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Novel DNA-launched Venezuelan equine encephalitis virus vaccine with rearranged genome

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ABSTRACT

Novel live-attenuated V4020 vaccine was prepared for Venezuelan equine encephalitis virus (VEEV), an alphavirus from the *Togaviridae* family. The genome of V4020 virus was rearranged, with the capsid gene expressed using a duplicate subgenomic promoter downstream from the glycoprotein genes. V4020 also included both attenuating mutations from the TC83 VEEV vaccine secured by mutagenesis to prevent reversion mutations. The full-length infectious RNA of V4020 vaccine virus was expressed from pMG4020 plasmid downstream from the CMV promoter and launched replication of live-attenuated V4020 *in vitro* or *in vivo*. BALB/c mice vaccinated with a single dose of V4020 virus or with pMG4020 plasmid had no adverse reactions to vaccinations and developed high titers of neutralizing antibodies. After challenge with the wild type VEEV, vaccinated mice survived with no morbidity, while all unvaccinated controls succumbed to lethal infection. Intracranial injections in mice showed attenuated replication of V4020 vaccine virus as compared to the TC83. We conclude that V4020 vaccine has safety advantage over TC83, while provides equivalent protection in a mouse VEEV challenge model.

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne alphavirus that causes human infections and epizootics in the North, Central, and South America, including a 1971 outbreak in Texas [1,2]. Climate, ecological changes, and international travel increase the risk of VEEV reemergence [3–5]. In addition, the virus can be aerosolized as a biological weapon [3]. The potentially threatening effects of the VEEV reemergence demand a safe and effective vaccine [6].

Live-attenuated vaccines have the advantage of rapidly inducing natural, long-lasting protective immunity after a single-dose vaccination, which makes them ideal candidates to contain outbreaks. However, because of the high rates of mutations in RNA viruses [7,8], there is a concern that live vaccines can potentially acquire reversion mutations leading to regeneration of the wild-type virulent phenotype. Such safety concerns have hindered the approval of many live-attenuated RNA virus vaccines including TC83, a live-attenuated VEEV vaccine developed in the 1960s [9]. The TC83 vaccine has been used for the immunization of medical personnel under an Investigational New Drug (IND) protocol [3,10,11]. The TC83 vaccine protects against many epizootic virus strains of the VEEV complex including IAB, IC, and IE [12]. However, it causes adverse reactions including headache and fever in approximately 23% of vaccine recipients, while approximately 18% do not develop sufficient neutralizing antibody titers [13]. Adverse reactions have been associated with genetic reversions [14].

The TC83 live-attenuated vaccine includes two attenuating mutations, 5′A3 and E2-Arg120, which can be included in the next generation VEEV vaccine due to the long record of their clinical use [14]. Previously, we described a DNA-launched, live-attenuated VEEV platform based on an iDNA® infectious clone, and a recombinant plasmid pTC83 encoding the entire genomic RNA of the TC83 vaccine virus under the transcriptional control of the CMV promoter [15]. When transfected *in vitro* or *in vivo*, the pTC83 plasmid initiated limited replication of a live-attenuated TC83-like vaccine virus [15]. However, although the vaccine virus was launched from a genetically stable plasmid, the design of additional measures to prevent reversion mutations would improve vaccine safety.

In this study, we report a novel, live-attenuated VEEV vaccine, V4020. In this vaccine, we used several novel technologies to improve vaccine safety including a genetically stable plasmid iDNA

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technology, rearrangement of the alphavirus genome, the TC83 attenuating mutations, and genetic stabilization of attenuating phenotype by using synonymous translational codon replacement.

2. Materials and methods

2.1. Cell line

Vero cell line from the American Type Culture Collection (Manassas, VA) was maintained in a humidified incubator at 37 °C in 5% CO_2 in αMEM supplemented with 10% fetal bovine serum (FBS) and gentamicin sulfate (10 μg/ml) (Life Technologies, Carlsbad, CA).

2.2. Plasmids

The pMG4020 plasmid was prepared using pUC-based vector and encoded the full-length rearranged V4020 vaccine virus genome (Supplementary Fig. 1). The capsid (C) gene was cloned downstream from the glycoprotein gene by PCR and expressed using the duplicate subgenomic promoter. The ATG codon was introduced at the 5′ of E3 gene within the glycoprotein (GP) genes, while a TGA stop codon was introduced at the 3′ of the C gene. The full-length genomic cDNA of V4020 virus was placed in the pMG4020 under transcriptional control of the CMV promoter. The hepatitis delta ribozyme was cloned downstream from the V4020 cDNA to ensure cleavage of the genomic RNA transcript after the synthetic poly(A) at the viral 3′ end. The cDNA of V4020 maintained both attenuating mutations 5′A3 (in the 5′ untranslated region) and E2-120Arg (in the E2 gene) derived from the TC83 sequence. Mutagenesis was performed to change codon AGA encoding E2-120Arg on the TC83 virus to a synonymous codon CGA in the pMG4020, which requires at least two mutations to revert to the wild-type VEEV E2-120Thr codon ACA. Plasmid pMG4017 was identical to the pMG4020 except E2-120Arg was encoded by an AGA codon, and an additional mutation E1-119Val was introduced in the E1 gene in place of E1-119Ala.

