 developed and produced using well-established manufacturing procedures and without the need
106 to propagate a pathogen or inactivate an infectious organism. DNA vaccines can also be readily
107 formulated to generate multi-agent vaccines (27). Importantly, DNA vaccines have also
108 exhibited a favorable safety profile in numerous human clinical trials (28). Despite these
109 promising characteristics, the primary limitation of this approach has been suboptimal
110 immunogenicity in humans when administered by conventional injection. To address this, we
111 have pursued a range of strategies for enhancing the potency of encephalitic alphavirus DNA

112 vaccines to include investigation of alternative delivery methods and refinement of the coding
113 sequences for the target antigens.

114 In our previous studies, a DNA vaccine expressing the structural proteins (C-E3-E2-6K-
115 E1) of VEEV subtype IAB (strain Trinidad donkey) from the wild-type genes administered by
116 particle-mediated epidermal delivery (PMED) or "gene gun" elicited strong virus-specific
117 antibody responses in multiple animal species; however, the virus-neutralizing antibody
118 responses were low and only partial protection against homologous VEEV aerosol challenge was
119 observed in mice and nonhuman primates (NHPs) (29-31). We subsequently employed directed
120 molecular evolution or "gene shuffling" of VEEV, WEEV, and EEEV envelope glycoprotein
121 genes in an attempt to improve the neutralizing antibody response to VEEV, WEEV, and EEEV
122 DNA vaccines. Although DNA vaccines expressing certain variant envelope glycoproteins
123 elicited increased VEEV IAB-neutralizing antibody titers compared to the wild-type parental
124 VEEV DNA vaccine and provided improved protection against VEEV IAB aerosol challenge in
125 mice when delivered by PMED, these studies failed to identify variant envelope glycoprotein
126 DNA vaccines exhibiting increased immunogenicity against WEEV and EEEV as compared to
127 the wild-type parental WEEV and EEEV DNA vaccines (30).

128 More recently, we optimized the VEEV DNA vaccine for increased mammalian expression
129 of the structural proteins by adapting the gene sequence to reflect the codon bias of highly-
130 expressed *Homo sapiens* genes, adjusting regions of very high (>80%) or very low (<30%)
131 guanine-cytosine content, and avoiding cis-acting motifs that can negatively impact mRNA
132 expression or stability. Because earlier studies by others indicated that the capsid protein of
133 VEEV and EEEV can be cytotoxic and can inhibit cellular transcription and nuclear import and
134 export in vertebrate cells (32-35), we also eliminated the capsid gene from this construct. When

135 delivered by intramuscular (IM) electroporation (EP), the optimized VEEV DNA vaccine
136 elicited significantly improved virus-specific antibody responses, including increased levels of
137 virus-neutralizing antibodies, in multiple animal species and provided complete protective
138 immunity against homologous VEEV aerosol challenge in mice and NHPs (36). Consequently, a
139 Phase 1 clinical trial to evaluate the safety, tolerability, and immunogenicity of this VEEV DNA
140 vaccine candidate delivered by EP in humans was initiated.

141 The primary objective of the studies reported here was to apply this approach in an attempt
142 to develop fully-protective DNA vaccines for WEEV and EEEV. However, our ultimate goal is
143 to develop a single multi-agent vaccine formulation capable of eliciting protective immunity
144 against VEEV, WEEV, and EEEV. Therefore, we performed a comparative evaluation of the
145 immunogenicity and protective efficacy of the individual optimized VEEV, WEEV, and EEEV
146 DNA vaccines with that of a 1:1:1 mixture of these vaccines, which we have termed the 3-EEV
147 DNA vaccine, when delivered by IM EP in mice. To directly compare the results obtained for the
148 DNA vaccines with those achieved with the vaccines currently used to protect at-risk personnel,
149 mice vaccinated with the live-attenuated VEEV IND vaccine TC-83 or the formalin-inactivated
150 WEEV or EEEV IND vaccines were also included in these studies. We also assessed the virus-
151 neutralizing antibody responses elicited by the individual VEEV, WEEV, and EEEV and 3-EEV
152 DNA vaccines delivered by IM EP in rabbits.

153

154 **MATERIALS AND METHODS**

155 **Ethics statement.** All animal research was conducted in compliance with the Animal Welfare
156 Act and other federal statutes and regulations relating to animals and experiments involving
157 animals and adheres to principles stated in the "Guide for the Care and Use of Laboratory

158 Animals," Institute for Laboratory Animal Research, Division of Earth and Life Studies,
159 National Research Council, National Academies Press, Washington, D.C., 2011. The
160 USAMRIID facility where this animal research was conducted is fully accredited by the
161 Association for the Assessment and Accreditation of Laboratory Animal Care International.

162 **Vaccines.** Codon-optimized VEEV, WEEV, and EEEV structural genes were generated
163 by subjecting the wild-type 26S structural gene sequences minus the capsid protein coding
164 region (E3-E2-6K-E1) of VEEV IAB strain Trinidad donkey (Genbank accession number
165 L01442), WEEV strain CBA87 (Genbank accession number DQ432026), and EEEV strain
166 FL91-4679 (Genbank accession number AY705241) to the GeneOptimizer™ bioinformatic
167 algorithm for optimized expression in *Homo sapiens* followed by synthesis of the codon-
168 optimized genes (Geneart). VEEV, WEEV, and EEEV DNA vaccine plasmids were constructed
169 by inserting the synthesized codon-optimized genes into the *Not*I and *Bgl*II restriction sites of the
170 eukaryotic expression vector pWRG7077, which has been described previously (37). Endotoxin-
171 free, research-grade plasmids used in these studies were manufactured by Aldevron. The live-
172 attenuated VEEV vaccine TC-83 used in these studies was manufactured by Merrell National
173 Laboratories (NDBR 102, Lot 4 Run 3). The inactivated WEEV (TSI-GSD-210, Lot 2-1-91) and
174 EEEV (TSI-GSD-104, Lot 2-1-89) vaccines used in these studies were manufactured by the
175 Government Services Division of the Salk Institute.

176 **Animals, vaccinations, and blood collections.** Female BALB/c mice (6-8 weeks old,
177 Charles River Laboratories) and New Zealand White rabbits (3-3.5 kg, Charles River
178 Laboratories) were vaccinated with plasmid DNA diluted to the appropriate concentration as
179 described in the text and shown in the figures in calcium- and magnesium-free phosphate
180 buffered saline (Invitrogen, Catalog # 10010-023) by IM EP using the TriGrid™ Delivery

181 System (Ichor Medical Systems) as described previously (38). Briefly, mice anesthetized with
182 IM injection of a diluted acepromazine/ketamine/xylazine mixture or with isoflurane gas were
183 injected into one tibialis anterior muscle with 20 µl of DNA solution using a 3/10 ml U-100
184 insulin syringe (Becton-Dickinson, Catalog # 328431) inserted into the center of a TriGrid™
185 electrode array with 2.5 mm electrode spacing. Rabbits anesthetized with isoflurane gas were
186 injected into one quadriceps muscle with 0.5 ml of DNA solution using a 1 ml syringe (Becton-
187 Dickinson, Catalog # 309602) inserted into the center of a TriGrid™ electrode array with 6.0
188 mm electrode spacing. Injection of DNA was followed immediately by electrical stimulation at
189 amplitude of 250 V/cm, and the total duration was 40 ms over a 400 ms interval. The live-
190 attenuated VEEV vaccine TC-83 and inactivated WEEV and EEEV vaccines were delivered to
191 mice as 0.5 ml doses by subcutaneous injection. At various times after vaccination as described
192 in the text and shown in the figures, blood samples were collected from anesthetized mice by
193 retro-orbital or submandibular vein bleed and from anesthetized rabbits by central auricular
194 artery bleed, and serum was recovered by centrifugation.

195 **ELISA assays.** Total IgG anti-VEEV, WEEV or EEEV antibody titers were determined
196 for serum samples by indirect enzyme-linked immunosorbent assay (ELISA) using sucrose-
197 purified, irradiated whole VEEV IAB strain Trinidad donkey, WEEV strain CBA87, or EEEV
198 strain FL91-4679 antigen as described previously (39). Briefly, twofold serial dilutions of sera
199 starting at 1:100 were incubated with 250 ng per well of antigen in 96-well plates. Horseradish
200 peroxidase (HRP)-conjugated anti-mouse IgG antibodies (Sigma-Aldrich, Catalog # A3673) and
201 ABTS peroxidase substrate (KPL, Catalog # 50-62-01) were used for detection. For antibody
202 isotype ELISA, HRP-conjugated anti-mouse IgG1 and anti-mouse IgG2a secondary antibodies
203 (Bethyl Laboratories, Catalog # A90-105P and A90-107P, respectively) were used. The optical

204 density at 405 nm was determined using a SpectraMax M2e microplate reader (Molecular
205 Devices) and the endpoint titers were calculated using a 4-parameter logistic curve fit in Softmax
206 Pro v5 (Molecular Devices).

207 **PRNT assays.** Virus-neutralizing antibody titers against VEEV subtypes IAB (strain
208 Trinidad donkey), IC (strain 6119), ID (strain 3880), and IE (strain 68U201) and MUCV, WEEV
209 (strain CBA87), and EEEV (strain FL91-4679) were determined for serum samples by plaque
210 reduction neutralization test (PRNT) as described previously (39). Briefly, twofold serial
211 dilutions of sera starting at 1:20 were mixed with equal volumes of medium containing ~200
212 PFU of virus and incubated for 24 h at 4°C. The virus/antibody mixtures were then used to infect
213 confluent monolayers of Vero cells contained in six-well plates for 1 h at 37°C after which an
214 overlay consisting of 0.6% agar (Genemate, Catalog # E-3121-125) in complete Eagle's Basal
215 Medium with Earle's salts (EBME) without phenol red (Invitrogen, Catalog # A15950DK) was
216 added. The plates were stained 24 h later by the addition of an overlay containing 5% neutral red
217 (Gibco, Catalog # 02-0066DG) and 0.6% agar in complete EBME without phenol red, and the
218 plaques were counted 24 h after staining. The neutralizing antibody titers were then calculated as
219 a reciprocal of the highest dilution resulting in an 80% reduction of the plaque number as
220 compared to virus-only control wells.