Plasmids were prepared in *E. coli* Stbl3 cells (Thermo, Carlsbad, CA) using standard LB medium in the presence of kanamycin, isolated by an endotoxin-free DNA isolation method (Qiagen, Valencia, CA) and formulated in phosphate-buffered saline (PBS) to a concentration of ∼1 mg/ml.

2.3. Viruses

Live-attenuated vaccine virus V4020 was prepared by transfecting Vero cells with pMG4020. Transfection was done by electroporation essentially as described previously [15]. Briefly, Vero cells were transfected with 100 ng of pMG4020 and incubated at 37 °C. The transfection medium was harvested at 24 h post-transfection, purified by ultracentrifugation and resuspended in PBS. The V4020 titer was determined by a standard plaque assay in Vero cell monolayers. The transfection medium was used to infect Vero cells at a multiplicity of infection of 0.01 to generate V4020 passage 1 (P1) virus. The titer of the V4020 P1 virus was determined, and the virus was aliquoted and stored at −80 °C until used *in vitro* or *in vivo*.

The TC83 live-attenuated vaccine (U.S. Army Medical Research and Materiel Command, Fort Detrick, MD) was similarly amplified once in Vero cells to generate P1 virus stock and stored at −80 °C until use.

The Trinidad donkey strain of VEEV (VEEV TrD), a 1943 subtype IAB isolate from an epidemic/epizootic [16], was obtained through the NIH Biodefense and Emerging Infectious Research Recourses Repository (NR-332, NIAID, NIH, BEI Resources, Manassas, VA), was similarly amplified once in Vero cells to generate P1 virus stock and stored at −80 °C in a BSL3 facility until use as a standard wild-type VEEV challenge stock.

2.4. Stability of plasmids in E. coli

Genetic stability of pMG4020 plasmid was evaluated by 10 consecutive passages in *E. coli*. From the 2 ml 24 h LB culture containing kanamycin, 10 μl of *E. coli* cell suspension was inoculated into another 2 ml culture, and incubation was continued for 24 h. Cell pellet was stored at −20 °C. Passages were repeated until 10th passage. Plasmid DNA was isolated from *E. coli* passages 1–10 and separated on 1% agarose gel to visualize plasmid bands. Identity of DNA was confirmed by DNA sequencing.

2.5. In vitro assays

To confirm replication of vaccine viruses, Vero cells were infected with 100–1000 PFU in 75 cm^2 flasks resulting in the multiplicity of infection (MOI) of ∼0.0001 to ∼0.001. Alternatively, Vero cells were electroporated with 1 ng to 100 ng of pMG4020 using ECM-830 electroporator (BTX Genetronics, San Diego, CA) as described previously [15]. Expression of viral antigens and detection of virus replication were monitored by immunofluorescence assay (IFA) infectious center assay (ICA), observation of cytopathic effect (CPE), and plaque assay in Vero cells [15,17]. For virus growth curves, virus samples were collected at 6 h intervals and quantitated in duplicates by plaque assay.

2.6. Immunizations and challenge of BALB/c mice

Research involving mice was done according to approved institutional animal protocols. BALB/c mice (4–8 week-old, 5 mice per group, Noble Life Sciences, Woodbine, MD) were anesthetized with isoflurane prior to vaccinations. Each experiment was done at least two times to ensure reproducibility. Mice were vaccinated subcutaneously (s.c.) with V4020 vaccine virus or with control TC83 vaccine in the dorsal area. Alternatively, vaccination was carried out intramuscularly (i.m.) with 50 μl of plasmids pMG4020 or control pMG4017 in the medial thighs, followed by *in vivo* electroporation at the site of injection using a two-pin electrode and a square wave ECM 830 electroporator [15]. After vaccinations, animals were observed daily for clinical signs of infection and body weights. Blood samples were collected from the retro-orbital sinus 2 days after vaccination to detect viremia, and on day 28 to detect immune response. Mice were then transferred into a Select Agent approved BSL3 facility and challenged with virulent VEEV TrD at a dose of 1×10^4 PFU in 20 μl by the s.c. route. After challenge, animals were observed daily for clinical signs of infection and body weight. Morbidity (body weight, behavior changes) and mortality (survival) were determined. Sera were taken on day 3 post challenge, and representative mice from each challenge group were sacrificed on day 3 to examine virus titers in the brain tissues.