221 **ELISpot assays.** Anti-VEEV cellular immune responses were analyzed by IFN-γ
222 enzyme-linked immunospot (ELISpot) assay using standard methods as described previously
223 (40). Briefly, splenocytes isolated from individual spleens obtained from vaccinated mice using
224 BD Falcon 100 μM nylon cell strainers (Corning, Catalog # 352360) were resuspended in
225 complete RPMI 1640 medium (Mediatech, Catalog # 10-040-CV). The resuspended splenocytes
226 from each spleen were then added at a concentration of 2×10^5 cells per well to triplicate wells

227 of MultiScreen$_{HTS}$ IP 0.45 µm PVDF filter 96-well plates (Millipore, Catalog # MSIPS4W10)
228 previously coated with mouse IFN-γ ELISpot capture antibody (Becton-Dickinson, Catalog # 51-
229 2003KZ). The splenocytes were then cultured with no peptide, 10 µg/ml of Concanavalin A
230 (Sigma-Aldrich, Catalog # C-5275), 20 µg/ml of β-galactosidase peptide TPHPARIGL (New
231 England Peptide), or 10 µg/ml of pooled 15-mer peptides with an 11-base overlap spanning the
232 VEEV IAB E2 or E1 envelope glycoprotein (Pepscan) for 24 h at 37°C with 5% CO_2. Secreted
233 IFN-γ was detected by aspirating the cell suspension and successively incubating the plate for 2 h
234 at room temperature with mouse IFN-γ ELISpot detection antibody (Becton-Dickinson, Catalog
235 # 552569b), for 1 h at room temperature with streptavidin-HRP (Becton-Dickinson, Catalog
236 #552569c), and for 20 min at room temperature with 3-amino-9-ethylcarbazole (AEC) substrate
237 (Becton-Dickinson, Catalog # 552569e). The substrate reaction was then stopped by washing the
238 plates with deionized H_2O, the plates were dried for 2 h at room temperature, and the spots were
239 enumerated.

240 **Aerosol challenge of mice.** Mice were placed into a Class III biological safety cabinet
241 located inside a biosafety level 3 containment suite and exposed in a whole-body aerosol
242 chamber to a VEEV, WEEV, or EEEV aerosol created by a Collison nebulizer for 10 min as
243 previously described (41). Sucrose-purified VEEV IAB strain Trinidad donkey, WEEV strain
244 CBA87, or EEEV strain FL91-4679 was diluted to an appropriate starting concentration in
245 Hank's Balanced Salt Solution (Gibco, Catalog # 14175-095) containing 1% fetal bovine serum
246 (Thermo Scientific, Catalog # SH30071.03) for use in aerosol generation. Samples collected
247 from the all-glass impinger attached to the aerosol chamber were analyzed by plaque assay on
248 Vero cells using standard methods as previously described to determine the inhaled dose of
249 VEEV, WEEV, or EEEV (42). The mice were monitored twice daily for clinical signs of illness

250 and death for 28 days post-challenge, and any animals found to be moribund were euthanized.
251 After the post-challenge observation period was completed, the protection data was used to
252 generate Kaplan-Meier survival curves.

253 **Statistical methods.** Log_{10} transformations were applied to whole-virus ELISA titers and
254 $PRNT_{80}$ titers for analyses. Mixed model analysis of variance (ANOVA) with post-hoc Tukey's
255 tests was used for pairwise comparisons of ELISA and $PRNT_{80}$ titers and ELISpot counts with
256 the same stimulation condition between groups at each time point. Paired t-tests were used to
257 compare ELISA and $PRNT_{80}$ titers and ELISpot counts for different stimulation conditions
258 within groups. Kaplan-Meier survival analysis and log-rank tests with stepdown Sidak
259 adjustment was used for comparison of survival curves between groups. Fisher's exact tests with
260 stepdown bootstrap adjustment were used to compare survival rates between groups. T-tests with
261 stepdown bootstrap adjustment were used to compare mean times-to-death between groups. The
262 effects of ELISA and $PRNT_{80}$ titers on the probability of survival were assessed using a
263 backwards-selection logistic regression model. Analyses were conducted using SAS v9.2 (SAS
264 Institute). Statistical significance was defined as $p < 0.05$ in all tests.

265

266 RESULTS

267 **VEEV-specific antibody responses of vaccinated mice.** To first compare the immunogenicity
268 and protective efficacy of the individual optimized VEEV DNA vaccine to that of a 1:1:1
269 mixture of the optimized VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine),
270 female BALB/c mice (n = 10 per group) were vaccinated on days 0 and 21 with 5 µg of the
271 VEEV plasmid or with 5 µg of each of the VEEV, WEEV, and EEEV plasmids (15 µg total) by
272 IM EP. Negative control mice (n = 10) were vaccinated on days 0 and 21 with 5 µg of the empty

273 vector plasmid by IM EP. To allow comparison to the live-attenuated VEEV IND vaccine, mice
274 (n = 10) received a single administration of the human dose of 0.5 ml of TC-83 (1×10^4 PFU) by
275 subcutaneous injection on day 0. Serum samples obtained on days 21 and 42 were assayed for
276 total IgG anti-VEEV antibodies by ELISA and for VEEV-neutralizing antibodies by PRNT.

277 Mice vaccinated with either the VEEV DNA or 3-EEV DNA developed a mean ELISA
278 titer that was significantly above background after a single vaccination ($p < 0.0001$) and that was
279 significantly boosted with a second vaccination ($p < 0.0001$) (Fig. 1A). In addition, the mean
280 titers of mice vaccinated with the VEEV DNA or the 3-EEV DNA were not significantly
281 different from one another on day 21 ($p = 0.7702$) or 42 ($p = 0.7328$). Although the day 21 mean
282 titer of mice that received TC-83 trended higher than that of mice that received the VEEV DNA
283 vaccine, the difference was not significant ($p = 0.1258$). By day 42, the mean titer of mice that
284 received a second dose of the VEEV DNA was significantly higher than that of mice that
285 received the single dose of TC-83 ($p = 0.0112$). Although the day 21 mean titer of mice
286 vaccinated with the 3-EEV DNA was significantly lower than that of mice vaccinated with TC-
287 83 ($p < 0.0111$), there was no significant difference between the day 42 mean titers of these
288 groups ($p = 0.1456$).

289 Mice vaccinated with the VEEV DNA developed a mean $PRNT_{80}$ titer that was
290 significantly above background on day 21 ($p = 0.0260$) (Fig. 1B). In contrast, the day 21 mean
291 titers of mice that received the 3-EEV DNA vaccine or the empty vector DNA were not
292 significantly different ($p = 0.9768$). Within groups vaccinated with either the VEEV DNA or 3-
293 EEV DNA, the mean titer was significantly higher on day 42 as compared to that on day 21 ($p <$
294 0.0001). Although the mean titers of mice that received the VEEV DNA or 3-EEV DNA were
295 not significantly different from one another on day 21 ($p = 0.0723$), the day 42 mean titer of mice

296 that received the VEEV DNA was significantly higher than that of mice that received the 3-EEV
297 DNA ($p = 0.0106$). In addition, although the mean titer of mice vaccinated with TC-83 was
298 significantly higher than that of mice vaccinated with the VEEV DNA ($p < 0.0007$) or the 3-EEV
299 DNA ($p < 0.0001$) on day 21, there was no significant difference between the day 42 mean titer
300 of mice vaccinated with TC-83 as compared to that of mice vaccinated with the VEEV DNA (p
301 = 0.5403) or 3-EEV DNA ($p = 0.2782$).

302 **VEEV aerosol challenge of vaccinated mice.** To compare the protective efficacy of these
303 vaccines, the mice from all groups were challenged on day 49 with 1×10^4 PFU (~10,000
304 median lethal doses [LD_{50}]) of VEEV IAB strain Trinidad donkey by the aerosol route. Negative
305 control mice that received the empty vector DNA all displayed clinical signs of disease including
306 ruffled fur, weight loss, inactivity, hunched posture, and hind limb paralysis, and all died or were
307 found morbid and were euthanized by day 9 post-challenge (Fig. 2). In contrast, mice vaccinated
308 with the VEEV DNA or 3-EEV DNA displayed no clinical signs of disease post-challenge and
309 all survived. Consistent with our previous results (30, 36), 90% of mice vaccinated with TC-83
310 displayed no clinical signs of disease post-challenge and survived, and the single mouse from
311 this group that did not survive the challenge had no detectable VEEV-specific antibody response
312 after vaccination. The survival of the VEEV DNA, 3-EEV DNA, and TC-83 groups was
313 significantly higher than that of the empty vector DNA group with respect to survival rate (p <
314 0.0001) and the survival curve ($p = 0.0003$).

315 **VEEV-specific cellular immune responses of vaccinated mice.** Previously, we showed
316 that delivery of the optimized VEEV DNA vaccine by IM EP was predicted to result in a
317 balanced type 1 helper T cell (Th1)/type 2 helper T cell (Th2) immune response in mice based on
318 IgG antibody isotype as determined by ELISA, and cellular immune responses directed against

319 the VEEV E2 and E1 proteins were detected by INFγ-ELISpot assay (36). To compare the
320 cellular responses elicited by the VEEV DNA vaccine and the 3-EEV DNA vaccine, female
321 BALB/c mice ($n = 6$ per group) were vaccinated on days 0 and 21 with 5 µg of empty vector
322 plasmid, 5 µg of the VEEV plasmid, or 5 µg of each of the VEEV**,** WEEV, and EEEV plasmids
323 (15 µg total) delivered by IM EP. On day 35, splenocytes isolated from the vaccinated mice were
324 restimulated with Concanavalin A, no peptide, an irrelevant β-galactosidase peptide, or pools of
325 overlapping peptides spanning the VEEV IAB strain Trinidad donkey E2 or E1 envelope
326 glycoproteins and analyzed by IFN-γ ELISpot. After restimulation with Concanavalin A,
327 splenocytes from mice from all groups produced spots that were too numerous to count (data not
328 shown). Splenocytes restimulated with no peptide ($p \geq 0.5964$) or with the β-galactosidase
329 peptide ($p \geq 0.1515$) failed to produce significant responses in this assay. After restimulation
330 with the VEEV E2 or E1 peptide pools, splenocytes obtained from mice vaccinated with the
331 VEEV DNA ($p < 0.0001$) or 3-EEV DNA ($p \leq 0.0010$) produced mean IFN-γ responses that
332 were significantly above background (Fig. 3). However, the mean IFN-γ responses of mice
333 receiving the VEEV DNA were significantly higher than those of mice receiving the 3-EEV
334 DNA against the E2 ($p = 0.0218$) and E1 ($p = 0.0180$) peptide pools. Consistent with our
335 previous results, the mean IFN-γ responses of splenocytes restimulated with the E2 peptides
336 were significantly higher than those restimulated with the E1 peptides for both the VEEV DNA
337 ($p = 0.0142$) and 3-EEV DNA ($p = 0.0010$) groups.

338 **WEEV-specific antibody responses of vaccinated mice.** To perform a comparative
339 evaluation of the immunogenicity and protective efficacy of the individual optimized WEEV
340 DNA and 3-EEV DNA vaccines, female BALB/c mice ($n = 10$ per group) were vaccinated on
341 days 0 and 21 with 5 µg of the WEEV plasmid or with 5 µg of each of the VEEV, WEEV, and

342 EEEV plasmids (15 µg total) by IM EP. Negative control mice (n = 10) were vaccinated on days
343 0 and 21 with 5 µg of the empty vector plasmid by IM EP. To allow comparison to the formalin-
344 inactivated WEEV IND vaccine, mice (n = 10) were vaccinated on days 0 and 21 with the human
345 dose of 0.5 ml of this vaccine by subcutaneous injection. Serum samples obtained on days 21 and
346 42 were assayed for total IgG anti-WEEV antibodies by ELISA and for WEEV-neutralizing
347 antibodies by PRNT.