To measure the magnitude, duration, phenotypic stability, and kinetics of virus replication of TC83 and V4020 attenuated viruses in the central nervous system, BALB/c mice (7–8 week-old, Jackson Laboratory, Bar Harbor, ME) were anesthetized with isoflurane prior to intracranial (i.c.) inoculation with 1×10^5 PFU of either V4020 or TC83 vaccine viruses. Mice were monitored daily for body weight and clinical scores as a composite 0–16 point measurement of four individual criteria (each scored 0–4) including activity, grimace scale, grooming and posturing, and neurological responses measured by gripping agility, tail strength, and trunk curl reflexes. Brain homogenates (10% w/v) prepared with an Omni TH Tissue Homogenizer in DMEM/F12, followed by clarification

with centrifugation at 4500*g* for 20 min, were tested for viral titer by standard plaque assay.

Viremia was determined on days 2–4 post-vaccination or post-challenge by either direct plaque assay, or by virus amplification in Vero cells. To confirm that the vaccine virus launched from the iDNA *in vivo* maintained the rearranged genome and an attenuating mutation, viral RNA was extracted by Trizol LS (Thermo). RT-PCR was done using the following primers: 5′-6904-CGCTGATT GAGGCGGCTTTCGGCGAAATTTCATCAATACA-6943-3′; and 5′-1003 8-GGTGCGTAGCCTGCTCTGTTGACTATAGTGTTATACGAGA-10077-3 ′ (TC83 numbering, GenBank L01443). The sequence of PCR fragments was determined by DNA sequencing to confirm the gene rearrangement and the attenuating mutations.

Antibody response was determined using sera collected on day 28 after vaccination. Neutralizing antibodies were determined by plaque reduction neutralization test (PRNT) in duplicates in Vero cells. Sera were heat inactivated and TC83 virus was mixed with serum dilutions before plaque assay. Dilution of serum required to cause 50% and 80% reduction of plaques was determined.

2.7. Statistical analysis

Results are reported as means ± SEM. Two-way ANOVA with Bonferroni's post-hoc test (for body weight data) or Mantel-Cox log rank test (for survival data) was used for the determination of statistical significance among treatment groups, as appropriate. Statistical analysis and graphics were performed using the GraphPad Prism version 7 for Windows package (GraphPad Software, LaJolla, CA).

3. Results

3.1. Design of the rearranged V4020 live-attenuated VEEV vaccine

The genomes of the VEEV, TC83, and V4020 viruses are schematically shown on Supplementary Fig. 1a. The functional RNA genome of the V4020 vaccine virus was encoded in the pMG4020 plasmid (Supplementary Fig. 1b). Compared to the TC83 vaccine, the V4020 vaccine virus contained several mutations and a genetic rearrangement within the genome. The structural gene region was split into two open reading frames (ORFs), one expressing GP genes E3-E2-6 K-E1, and the other expressing the C gene only. Each structural ORF was expressed from its own 26S subgenomic promoter, and included translational start and stop codons. Furthermore, the genes were rearranged, with the GP genes placed in front of the C gene in the V4020 (Supplementary Fig. 1a). Artificially rearranged genomes lead to the attenuation of many viruses including VEEV and are resistant to reversions because many independent mutations would be required to restore the wild type virus sequence [18–20]. The V4020 also included both 5′A3 and E2-120Arg attenuating mutations derived from the TC83 vaccine. However, the E2-120Arg was encoded in the V4020 and in pMG4020 by a CGA codon instead of AGA in the TC83 virus. Therefore, in the V4020 and in pMG4020, at least two mutations would be needed to revert to the wild type ACA (E2-120Thr). In contrast, in the TC83 vaccine, an AGA codon encodes the attenuating mutation E2-120Arg, and a single point mutation would be sufficient to revert to the VEEV wild type ACA (E2-120Thr).

The full-length RNA of V4020 vaccine virus was placed downstream from the CMV promoter in the pMG4020 plasmid to launch replication of live-attenuated V4020 virus *in vitro* or *in vivo* (Supplementary Fig. 1b) [15]. The CMV promoter directed transcription of the functional viral genomic RNA starting from the 5′ terminus to the 3′-terminal poly-A sequence. A control plasmid pMG4017 contained a similarly rearranged genome; however, in addition to the two TC83 attenuating mutations 5′A3 and E2-120Arg, an additional mutation, E1-119Val, not present in any VEEV isolates, was introduced in the E1 gene in place of the TC83 E1-119Ala.

3.2. Stability of pMG4020 in E. coli

The pMG4020 plasmid is ~15 kB long and contains the full-length rearranged RNA genome of live-attenuated V4020 virus, including duplicated subgenomic promoters (Supplementary Fig. 1b). To confirm genetic stability, pMG4020 was passed ten times in *E. coli* Stbl3 cells. Plasmid DNA was isolated from passages 1–10 and compared for size, yields, restriction enzyme digest pattern, DNA sequencing, and the ability to launch live V4020 virus *in vitro*. No considerable passage-to-passage variation of size, restriction enzyme digest pattern or yield of the plasmids was detected (Supplementary Fig. 1b, bottom panel) suggesting that plasmids are relatively stable in *E. coli*. The observed minor variations can be explained by sample-to-sample differences in the DNA isolation. Sequence of pMG4020 plasmid derived from passage 10 virus was find identical to that of parent pMG4020 before passages, including rearranged genes and attenuating mutations derived from the TC83 vaccine (Fig. 1). The passage 10 pMG4020 was also confirmed to launch V4020 virus *in vitro* as described below.

3.3. V4020 Vaccine virus is launched from pMG4020 plasmid in vitro

To evaluate if V4020 live-attenuated virus can be launched *in vitro* from the plasmid, pMG4020 was transfected into Vero cells. Expression of virus was confirmed by IFA in the cytoplasm of trans-

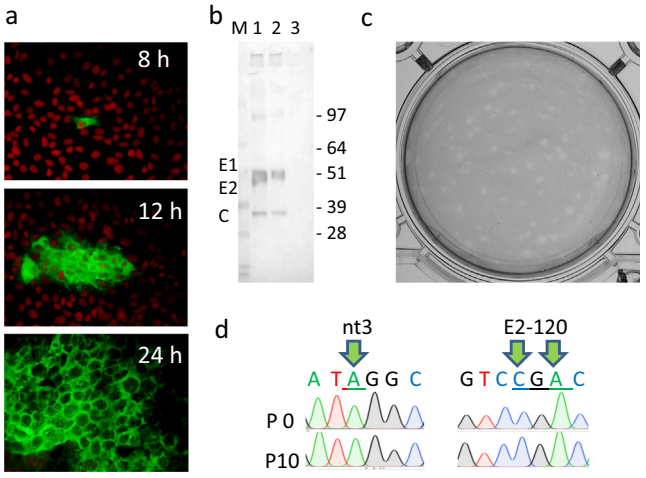

Fig. 1. Replication of iDNA-derived V4020 virus in pMG4020-transfected Vero cells. (a) Vero cells were transfected with pMG4020 iDNA plasmid and probed in IFA with TC83-specific antiserum followed by FITC-conjugated secondary antiserum. Mounting medium containing propidium iodide counterstain (Vector Labs, Burlingame, CA) was used to visualize nuclei of the cells. (b) Western blot of V4020 vaccine virus isolated from Vero cells transfected with pMG4020 iDNA. As a control, the TC83 vaccine was used. M, Marker SeeBlue Plus2 (Thermo). 1, TC83 virus control; 2, V4020 virus; 3, uninfected Vero cells. Western blotting was done using vaccine viruses purified by ultracentrifugation (5 h at 26,000 rpm, SW28 rotor) from the supernatant of Vero cells transfected with pMG4020 or infected with TC83 vaccine. Proteins were separated using 4–12% gradient SDS-PAGE, transferred to a nitrocellulose membrane and probed with reference anti-TC83 mouse antisera at 1:1000 dilution. (c) Infectious center assay (ICA) of Vero cells transfected with pMG4020 after passage 10 (Supplementary Fig. 1). Vero cells transfected with pMG4020 were allowed to adhere for 6 h, covered with agarose to develop plaques for 24 h at 37 °C, and stained with neutral red. (d) Sequencing of pMG4020 to confirm attenuating mutations (shown with arrows) at nt 3 and E2-120 after passages 0 and 10. Attenuating nt3 and E2-120 codon CGA are underlined.

fected cells at 8–24 h post transfection using TC83-specific antiserum. By 24 h post-transfection, the majority of cells were positive for vaccine antigens (Fig. 1a). At 24 h post-transfection, many cells were detached and CPE was clearly visible. The V4020 virus was isolated from the supernatant of transfected cells and probed in western blot along with the TC83 vaccine. By western blot, no difference in protein processing was detected between V4020 and TC83 (Fig. 1b). Infectious centers were observed by ICA as plaques at ~30 h post-transfection (Fig. 1c). Replicating virus was also detected by direct plaque assay of the media samples from pMG4020-transfected Vero cells (Fig. 2).

3.4. V4020 vaccine replication in virus-infected and DNA-transfected Vero cells

In the next experiment, we compared replication dynamics of the V4020 vaccine virus in the V4020-infected and in pMG4020-transfected Vero cells. Cells were either infected with 100–1000 PFU of V4020 virus (Fig. 2) or transfected with 100 ng, 10 ng, and 1 ng of pMG4020. As controls, cells were infected with 1000 PFU of TC83 vaccine. Samples of the growth medium were collected from transfected and infected cells every 6 h and the viruses were quantitated by plaque assay (Fig. 2). Transfection with 1, 10 and 100 ng of pMG4020 resulted in the rapid replication of V4020 virus. This is in agreement with our previous study [15] showing that less than 10 ng of iDNA plasmid is sufficient to launch the virus. As expected for rapidly replicating alphavirus, growth curves showed mostly single-step growth kinetics, with the exception of transfection with 1 ng of pMG4020. Transfection of 100 ng of iDNA and infection with 100 PFU of V4020 virus resulted in a comparable replication kinetics (Fig. 2a). Plaques of V4020 appeared to be relatively uniform in size, with average size of ~0.6 ± 0.2 mm (Fig. 2b).