348 Mice that received the WEEV DNA vaccine, 3-EEV DNA vaccine, or WEEV IND vaccine
349 developed mean ELISA titers that were significantly above background after a single vaccination
350 ($p < 0.0001$) and that were significantly boosted with a second vaccination ($p \leq 0.0007$) (Fig.
351 4A). The mean titers of mice vaccinated with the WEEV DNA or 3-EEV DNA were not
352 significantly different from one another on day 21 ($p = 0.1435$) or 42 ($p = 0.4116$). In addition,
353 the mean titers of mice vaccinated with the WEEV DNA or 3-EEV DNA were statistically
354 higher than that of mice receiving the WEEV IND vaccine at day 21 ($p \leq 0.0004$) and 42 ($p <$
355 0.0001). Because we lacked the WEEV E2 and E1 peptides necessary to perform an IFN-γ
356 ELISpot assay as done for VEEV, we indirectly measured the potential for these WEEV
357 vaccines to elicit cell-mediated immune responses by determining the IgG1 and IgG2a subtype
358 anti-WEEV antibody titers by ELISA using pooled day 42 sera from each group. This analysis
359 revealed that mice receiving the WEEV DNA, 3-EEV DNA, or WEEV IND vaccine would be
360 predicted to have similarly balanced Th1/Th2 immune responses based on the ratio of IgG2a to
361 IgG1 titers (Fig. 4B).

362 Mice vaccinated with the WEEV DNA developed a mean $PRNT_{80}$ titer that was
363 significantly above background after a single vaccination ($p < 0.0001$) and that was significantly
364 boosted with a second vaccination ($p = 0.0011$) (Fig. 4C). In contrast, although mice that

365 received a single vaccination with the 3-EEV DNA did not develop a mean titer that was
366 significantly above background ($p = 0.4304$), the mean titer of these mice was significantly
367 boosted ($p = 0.0004$) and was significantly above background after a second vaccination ($p <$
368 0.0001). Although the mean titer of mice that received the WEEV IND vaccine was significantly
369 above background after a single vaccination ($p < 0.0001$), the mean titer was not significantly
370 boosted with a second vaccination ($p = 0.0596$). In comparing the mean titers between groups,
371 the titers of mice that received the WEEV DNA or WEEV IND vaccine were not significantly
372 different on day 21 ($p = 0.8361$) or 42 ($p = 0.1557$). However, the mean titer of mice that
373 received the 3-EEV DNA vaccine was significantly lower than those of mice that received the
374 WEEV DNA or WEEV IND vaccine at day 21 ($p < 0.0001$) and 42 ($p \leq 0.0004$).

375 **WEEV aerosol challenge of vaccinated mice.** To perform a comparative evaluation of
376 the protective efficacy of these vaccines, the mice from all groups were challenged on day 49
377 with 2×10^4 PFU (~500 LD_{50}) of WEEV strain CBA87 by the aerosol route. Negative control
378 mice that received the empty vector DNA all displayed clinical signs of disease including ruffled
379 fur, weight loss, inactivity, hunched posture, and hind limb paralysis and all died or were found
380 morbid and were euthanized by day 7 post-challenge (Fig. 5). In contrast, mice vaccinated with
381 the WEEV DNA or 3-EEV DNA displayed no clinical signs of disease post-challenge and all
382 survived. Consistent with our previous unpublished results, only 30% of the mice that received
383 the WEEV IND vaccine survived the challenge. The survival of the WEEV DNA and 3-EEV
384 DNA groups was significantly higher than that of the WEEV IND group with respect to the
385 survival rate ($p = 0.0030$) and survival curve ($p = 0.0056$). In addition, the survival of the empty
386 vector DNA and WEEV IND groups were not significantly different with respect to the survival
387 rate ($p = 0.2101$), mean time-to-death ($p = 0.8420$), and survival curve ($p = 0.2856$).

388 **EEEV-specific antibody responses of vaccinated mice.** We also completed a
389 comparative evaluation of the immunogenicity and protective efficacy of the individual
390 optimized EEEV DNA and 3-EEV DNA vaccines delivered by IM EP in mice. In our
391 unpublished studies, it has proven difficult to elicit protective immunity in mice against EEEV
392 aerosol challenge. Consequently, for this study, we vaccinated female BALB/c mice (n = 10 per
393 group) three times, instead of twice, on days 0, 21, and 42 with 5 µg of the EEEV plasmid or
394 with 5 µg of each of the VEEV, WEEV, and EEEV plasmids (15 µg total) by IM EP. Negative
395 control mice (n = 10) were vaccinated on days 0, 21, and 42 with 5 µg of the empty vector
396 plasmid by IM EP. To allow comparison to the formalin-inactivated EEEV IND vaccine, mice (n
397 = 10) were vaccinated on days 0, 21, and 42 with the human dose of 0.5 ml of this vaccine by
398 subcutaneous injection. Serum samples obtained on days 21, 42, and 63 were assayed for total
399 IgG anti-EEEV antibodies by ELISA and for EEEV-neutralizing antibodies by PRNT.

400 Mice that received the EEEV DNA vaccine, 3-EEV DNA vaccine, or EEEV IND vaccine
401 developed mean ELISA titers that were significantly above background after a single vaccination
402 ($p < 0.0001$) and that were significantly boosted with a second vaccination ($p \leq 0.0040$) (Fig.
403 6A). While the mean titer of mice vaccinated with the EEEV DNA was not significantly boosted
404 with a third vaccination ($p = 0.0508$), those of mice that received the 3-EEV DNA or EEEV IND
405 vaccine were significantly higher on day 63 as compared to day 42 ($p \leq 0.0432$). In comparing
406 the mean titers between groups, the titers of mice vaccinated with the EEEV DNA or 3-EEV
407 DNA were not significantly different from one another on day 21 ($p = 0.9280$), 42 ($p = 0.7396$),
408 or 63 ($p = 0.1267$). In addition, the mean titers of mice vaccinated with the EEEV DNA or 3-
409 EEV DNA were significantly higher than that of mice receiving the EEEV IND vaccine on day
410 21 ($p \leq 0.0021$), 42 ($p < 0.0001$), and 63 ($p < 0.0001$). Because we lacked the EEEV E2 and E1

411 peptides necessary to perform an IFN-γ ELISpot assay as done for VEEV, we indirectly
412 measured the potential for these EEEV vaccines to elicit cell-mediated immune responses by
413 determining the IgG1 and IgG2a subtype anti-EEEV antibody titers by ELISA using pooled day
414 63 sera from each group. This analysis revealed that although mice vaccinated with the EEEV
415 DNA or 3-EEV DNA would be predicted to have balanced Th1/Th2 immune responses based on
416 the ratio of IgG2a to IgG1 titers, mice receiving the EEEV IND vaccine would be predicted to
417 have an immune response that is more skewed toward a Th2 response (Fig. 6B).

418 Mice that received the EEEV DNA vaccine developed a mean $PRNT_{80}$ titer that was
419 significantly above background after a single vaccination ($p = 0.0030$) and significantly boosted
420 with a second vaccination ($p < 0.0001$), but not significantly boosted with a third vaccination (p
421 $= 0.4473$) (Fig. 6C). Although the mean titers of mice that received the 3-EEV DNA or EEEV
422 IND vaccine were not significantly above background after a single vaccination ($p \geq 0.0538$),
423 they were significantly boosted ($p \leq 0.0002$) and significantly above background after a second
424 vaccination ($p < 0.0001$). The mean titers of the 3-EEV DNA or EEEV IND vaccine groups were
425 also significantly boosted with a third vaccination ($p \leq 0.0310$). In comparing the mean titers
426 between groups, the titers of mice vaccinated with the EEEV DNA or 3-EEV DNA were not
427 significantly different from one another on day 21 ($p = 0.0533$) and 63 ($p = 0.5463$), while the
428 day 42 titer of the EEEV DNA group was significantly higher than that of the 3-EEV DNA
429 group ($p = 0.0346$). In addition, the mean titer of mice that received the EEEV IND vaccine was
430 not significantly different from those of mice vaccinated with the EEEV DNA or 3-EEV DNA at
431 day 21 ($p \geq 0.4041$), 42 ($p \geq 0.0927$), or 63 ($p \geq 0.2960$).

432 **EEEV aerosol challenge of vaccinated mice.** To perform a comparative evaluation of the
433 protective efficacy of these vaccines, the mice from all groups were challenged on day 70 with 1

434 $\times 10^5$ PFU (~3,000 LD_{50}) of EEEV strain FL91-4679 by the aerosol route. Negative control mice
435 that received the empty vector DNA all displayed clinical signs of disease including ruffled fur,
436 weight loss, inactivity, hunched posture, and hind limb paralysis, and all died or were found
437 morbid and were euthanized by day 5 post-challenge (Fig. 7). In contrast, mice vaccinated with
438 the EEEV DNA or 3-EEV DNA displayed no clinical signs of disease post-challenge and all
439 survived. Consistent with our previous unpublished results, only 40% of the mice that received
440 the EEEV IND vaccine survived the challenge. The survival rates of the EEEV DNA and 3-EEV
441 DNA groups were significantly higher than that of the EEEV IND group ($p = 0.0329$). Although
442 the survival rates of mice receiving the EEEV IND group and the empty vector DNA group were
443 not statistically different ($p = 0.3025$), the survival of the EEEV IND group was significantly
444 enhanced relative to that of the empty vector DNA group with respect to the mean time-to-death
445 ($p = 0.0452$) and the survival curve ($p = 0.0066$). Of note, mice that received only two
446 vaccinations with the EEEV DNA vaccine were also completely protected from challenge (data
447 not shown).