As expected, infection of Vero cells with 1000 PFU of TC83 virus also resulted in the rapid replication of TC83 virus reaching 10^{10} PFU/ml in 24 h post-infection. The TC83 plaques showed more variation in size than V4020 plaques, with average size of ~1.1 ± 0.3 mm, and ~88% of plaques were larger than the average size of V4020 plaques (Fig. 2b). The results suggested the potentially attenuated growth and improved homogeneity of the

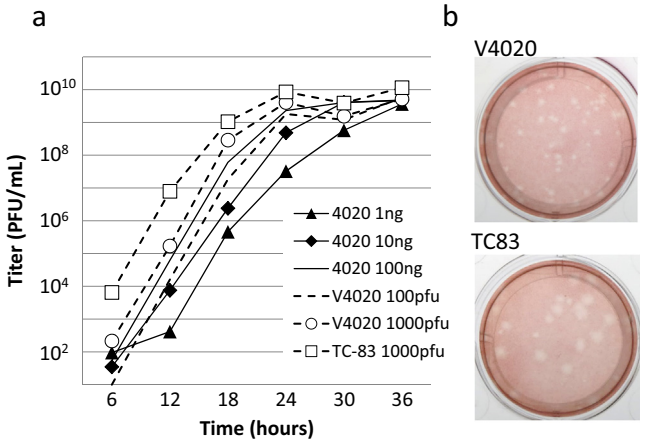

Fig. 2. (a) Replication kinetics of V4020 virus in virus-infected, and pMG4020-transfected, Vero cells. Vero cells were either infected with 10^2 or 10^3 PFU of indicated virus, or transfected with indicated amounts of pMG4020 iDNA. Infection with TC83 vaccine virus was used as a control. Plaque titer was determined in duplicates, error bars are not visible at the scale shown. Limit of detection is 5 PFU/ml. (b) Morphology of V4020 and TC83 plaques in Vero cell monolayers, by plaque assay.

Table 1
Immunogenicity and Efficacy of V4020 vaccine in BALB/c mice.

Vaccine Virus[a]	Viremia direct, % on day 2	Viremia, by amplification, % on day 2	$PRNT_{80}$, % on day 28 [mean log2] (Range)	$PRNT_{50}$, % on day 28 [mean log2] (Range)	Survival[b]	Morbidity	Viremia, after challenge, % (Range, PFU/ml)	Virus titer, brain tissue, % (Range, PFU/g)
V4020	0	20	100 [8.9] (160–1280)	100 [9.9] (320–2560)	100	0	0	0
TC83	20	80	100 [9.3] (320–1280)	100 [9.7] (320–1280)	100	0	0	0
Negative Control	0	0	0 (N/A)	0 (N/A)	0	100	100 (10^5–10^6)	100 (10^7–10^8)

[a] Vaccines were administered to BALB/c mice (n = 5) s.c. using a single dose of 10^4 PFU. The data are average of two experiments. For PRNT, % of seroconverted animals, mean log2 titer, and the range of PRNT titer in the individual animals are shown.
[b] Challenge was done with 1×10^4 PFU of VEEV TrD virus in 20 μl.

V4020 vaccine virus in comparison with the TC83 virus; however, additional research is needed.

3.5. Immunogenicity and efficacy of V4020 vaccine in mice

BALB/c mice were vaccinated with a single s.c. dose of 10^4 PFU of V4020 virus prepared from the growth medium of pMG4020-transfected Vero cells. Similarly, 10^4 PFU of TC83 vaccine virus was administered as control (n = 5 per group, two independent repeats). After injections, all mice remained healthy with no detectable adverse effects such as changes in weight or behavior. Viremia could not be detected in V4020-vaccinated mice by direct plaque assay (detection limit 25 PFU/ml). However, replicating virus was detected in the serum of ~20% animals by amplification in Vero cells followed by CPE analysis and plaque assay (Table 1). In contrast, viremia was detectable in the TC83-vaccinated mice by both direct assay and by amplification. At day 28, all mice seroconverted as determined by IFA and PRNT, with high titers of neutralizing antibodies, with $PRNT_{50}$ titer reaching 2560.

On day 28, animals were transferred to a BSL3 laboratory and challenged with 10^4 PFU of the wild type VEEV TrD. All V4020-vaccinated animals survived challenge with no morbidity. No weight loss was detected in V4020-vaccinated mice (Fig. 3a). Moreover, no viremia was detected in the serum, and no virus was detected in the brain tissues in V4020-vaccinated mice after challenge. In contrast, control mice vaccinated with unrelated control flavivirus rapidly lost weight, succumbed to lethal disease and died with high viremia and high virus load in the brain tissues (Table 1, Fig. 3a).