448 **Virus-specific antibody responses of vaccinated rabbits.** To perform a comparative
449 evaluation of the immunogenicity of the individual optimized VEEV, WEEV, and EEEV DNA
450 vaccines and the 3-EEV DNA vaccine in an additional animal model that permits administration
451 of higher DNA doses that are more similar to those expected to be delivered to humans and is
452 better-suited to assessment of antibody durability, we also completed a study in rabbits. New
453 Zealand White rabbits ($n = 5$ per group) were vaccinated on days 0, 28, and 230 with 0.5 mg of
454 the VEEV, WEEV, or EEEV plasmid or with 0.5 mg each of the VEEV, WEEV, and EEEV
455 DNA plasmids (1.5 mg total) delivered by IM EP. Serum samples obtained on days 27, 42, 230,
456 266 and 349 were assayed for neutralizing antibodies against VEEV, WEEV, or EEEV by

457 PRNT.

458 Rabbits that received the VEEV DNA vaccine or 3-EEV DNA vaccine developed mean
459 $PRNT_{80}$ titers against VEEV that were significantly above background after a single vaccination
460 ($p < 0.0001$) and significantly boosted with a second vaccination ($p < 0.0001$) (Fig. 8A). While
461 the day 230 mean titer of rabbits vaccinated with the VEEV DNA was significantly lower than
462 that on day 42 ($p = 0.0004$), there was no significant difference in the day 42 and day 230 mean
463 titers for rabbits vaccinated with the 3-EEV DNA ($p = 0.2827$). The mean titer of rabbits that
464 received the VEEV DNA was also significantly boosted with the long-range boosting
465 vaccination performed on day 230 ($p = 0.0133$). Although the long-range boosting vaccination
466 increased the mean $\log_{10}$ titer of rabbits that received the 3-EEV DNA from 2.80 on day 230 to
467 2.97 on day 266, this increase was not statistically significant ($p > 0.9999$). In addition, there was
468 no significant difference in the day 266 and day 349 mean titers of rabbits vaccinated with the
469 VEEV DNA or 3-EEV DNA within these groups ($p > 0.9999$). In comparing the mean titers
470 between groups, there was no significant difference in the titers of rabbits vaccinated with the
471 VEEV DNA or 3-EEV DNA at day 27 ($p = 0.523$), 42 ($p = 0.3935$), and 230 ($p > 0.9999$).
472 However, after the long-range boosting vaccination, the mean titers of rabbits that received the
473 VEEV DNA vaccine were significantly higher than those of rabbits that received the 3-EEV
474 DNA vaccine at day 266 ($p = 0.0252$) and 349 ($p = 0.0464$).

475 To assess the potential for the subtype IAB-based VEEV DNA vaccine to provide
476 protection against heterologous VEEV strains, we measured the neutralizing activity of the day
477 42 samples from rabbits vaccinated with the VEEV DNA or 3-EEV DNA against VEEV
478 subtypes IC, ID, and IE and MUCV (formerly VEEV IIIA). Within groups receiving the VEEV
479 DNA or 3-EEV DNA, there was no significant difference in the mean $PRNT_{80}$ titers against

480 VEEV subtypes IAB, IC, ID or IE or MUCV ($p \geq 0.0587$) (Fig. 8B). In comparing the mean
481 titers between groups, there was no significant difference in the titers of rabbits vaccinated with
482 the VEEV DNA or 3-EEV DNA against VEEV subtypes IAB, IC, ID, or IE or MUCV ($p \geq$
483 0.2802).

484 Rabbits that received the WEEV DNA vaccine or 3-EEV DNA vaccine developed mean
485 $PRNT_{80}$ titers against WEEV that were significantly above background after a single vaccination
486 ($p < 0.0001$) (Fig. 8C). Although the mean titer of rabbits vaccinated with the WEEV DNA was
487 significantly boosted with a second vaccination ($p = 0.005$), there was no significant difference
488 in the day 27 and day 42 mean titers of rabbits vaccinated with the 3-EEV DNA ($p = 0.394$).
489 There was also no significant difference in the day 42 and day 230 mean titers for rabbits
490 vaccinated with the WEEV DNA ($p = 0.7824$) or 3-EEV DNA ($p = 0.9976$). Although the long-
491 range boosting vaccination increased the mean log_{10} titer from 3.10 on day 230 to 3.93 on day
492 266 for rabbits receiving the WEEV DNA and from 2.53 on day 230 to 3.50 on day 266 for
493 rabbits receiving the 3-EEV DNA, these increases were not statistically significant ($p \geq 0.1551$).
494 In addition, there was no significant difference in the day 266 and day 349 mean titers of rabbits
495 vaccinated with the WEEV DNA or 3-EEV DNA within these groups ($p \geq 0.9917$). In
496 comparing the mean titers between groups, there was no significant difference in the titers of
497 rabbits vaccinated with the WEEV DNA or 3-EEV DNA at any of the time points ($p \geq 0.3404$).

498 Rabbits that received the EEEV DNA vaccine or 3-EEV DNA vaccine developed mean
499 $PRNT_{80}$ titers against EEEV that were significantly above background after a single vaccination
500 ($p \leq 0.0013$) (Fig. 8D). Although the mean titer of rabbits vaccinated with the EEEV DNA was
501 significantly boosted with a second vaccination ($p = 0.048$), there was no significant difference
502 in the mean titers at day 27 and day 42 for rabbits vaccinated with the 3-EEV DNA ($p = 0.135$).

503 There was also no significant difference in the day 42 and day 230 mean titers for rabbits
504 vaccinated with the EEEV DNA ($p = 0.4883$) or 3-EEV DNA ($p = 0.3987$). Although the long-
505 range boosting vaccination increased the mean $\log_{10}$ titer from 2.67 on day 230 to 3.18 on day
506 266 for rabbits receiving the EEEV DNA and from 1.94 on day 230 to 2.14 on day 266 for
507 rabbits receiving the 3-EEV DNA, these increases were not statistically significant ($p \geq 0.9108$).
508 In addition, there was no significant difference in the day 266 and day 349 mean titers of rabbits
509 vaccinated with the EEEV DNA or 3-EEV DNA within these groups ($p > 0.9999$). In comparing
510 the mean titers between groups, there was no significant difference in the titers of rabbits
511 vaccinated with the EEEV DNA or 3-EEV DNA at any of the time points ($p \geq 0.1383$).
512

513 DISCUSSION

514 The results of our previous studies demonstrated that a strategy that encompassed optimization
515 of the construct for increased antigen expression and EP-based delivery successfully improved
516 the immunogenicity and protective efficacy of a VEEV DNA vaccine (36). Consistent with those
517 results, mice that received two doses of the optimized VEEV DNA vaccine delivered by IM EP
518 in the present studies developed robust virus-specific total IgG and virus-neutralizing antibody
519 responses. Comparative evaluation against mice that received a single vaccination with a human
520 dose of the live-attenuated VEEV IND vaccine TC-83 revealed that the virus-specific total IgG
521 titers elicited by the VEEV DNA vaccine were significantly higher than those observed for TC-
522 83, while the virus-neutralizing antibody responses were similar between these two vaccination
523 regimens. Also consistent with our previous results, mice that received the VEEV DNA vaccine
524 were completely protected against lethal VEEV aerosol challenge, whereas 90% of mice
525 receiving TC-83 were protected. In a similar manner, mice that received the optimized WEEV or

526 EEEV DNA vaccine delivered by IM EP developed robust virus-specific total IgG and virus-
527 neutralizing antibody responses. Comparative evaluation against mice that received the same
528 number of vaccinations with human doses of the formalin-inactivated WEEV or EEEV IND
529 vaccine revealed that the virus-specific total IgG titers elicited by the WEEV or EEEV DNA
530 vaccine were significantly higher than those observed for the respective WEEV or EEEV IND
531 vaccine, while the virus-neutralizing antibody responses were similar between these vaccination
532 regimens. Mice that received the WEEV or EEEV DNA vaccine were also completely protected
533 from lethal homologous WEEV or EEEV aerosol challenge and exhibited significantly higher
534 survival rates than mice that received the WEEV or EEEV IND vaccine, which only protected
535 30% and 40% of vaccinated mice, respectively. These results demonstrate that this vaccination
536 strategy was also successful in developing protective DNA vaccines for WEEV and EEEV that
537 provide significantly increased protection against lethal viral aerosol challenge in mice compared
538 to the formalin-inactivated IND vaccines.

539 In the present studies, we also evaluated whether the optimized VEEV, WEEV, and EEEV
540 DNA vaccines could be administered in a multi-agent formulation without a significant reduction
541 in immunogenicity or protective efficacy compared to the individual DNA vaccines. While the
542 virus-specific total IgG antibody titers of mice that received the individual VEEV, WEEV, or
543 EEEV DNA vaccine were similar to those of mice that received the 3-EEV DNA vaccine, the
544 virus-neutralizing antibody titers were significantly lower in mice that received the 3-EEV DNA
545 vaccine compared to those that received the individual VEEV or WEEV DNA vaccine. Despite
546 the putative immune interference that we observed, it is encouraging that all of the mice that
547 received the 3-EEV DNA vaccine had detectable neutralizing antibody responses against VEEV,
548 WEEV, and EEEV and were completely protected against lethal VEEV, WEEV, and EEEV

549 aerosol challenge. As observed for the individual VEEV, WEEV and EEEV DNA vaccines, the
550 3-EEV DNA vaccine also provided similar levels of protection against lethal VEEV aerosol
551 challenge as compared to TC-83 and significantly increased protection against lethal WEEV and
552 EEEV aerosol challenge as compared to the formalin-inactivated WEEV and EEEV IND
553 vaccines in mice. Furthermore, there was no significant difference in the neutralizing antibody
554 responses against VEEV, WEEV, and EEEV elicited by the individual DNA vaccines or 3-EEV
555 DNA vaccine after the initial vaccination series in rabbits. These results provide important
556 preliminary evidence to support the potential use of the 3-EEV DNA as a single multi-agent
557 vaccine formulation capable of eliciting protective immunity against VEEV, WEEV, and EEEV.

558 Of note, there have been previous published reports on the evaluation of WEEV DNA
559 vaccines in mice. In one report, a DNA vaccine expressing the structural proteins (C-E3-E2-6K-
560 E1) of WEEV strain 71V-1658 from the wild-type genes administered in four 5 μg doses by
561 PMED provided complete protection against homologous intranasal challenge with 1.5×10^3
562 PFU (25 LD_{50}) of virus (43). However, this vaccine provided only partial protection against
563 similar challenges with the heterologous WEEV strains CBA87 and Fleming. Although cell-
564 mediated immune responses against the E2 and E1 antigens were elicited by this DNA vaccine
565 as measured by lymphocyte proliferation assays, no virus-specific antibody responses were
566 detected by ELISA. In a subsequent report by this group, DNA vaccines expressing the C-E3-
567 E2-6K-E1, E3-E2-6K-E1, or 6K-E1 proteins of WEEV strain 71V-1658 from the wild-type
568 genes administered in three 2 μg doses by PMED provided complete protection against
569 homologous intranasal challenge with the same 1.5×10^3 PFU (25 LD_{50}) dose of virus, while a
570 DNA vaccine expressing the E3-E2 proteins did not provide any protection (44). Although the
571 DNA vaccines expressing the C-E3-E2-6K-E1, E3-E2-6K-E1, or 6K-E1 proteins provided

572 significant protection against a similar challenge with the CBA87 strain, only the DNA vaccines
573 expressing the C-E3-E2-6K-E1 and E3-E2-6K-E1 proteins provided significant protection
574 against the Fleming strain. In addition, the DNA vaccine expressing the E3-E2-6K-E1 proteins
575 provided better protection against this strain than the DNA vaccine expressing C-E3-E2-6K-E1.
576 In our studies, we showed that two administrations of a 5 μg dose of a DNA vaccine expressing
577 E3-E2-6K-E1 proteins of WEEV CBA87 from codon-optimized genes delivered by IM EP
578 provided complete protection against aerosol challenge with 2×10^4 PFU (~500 LD_{50}) of
579 homologous virus. Taken together, the described results of the studies previously performed by
580 others and of those reported here support the use of E3-E2-6K-E1 as the most appropriate target
581 antigens for a successful DNA vaccination strategy against encephalitic alphaviruses. However,
582 our results indicate that it is likely that codon optimization of the structural genes in the construct
583 along with the efficiency of EP-based delivery contributed to the ability of the DNA vaccine
584 evaluated here to protect against the higher challenge dose with fewer DNA administrations.
585 Because no immunogenicity results were provided in the report by Gauci et al., it is not possible
586 to make an indirect comparison of the immunogenicity of the previously tested WEEV DNA
587 vaccines with that of the one we evaluated here.

588 It should also be noted that evaluation of individual and combined VEEV, WEEV, and
589 EEEV virus replicon particle (VRP) vaccines in mice and NHPs has also been recently reported.
590 In these experiments, the individual VRP vaccines delivered twice at a dose of 1×10^7 infectious
591 units elicited strong and durable virus-specific antibody responses in mice as measured by
592 ELISA and PRNT and provided complete protection against homologous lethal VEEV, WEEV,
593 and EEEV aerosol challenges (45). The VEEV VRP vaccine based on the IAB strain was also
594 shown to elicit durable protective immunity in mice against lethal aerosol challenge with the

595 heterologous VEEV strain IE and MUCV. In the murine studies, there were also no significant
596 differences in the antibody or protection levels when the VRP vaccines were administered in
597 combination. While the individual VEEV and EEEV and combination VRP vaccines protected
598 NHPs against homologous VEEV and EEEV aerosol challenge, the protection elicited by the
599 WEEV or combination VRP vaccines against WEEV aerosol challenge was not significantly
600 different from that of mock-vaccinated controls. The DNA vaccines evaluated in our studies
601 reported here compare favorably to the VRP vaccines in that complete protection in mice against
602 the same challenge doses of aerosolized VEEV, WEEV, and EEEV was also afforded by the
603 individual and 3-EEV DNA vaccines. Although we did not directly assess the duration of
604 protective immunity elicited by the individual and 3-EEV DNA vaccines in the mouse studies
605 reported here, our results in rabbits demonstrated that virus-neutralizing antibody titers elicited
606 by these vaccines remained significantly above background out to 349 days after the initial
607 vaccination. We also showed that sera from rabbits that received the subtype IAB-based VEEV
608 DNA vaccine administered individually or in the 3-EEV DNA formulation had high levels of
609 neutralizing activity against heterologous VEEV subtypes IC, ID, and IE and MUCV. While
610 these results are indicative of the potential for the individual and 3-EEV DNA vaccines to elicit
611 durable protective immunity and for the VEEV DNA and 3-EEV DNA vaccines to protect
612 against heterologous VEEV subtypes, we are currently completing studies to directly evaluate
613 these possibilities. We are also currently completing studies to evaluate the immunogenicity and
614 protective efficacy of the individual and 3-EEV DNA vaccines delivered by EP against VEEV,
615 WEEV, and EEEV aerosol challenge in NHPs. The results of these studies will be important for
616 further comparisons to the VRP and other next-generation alphavirus vaccine candidates.
617 The most widely accepted correlate of protection against the encephalitic alphaviruses is

618 neutralizing antibodies directed against the envelope glycoproteins (46-50). However,
619 neutralizing antibody titers are not always significantly associated with protection against
620 encephalitic alphavirus challenge by the aerosol route (51-53). In the studies reported here, the
621 VEEV, WEEV, and EEEV DNA vaccines elicited robust virus-specific antibody responses, to
622 include detectable levels of virus-neutralizing antibodies, when delivered individually or in a
623 multi-agent formulation. Although we observed that mice that received the individual WEEV
624 DNA or WEEV IND vaccine had similar virus-neutralizing antibody titers, those that received
625 the WEEV DNA vaccine were completely protected from WEEV aerosol challenge and had
626 significantly improved protection as compared to mice that received the WEEV IND vaccine.
627 More strikingly, mice that received the 3-EEV DNA vaccine were also completely protected
628 from WEEV aerosol challenge and had significantly improved protection as compared to mice
629 that received the WEEV IND vaccine despite having significantly lower virus-neutralizing
630 antibody titers. Similarly, although mice that received the individual EEEV DNA, the 3-EEV
631 DNA, or the EEEV IND vaccine had similar virus-neutralizing antibody titers, those that
632 received the EEEV DNA or 3-EEV DNA vaccine were completely protected from EEEV aerosol
633 challenge and had significantly improved protection as compared to mice that received the
634 EEEV IND vaccine. The ability of non-neutralizing antibodies to also mediate protection against
635 encephalitis caused by alphaviruses has been previously documented (54, 55). Therefore, it is
636 possible that non-neutralizing antibody responses elicited by the individual VEEV, WEEV, and
637 EEEV DNA vaccines and 3-EEV DNA vaccine also contributed to the protection levels
638 observed in the present studies. This is supported by our observation that mice that received the
639 individual WEEV, individual EEEV, or 3-EEV DNA vaccine had significantly higher virus-
640 specific total IgG antibody titers than mice receiving the respective IND vaccine.

641 Although cytotoxic T cell activity was not observed in previous studies with TC-83, more
642 recent studies have also demonstrated an importance for certain populations of T cells in
643 protection against lethal encephalitis caused by VEEV in mice (56-59). In our previous studies,
644 we demonstrated that the optimized VEEV DNA vaccine delivered by IM EP was predicted to
645 elicit a balanced Th1/Th2 immune response in mice as determined by antibody isotype ELISA,
646 and significant cell-mediated immune responses against the VEEV E2 and E1 glycoproteins
647 were measured by IFN-γ ELISpot assay (36). The ELISpot assay results obtained for the
648 individual VEEV DNA vaccine in our current studies were consistent with those previous
649 results. Although the 3-EEV DNA vaccine elicited significantly lower responses against the
650 VEEV E2 and E1 proteins as compared to the individual VEEV DNA vaccine in this assay, they
651 remained at significant levels. Therefore, it is possible that cell-mediated immune responses
652 elicited by the 3-EEV DNA vaccine also contributed to the protection against VEEV aerosol
653 challenge observed here. Although IFN-γ ELISpot assays required to directly measure cell-
654 mediated immune responses against WEEV and EEEV remain under development in our
655 laboratory, our current studies demonstrated that the individual WEEV, individual EEEV, and 3-
656 EEV DNA vaccines are also predicted to elicit balanced Th1/Th2 immune responses in mice as
657 determined by antibody isotype ELISA. Although a caveat of this analysis was the use of pooled
658 sera instead of serum samples from individual mice, which was necessitated by the limited
659 amount of serum that could be obtained from the mice, these results are indicative of the
660 potential for the individual WEEV, individual EEEV, and 3-EEV DNA vaccines to also elicit
661 virus-specific cell-mediated immune responses. Roles for mucosal immune responses and
662 antibody-dependent cellular cytotoxicity in protection against aerosol VEEV challenge in mice
663 have also been documented (60-62). Therefore, we are currently performing a more thorough

664 characterization of the various immune responses elicited by the individual VEEV, WEEV, and
665 EEEV DNA vaccines and 3-EEV DNA vaccine to further elucidate the contributing role of these
666 responses in the protection observed for these vaccines against VEEV, WEEV, and EEEV
667 aerosol challenge.

668 The results of our previous studies demonstrated that IM EP delivery clearly enhanced the
669 immunogenicity and protective efficacy of the VEEV DNA vaccine in mice (36). The results of
670 our current studies reported here further support IM EP as an efficient means of administering
671 encephalitic alphavirus DNA vaccines, as the individual VEEV, WEEV, and EEEV DNA
672 vaccines and 3-EEV DNA vaccine delivered by this method elicited robust and completely
673 protective immune responses with relatively low DNA doses and few vaccinations. Despite the
674 promise for this delivery platform as demonstrated by our results and those from numerous
675 studies performed by others, there remains some concern about the tolerability of this
676 administration procedure for widespread clinical use (63). However, to date this device has been
677 utilized in over 20 Phase 1 and 2 clinical trials for a wide variety of DNA vaccines, has been
678 used to administer DNA vaccines in over 600 human subjects, and is currently being refined for
679 late-stage clinical testing and eventual commercial use (D. Hannaman, personal communication).
680 Our Phase 1 clinical trial also includes the first human testing of a recently-developed device for
681 EP-mediated intradermal (ID) delivery of the VEEV DNA vaccine. Therefore, the results of this
682 study will also allow us to evaluate the possibility that alternative routes of DNA vaccine
683 administration could have beneficial effects on tolerability and/or immunogenicity relative to IM
684 EP.

685 Taken together, the results of our studies described here provide substantial evidence to
686 demonstrate that the individual VEEV, WEEV, and EEEV DNA vaccines and 3-EEV DNA

vaccine delivered by IM EP are capable of eliciting protective immunity against aerosol exposure with encephalitic alphaviruses. To our knowledge, this is also the first report of a single nucleic acid-based multi-agent vaccine formulation that can provide protection against VEEV, WEEV, and EEEV aerosol challenge in mice. Consequently, these DNA vaccines may represent a viable next-generation alternative to the current alphavirus IND vaccines. The DNA vaccine platform used here also avoids issues with manufacturing, boosting potential, stability, and safety that can be problematic for other approaches to develop next-generation vaccines. The results from our currently ongoing Phase 1 clinical trial will provide critical information regarding the safety, tolerability, and immunogenicity of the VEEV DNA vaccine candidate delivered by IM or ID EP in humans. We are also currently completing studies to evaluate and compare the immunogenicity and protective efficacy of the individual VEEV, WEEV, and EEEV and 3-EEV DNA vaccines delivered by IM or ID EP in NHPs. Should protective efficacy be successfully demonstrated in these studies, then the individual EEEV, individual WEEV, and 3-EEV DNA vaccines will also be well poised for transition to clinical development.