3.6. Vaccination with V4020 delivered by transfection with pMG4020 in vivo

To determine if the delivery of V4020 vaccine virus *in vivo* using pMG4020 results in induction of protective immune responses, BALB/c mice were vaccinated with a single dose of 5 μg or 0.5 μg of pMG4020 plasmid iDNA intramuscularly (i.m.) followed by *in vivo* electroporation. An additional group of mice received vaccination with the control pMG4017containing additional mutation. Animals showed no adverse effects and remained healthy after vaccinations, similarly to the controls inoculated with unrelated plasmid of similar size. Serum samples were collected on days 2–4 post vaccination for detection of viremia, as well as at day 28 for seroconversion. Viremia post-vaccination was not detectable in any animals by direct plaque assay. One tenth of serum samples from the pMG4020-vaccinated animals at 2 days post-vaccination

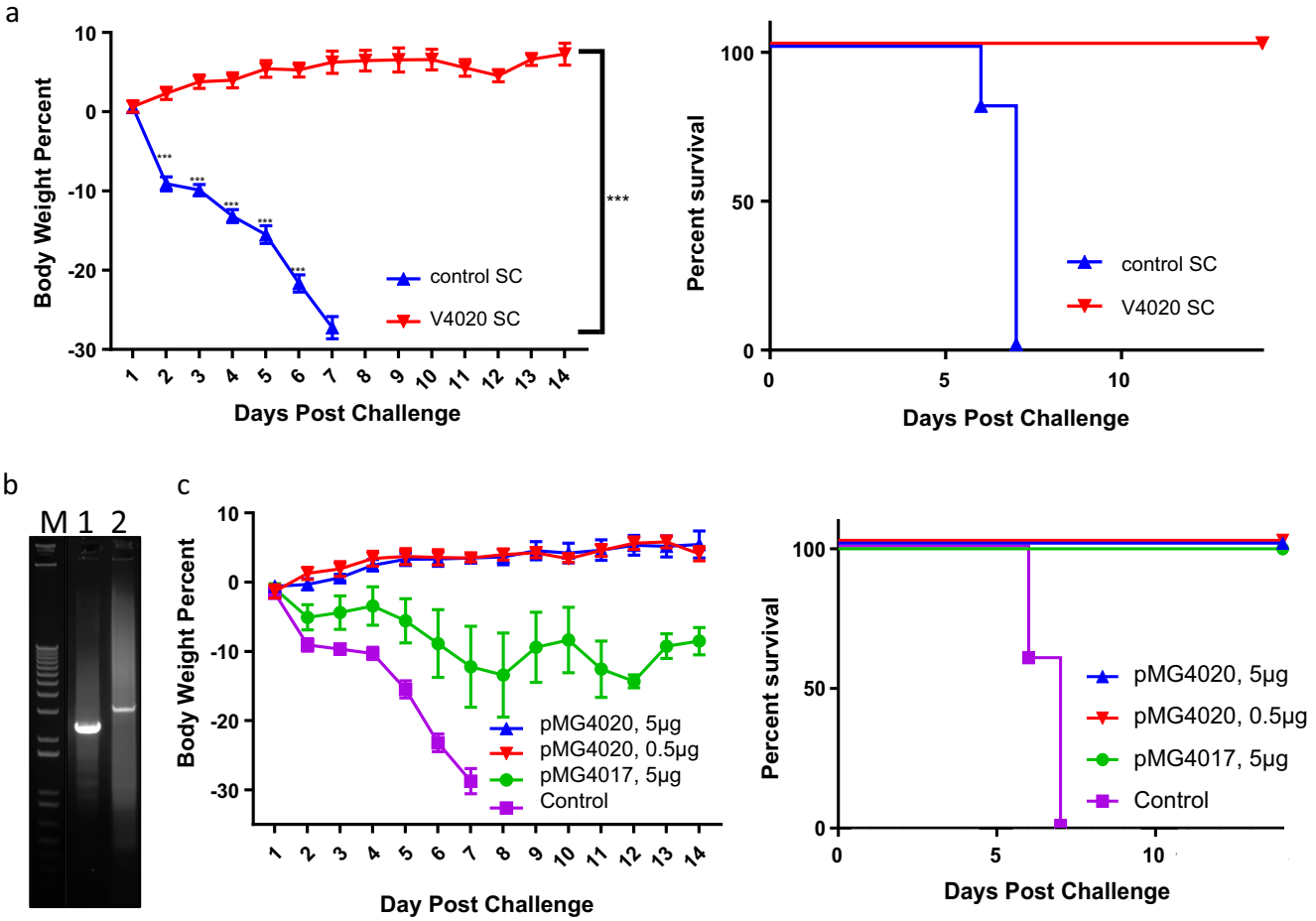

Fig. 3. (a) Survival and morbidity of BALB/c mice vaccinated s.c. with 10^4 PFU of V4020 and challenged with VEEV (Table 1). On day 28 after vaccination, mice were transferred into BSL3 facility and challenged s.c. with 10^4 PFU of VEEV TrD virus. Animals were daily monitored for weight loss (left panel) and survival (right panel) after challenge. For weight loss, *** $p < 0.001$ tested by 2-way ANOVA with Bonferroni's post-hoc analysis by day. For survival, $p < 0.001$ tested by Mantel-Cox log rank test. (b) RT-PCR to confirm rearrangement in V4020 RNA from viremic mouse (lane 1, expected size 2.4 Kb) in comparison to the TC83 RNA (lane 2, expected size 3.2 Kb). (c) Survival and morbidity of BALB/c mice vaccinated with pMG4020 and challenged with VEEV. Mice were vaccinated using 5 μg or 0.5 μg of pMG4020 i.m. followed by electroporation (Table 2). On day 28 after vaccination, mice were transferred into BSL3 facility and challenged s.c. with 10^4 PFU of VEEV virus. Mice were daily monitored for weight loss (left panel) and survival (right panel. $p < 0.001$, tested by Mantel-Cox log rank test.

Table 2
Immunogenicity and Efficacy of pMG4020 iDNA vaccine in BALB/c mice.

Vaccine plasmid[a]	Viremia direct, % on day 2	Viremia, by amplification, % on day 2	$PRNT_{80}$, % on day 28 [mean log2] (Range)	$PRNT_{50}$, % on day 28 [mean log2] (Range)	Survival[b]	Morbidity	Viremia, after challenge, % (Range, PFU/ml)	Virus titer, brain tissue, % (Range, PFU/g)
pMG4020 5 μg	0	20	100 [9.3] (320–>640)	100 [9.9] (640–1280)	100	0	0	0
pMG4020 0.5 μg	0	40	100 [9.1] (320–>1280)	100 [9.7] (320–>1280)	100	0	0	0
pMG4017 5 μg	0	0	0	10 (10)	100	60	60 (10^5–10^6)	0
Negative Control plasmid 20 μg	0	0	0	0	0	100	100 (10^5–10^6)	100 (10^7–10^8)