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898
899 ## FIGURE LEGENDS
900
901 **Figure 1.** VEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10
902 per group) were vaccinated on days 0 and 21 with 5 µg of empty vector DNA, 5 µg of the VEEV
903 DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA
904 vaccine) delivered by IM EP or on day 0 with 0.5 ml of the live-attenuated VEEV IND vaccine
905 TC-83 (1 x 10^4 PFU) delivered by subcutaneous injection. Serum samples obtained on days 21
906 and 42 were assayed for total IgG anti-VEEV antibodies by ELISA and for VEEV-neutralizing
907 antibodies by PRNT. The group mean $\log_{10}$ ELISA (Fig. 1A) and $PRNT_{80}$ (Fig. 1B) titers along
908 with the standard error of the mean (SEM) are shown. *$p < 0.05$ for comparison of titers between
909 groups.
910
911 **Figure 2.** Survival of vaccinated mice challenged with VEEV. Female BALB/c mice (n = 10 per
912 group) vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the VEEV
913 DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA
914 vaccine) delivered by IM EP or with a single administration of 0.5 ml of the live-attenuated

915 VEEV IND vaccine TC-83 (~1 x 10^4 PFU) delivered by subcutaneous injection were challenged
916 4 weeks after the final vaccination with 1 × 10^4 PFU (~10,000 LD_{50}) of VEEV IAB strain
917 Trinidad donkey by the aerosol route. Kaplan-Meier survival curves indicating the percentage of
918 surviving mice at each day of the 28-day post-challenge observation period are shown. *$p < 0.05$
919 for survival rate and survival curve as compared to negative control group.
920
921 **Figure 3.** VEEV-specific cellular immune responses of vaccinated mice. Female BALB/c mice
922 (n = 6 per group) were vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5
923 µg of the VEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-
924 EEV DNA vaccine) delivered by IM EP. Two weeks after the second vaccination, splenocytes
925 were isolated and restimulated with no peptide, a peptide from the unrelated β-Galactosidase
926 protein, or pools of overlapping peptides spanning the VEEV IAB E2 or E1 envelope
927 glycoproteins and analyzed by IFN-γ ELISpot assay. The mean spot forming units (SFU) per 10^6
928 cells along with the SEM are shown for each group. *$p < 0.05$ for comparison of spot counts
929 between groups.
930
931 **Figure 4.** WEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10
932 per group) were vaccinated on days 0 and 21 with 5 µg of empty vector DNA, 5 µg of the
933 WEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV
934 DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated WEEV IND vaccine
935 delivered by subcutaneous injection. Serum samples obtained on days 21 and 42 were assayed
936 for total IgG anti-WEEV antibodies by ELISA and for WEEV-neutralizing antibodies by PRNT.
937 The group mean log_{10} ELISA (Fig. 6A) and $PRNT_{80}$ (Fig. 6C) titers along with the SEM are

938 shown. Pooled day 42 serum samples from each group were assayed for IgG1 and IgG2a anti-
939 WEEV antibodies by ELISA and the ratios of IgG2a to IgG1 antibody titers are shown (Fig. 6B).
940 $^{*}p < 0.05$ for comparison of titers between groups.
941
942 **Figure 5.** Survival of vaccinated mice challenged with WEEV. Female BALB/c mice (n = 10
943 per group) vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the
944 WEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV
945 DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated WEEV IND vaccine
946 delivered by subcutaneous injection were challenged 4 weeks after the final vaccination with $2 \times$
947 10^4 PFU (~500 LD_{50}) of WEEV strain CBA87 by the aerosol route. Kaplan-Meier survival
948 curves indicating the percentage of surviving mice at each day of the 28-day post-challenge
949 observation period are shown. $^{*}p < 0.05$ for survival rate as compared to negative control group.
950
951 **Figure 6.** EEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10
952 per group) were vaccinated on days 0, 21, and 42 with 5 µg of empty vector DNA, 5 µg of the
953 EEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV
954 DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated EEEV IND vaccine
955 delivered by subcutaneous injection. Serum samples obtained on days 21, 42, and 63 were
956 assayed for total IgG anti-EEEV antibodies by ELISA and for EEEV-neutralizing antibodies by
957 PRNT. The group mean $\log_{10}$ ELISA (Fig. 6A) and $PRNT_{80}$ (Fig. 6C) titers along with the SEM
958 are shown. Pooled day 63 serum samples from each group were assayed for IgG1 and IgG2a
959 anti-EEEV antibodies by ELISA and the ratios of IgG2a to IgG1 antibody titers are shown (Fig.
960 6B). $^{*}p < 0.05$ for comparison of titers between groups.

961

962 **Figure 7.** Survival of vaccinated mice challenged with EEEV. Female BALB/c mice (n = 10 per
963 group) vaccinated three times at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the
964 EEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV
965 DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated EEEV IND vaccine
966 delivered by subcutaneous injection were challenged 4 weeks after the final vaccination with $1 \times$
967 10^5 PFU (~3,000 LD_{50}) of EEEV strain FL91-4679 by the aerosol route. Kaplan-Meier survival
968 curves indicating the percentage of surviving mice at each day of the 28-day post-challenge
969 observation period are shown. *$p < 0.05$ for survival rate as compared to negative control group.

970

971 **Figure 8.** Virus-neutralizing antibody responses of vaccinated rabbits. New Zealand White
972 rabbits (n = 5 per group) were vaccinated on days 0, 28, and 230 with 0.5 mg of the VEEV,
973 WEEV, or EEEV DNA vaccine or with 0.5 mg each of the VEEV, WEEV, and EEEV DNA
974 vaccines (3-EEV DNA vaccine) delivered by IM EP. Serum samples obtained on days 27, 42,
975 230, 266 and 349 were assayed for neutralizing antibodies against VEEV IAB (Fig. 8A), WEEV
976 (Fig. 8C), or EEEV (Fig. 8D) by PRNT. The day 42 serum samples from rabbits vaccinated with
977 the VEEV DNA or 3-EEV DNA were also assayed for neutralizing activity against heterologous
978 VEEV subtypes IC, ID, and IE and MUCV (Fig. 8B) by PRNT. The group mean $\log_{10}$ $PRNT_{80}$
979 titers along with the SEM are shown. *$p < 0.05$ for comparison of titers between groups.

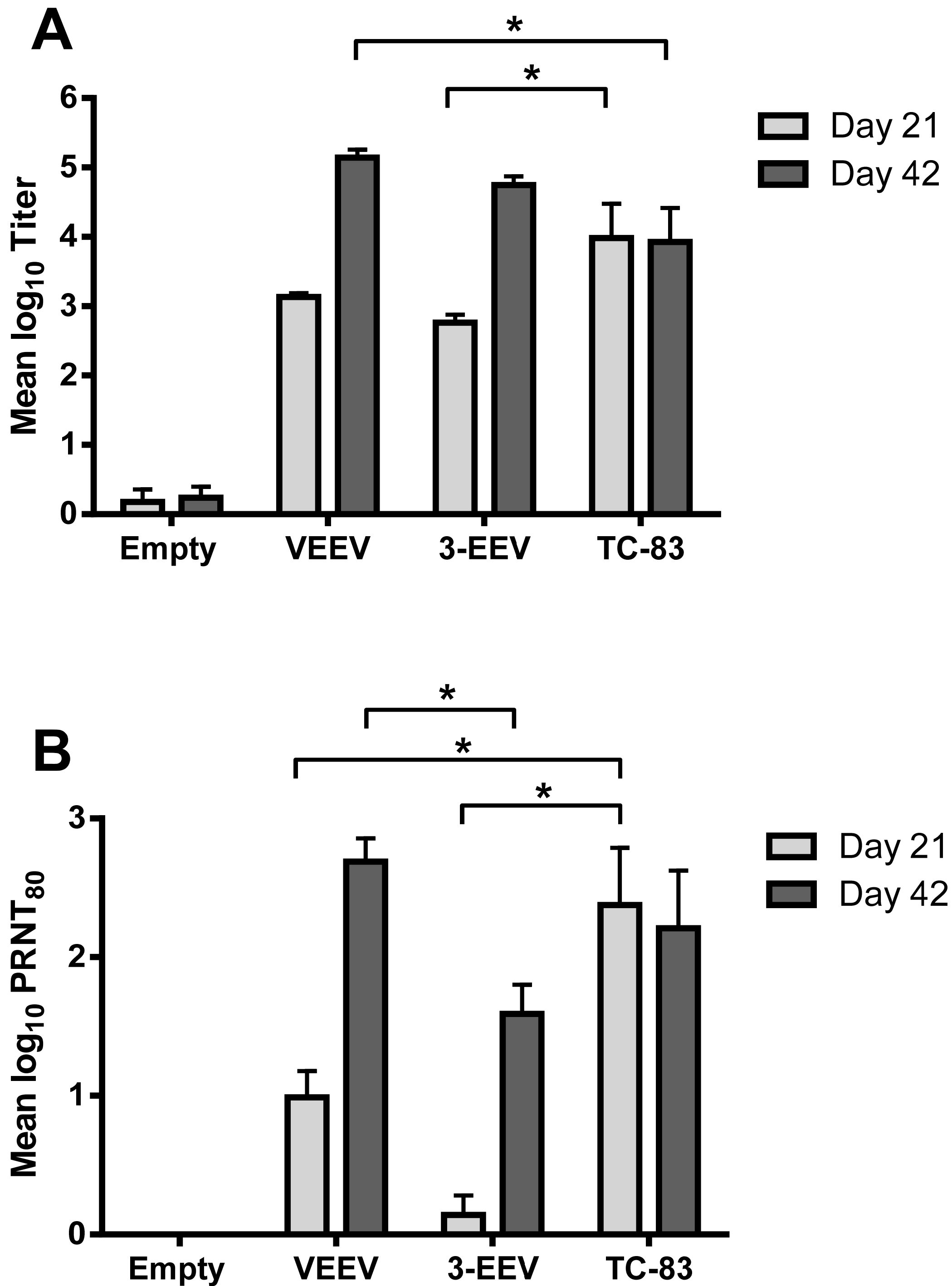

Figure 1. VEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10 per group) were vaccinated on days 0 and 21 with 5 µg of empty vector DNA, 5 µg of the VEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or on day 0 with 0.5 ml of the live-attenuated VEEV IND vaccine TC-83 (1×10^4 PFU) delivered by subcutaneous injection. Serum samples obtained on days 21 and 42 were assayed for total IgG anti-VEEV antibodies by ELISA and for VEEV-neutralizing antibodies by PRNT. The group mean $\log_{10}$ ELISA (Fig. 1A) and $PRNT_{80}$ (Fig. 1B) titers along with the standard error of the mean (SEM) are shown. $^*p < 0.05$ for comparison of titers between groups.

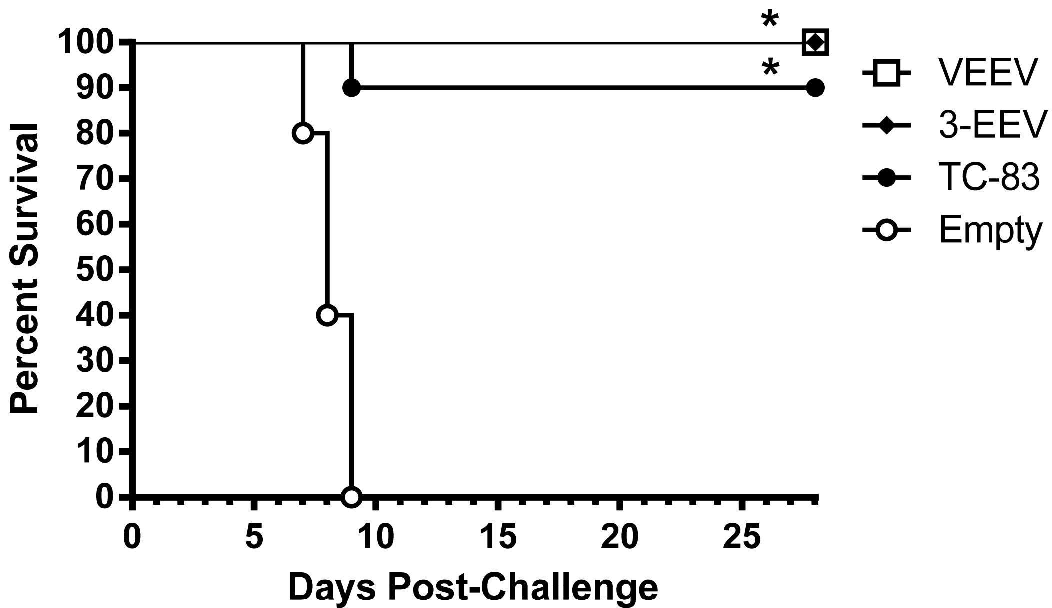

Figure 2. Survival of vaccinated mice challenged with VEEV. Female BALB/c mice (n = 10 per group) vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the VEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or with a single administration of 0.5 ml of the live-attenuated VEEV IND vaccine TC-83 (~1 x 10^4 PFU) delivered by subcutaneous injection were challenged 4 weeks after the final vaccination with 1 × 10^4 PFU (~10,000 LD_{50}) of VEEV IAB strain Trinidad donkey by the aerosol route. Kaplan-Meier survival curves indicating the percentage of surviving mice at each day of the 28-day post-challenge observation period are shown. *$p < 0.05$ for survival rate and survival curve as compared to negative control group.

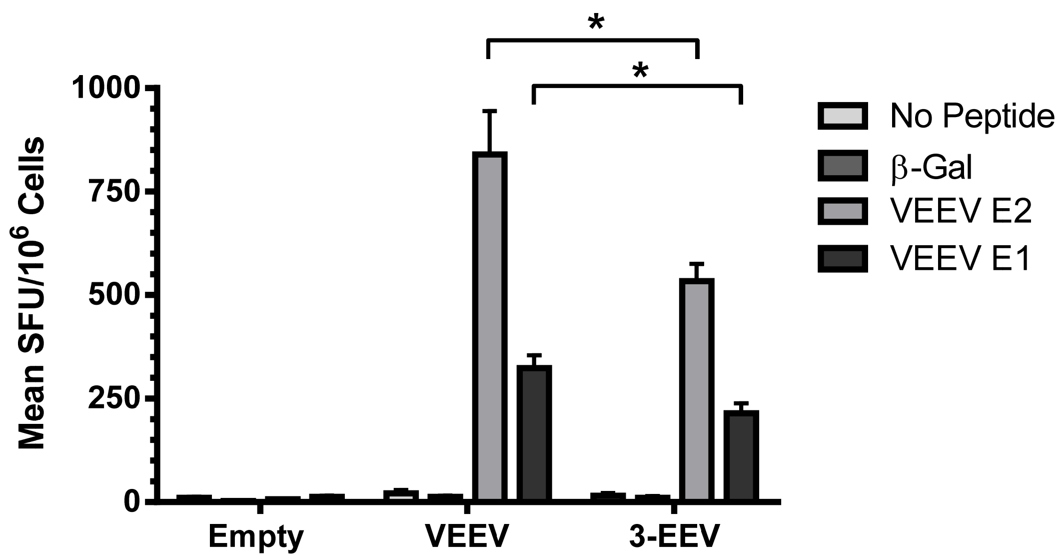

Figure 3. VEEV-specific cellular immune responses of vaccinated mice. Female BALB/c mice (n = 6 per group) were vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the VEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP. Two weeks after the second vaccination, splenocytes were isolated and restimulated with no peptide, a peptide from the unrelated β-Galactosidase protein, or pools of overlapping peptides spanning the VEEV IAB E2 or E1 envelope glycoproteins and analyzed by IFN-γ ELISpot assay. The mean spot forming units (SFU) per 10^6 cells along with the SEM are shown for each group. *$p < 0.05$ for comparison of spot counts between groups.

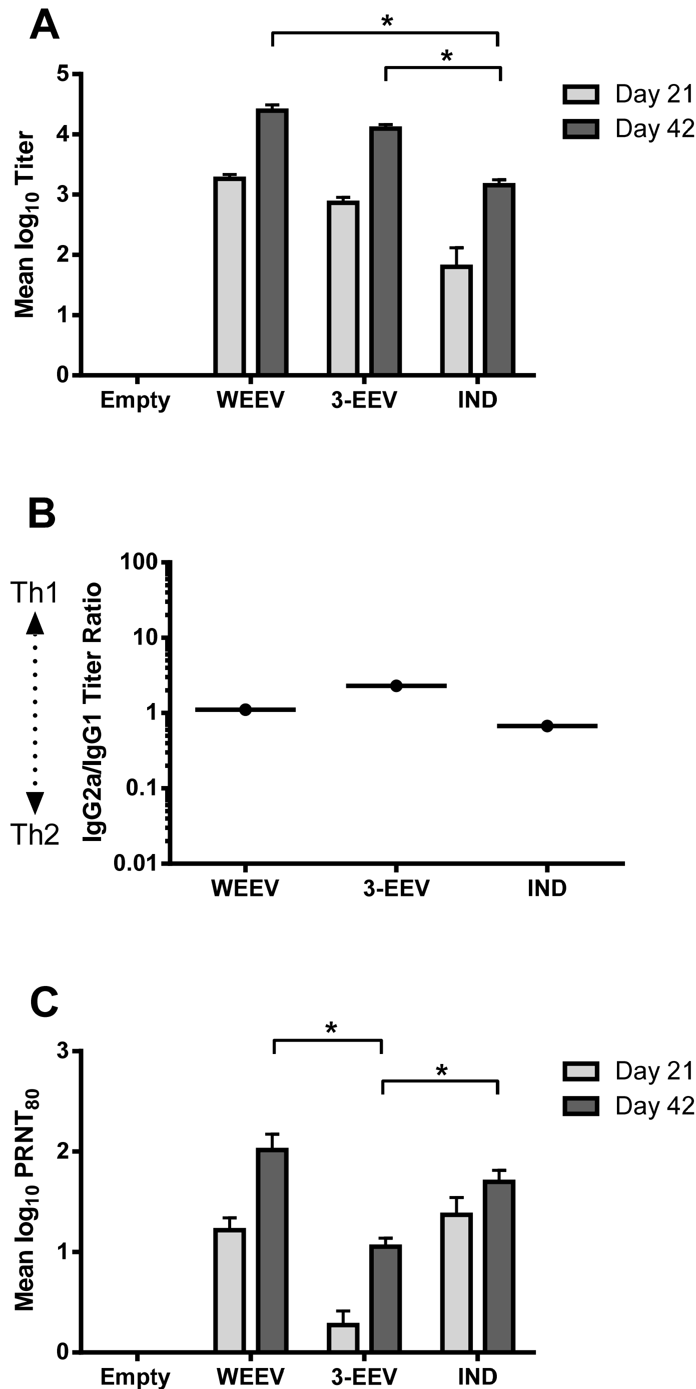

Figure 4. WEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10 per group) were vaccinated on days 0 and 21 with 5 µg of empty vector DNA, 5 µg of the WEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated WEEV IND vaccine delivered by subcutaneous injection. Serum samples obtained on days 21 and 42 were assayed for total IgG anti-WEEV antibodies by ELISA and for WEEV-neutralizing antibodies by PRNT. The group mean $\log_{10}$ ELISA (Fig. 6A) and $PRNT_{80}$ (Fig. 6C) titers along with the SEM are shown. Pooled day 42 serum samples from each group were assayed for IgG1 and IgG2a anti-WEEV antibodies by ELISA and the ratios of IgG2a to IgG1 antibody titers are shown (Fig. 6B). $*p < 0.05$ for comparison of titers between groups.

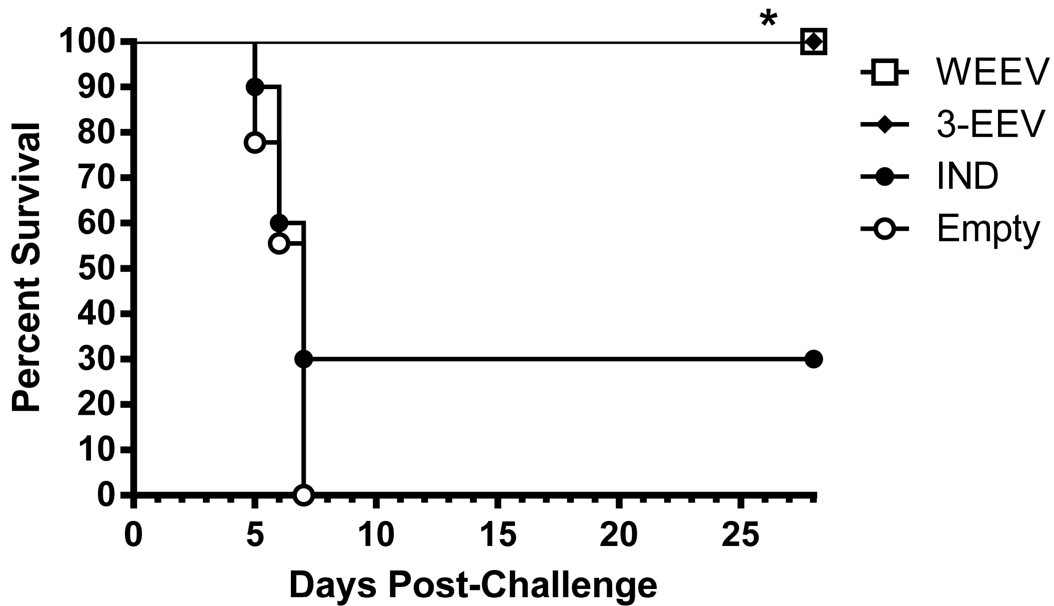

Figure 5. Survival of vaccinated mice challenged with WEEV. Female BALB/c mice (n = 10 per group) vaccinated twice at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the WEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated WEEV IND vaccine delivered by subcutaneous injection were challenged 4 weeks after the final vaccination with 2×10^4 PFU (~500 LD_{50}) of WEEV strain CBA87 by the aerosol route. Kaplan-Meier survival curves indicating the percentage of surviving mice at each day of the 28-day post-challenge observation period are shown. *$p < 0.05$ for survival rate as compared to negative control group.