[a] Vaccines were administered to BALB/c mice (n = 5) i.m. and electroporation *in vivo* using an indicated dose of pMG4020. The data are average of two experiments. For PRNT, % of seroconverted animals, mean log2 titer, and the range of PRNT titer in the individual animals are shown.
[b] Challenge was done with 1×10^4 PFU of VEEV TrD virus in 20 μl.

showed the presence of vaccine virus by virus amplification in Vero cells. RNA was isolated from the recovered amplified virus, and PCR fragments containing partial sequences of GP and C genes were generated by RT-PCR. The expected sequence was confirmed by PCR and DNA sequencing including rearrangement of the GP and C genes (Fig. 3b) and the major attenuating mutation E2-120Arg [21].

By day 28, all pMG4020-vaccinated mice seroconverted as determined by IFA and PRNT with high titers of neutralizing antibodies (Table 2). However, seroconversion was undetectable in V4017-vaccinated mice except one serum showing $PRNT_{50}$ titer of 10. As expected, no viremia or seroconversion was detected in negative controls (Table 2).

On day 28 post-vaccination, animals were transferred to the BSL3 facility and challenged s.c. with 10^4 PFU of virulent VEEV TrD. The pMG4017-vaccinated mice were protected from lethal disease, however, more than half mice showed morbidity with weight loss (Fig. 3c). These mice also showed viremia after challenge, although no virus was detected in the brain tissues. In contrast, all pMG4020-vaccinated animals survived challenge with no detectable morbidity (Fig. 4c). Furthermore, challenged animals did not have any viremia after challenge, and there was no detectable virus in the brain tissues (Table 2). In contrast, all unvaccinated control animals developed high levels of viremia, lost weight, and died after challenge with high titers of virus in blood and brain tissues (Fig. 3c; Table 2).

3.7. V4020 shows attenuated replication in brain of vaccinated mice

BALB/c mice were inoculated i.c. to compare the replication of V4020 versus TC83 in the central nervous system. No significant differences in body weight were observed in V4020- and TC83-inoculated mice. However, TC83-inoculated mice tended to have lower body weights on days 6–7 post-challenge (Fig. 4a), which corresponds to the typical peak of disease observed with VEEV TrD s.c. challenge. Furthermore, the TC83 mice displayed increased clinical signs of infection on days 6–7 (Fig. 4b). Notably, mice infected with V4020 had no detectable virus after 5 days post-challenge, while in TC83 group the virus load was $>10^5$ PFU/g (Figure 5c) and persisted till day 9 (data is not shown).