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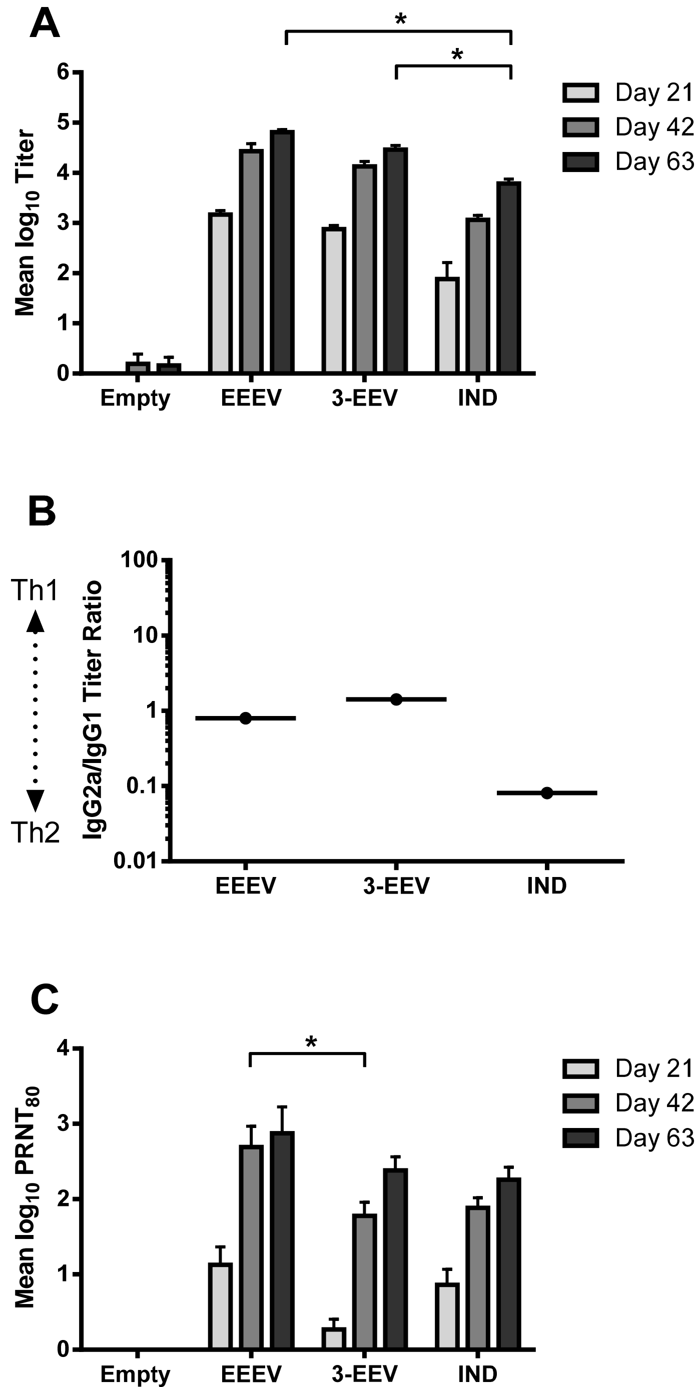

Figure 6. EEEV-specific antibody responses of vaccinated mice. Female BALB/c mice (n = 10 per group) were vaccinated on days 0, 21, and 42 with 5 µg of empty vector DNA, 5 µg of the EEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated EEEV IND vaccine delivered by subcutaneous injection. Serum samples obtained on days 21, 42, and 63 were assayed for total IgG anti-EEEV antibodies by ELISA and for EEEV-neutralizing antibodies by PRNT. The group mean $\log_{10}$ ELISA (Fig. 6A) and $PRNT_{80}$ (Fig. 6C) titers along with the SEM are shown. Pooled day 63 serum samples from each group were assayed for IgG1 and IgG2a anti-EEEV antibodies by ELISA and the ratios of IgG2a to IgG1 antibody titers are shown (Fig. 6B). *$p < 0.05$ for comparison of titers between groups.

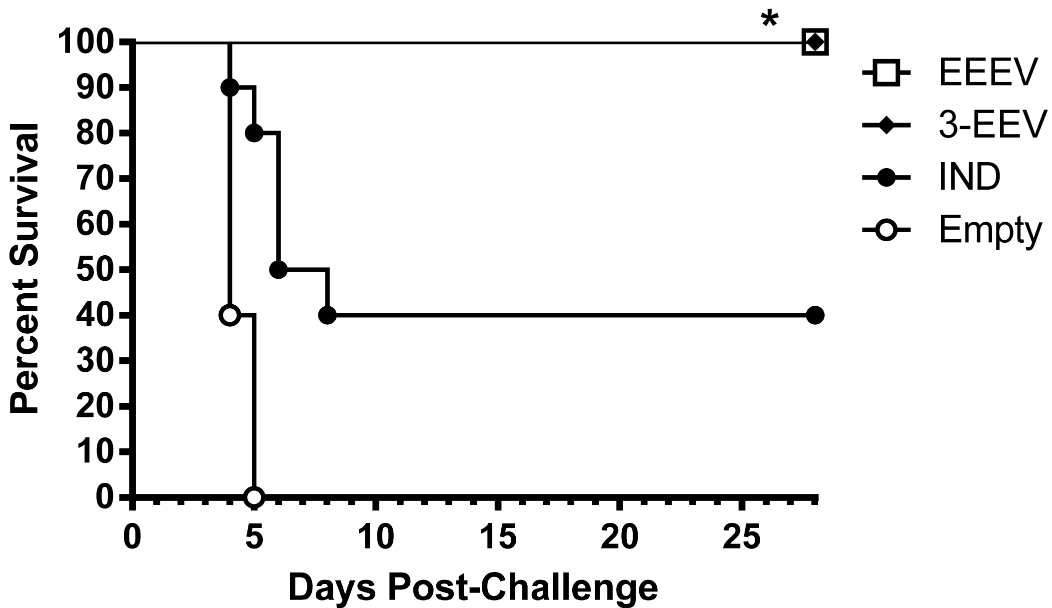

Figure 7. Survival of vaccinated mice challenged with EEEV. Female BALB/c mice (n = 10 per group) vaccinated three times at a 3-week interval with 5 µg of empty vector DNA, 5 µg of the EEEV DNA vaccine, or 5 µg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP or 0.5 ml of the formalin-inactivated EEEV IND vaccine delivered by subcutaneous injection were challenged 4 weeks after the final vaccination with 1×10^5 PFU (~3,000 LD_{50}) of EEEV strain FL91-4679 by the aerosol route. Kaplan-Meier survival curves indicating the percentage of surviving mice at each day of the 28-day post-challenge observation period are shown. *$p < 0.05$ for survival rate as compared to negative control group.

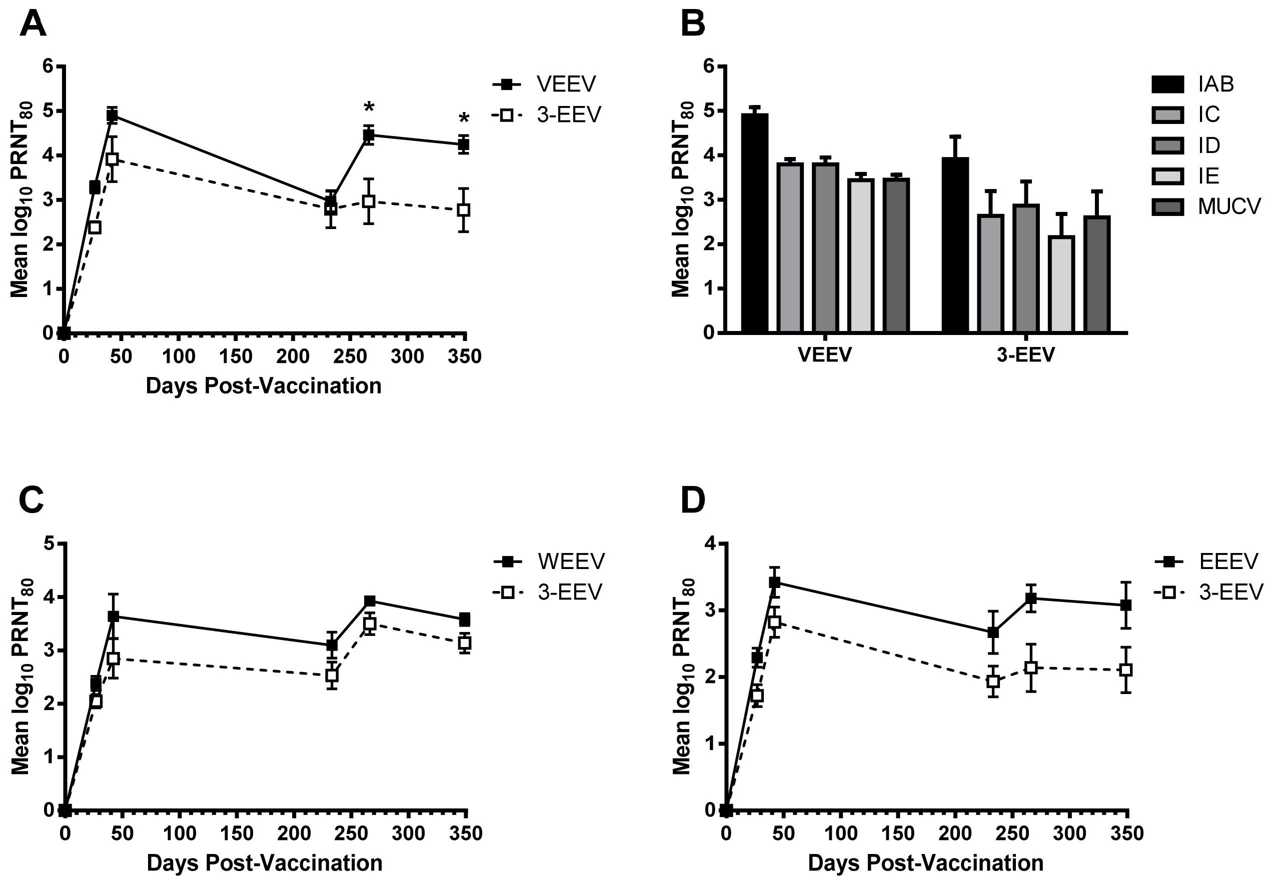

Figure 8. Virus-neutralizing antibody responses of vaccinated rabbits. New Zealand White rabbits (n = 5 per group) were vaccinated on days 0, 28, and 230 with 0.5 mg of the VEEV, WEEV, or EEEV DNA vaccine or with 0.5 mg each of the VEEV, WEEV, and EEEV DNA vaccines (3-EEV DNA vaccine) delivered by IM EP. Serum samples obtained on days 27, 42, 230, 266 and 349 were assayed for neutralizing antibodies against VEEV IAB (Fig. 8A), WEEV (Fig. 8C), or EEEV (Fig. 8D) by PRNT. The day 42 serum samples from rabbits vaccinated with the VEEV DNA or 3-EEV DNA were also assayed for neutralizing activity against heterologous VEEV subtypes IC, ID, and IE and MUCV (Fig. 8B) by PRNT. The group mean log_{10} $PRNT_{80}$ titers along with the SEM are shown. *$p < 0.05$ for comparison of titers between groups.