4. Discussion

VEEV can be spread by many mosquito vectors including *Culex*, *Mansonia*, *Psorophora*, and *Aedes* species and is capable of causing explosive outbreaks [5,22–24]. When rapid containment of outbreaks is needed, live-attenuated vaccines have an advantage because they induce rapid and strong immunity with a single vaccination. Furthermore, live-attenuated vaccines are among the most cost-effective and broadly used public health interventions representing approximately half of all licensed vaccines. In the recent years, live vaccines Zostavax, FluMist and others have been licensed for human use demonstrating that live vaccines can be configured to meet stringent FDA safety standards [25].

The TC83 vaccine for VEEV was prepared by classic virology using multiple passages in tissue culture to select attenuating mutations [9,21]. However, only two attenuating mutations have been identified in the TC83 vaccine [21]. Adverse events in people who have received the TC83 vaccine have been associated with reversion mutations [13,14,26]. Another live-attenuated vaccine, V3526, was one of the first rationally-designed VEEV vaccines prepared using the infectious clone technology utilizing transcription *in vitro* to generate infectious RNA genomes [27,28]. Other rationally designed, live-attenuated VEEV vaccine approaches included chimeric viruses [29,30], ribosomal frameshifting [31], IRES-

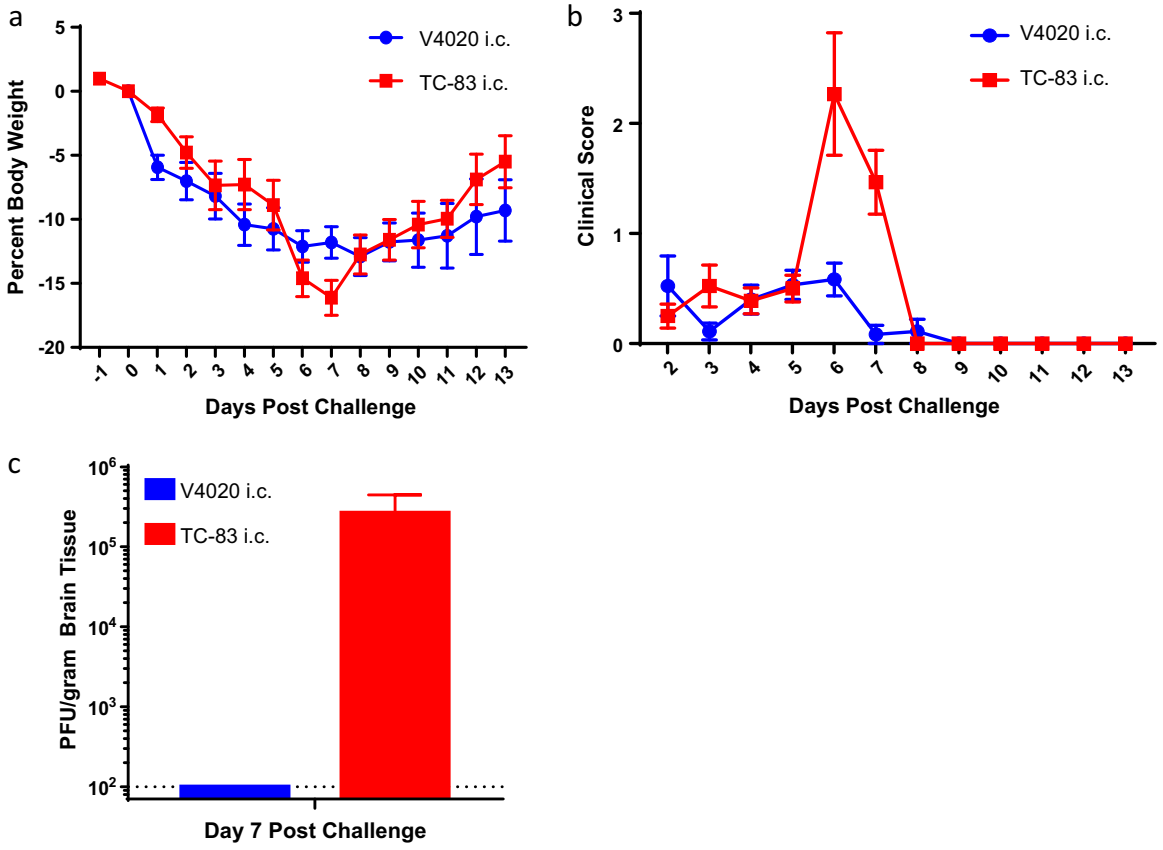

Fig. 4. (a) Body weight and (b) clinical score of BALB/c mice inoculated i.c. with 10^5 PFU of either V4020 or TC83 vaccine viruses. (c) Brain tissue was collected from 3 mice per group, homogenized, and tested by standard plaque assay to determine the viral titer in the brain on day 7 with a 10^2 PFU limit of detection.

based vaccine [32] and a host-restricted, mosquito-borne chimeric Eilat virus engineered to express VEEV structural proteins [33]. Experimental VEEV vaccine approaches also included replication-defective vaccines such as MVA and replicon vectors, VLPs, recombinant subunit antigens [34], inactivated viruses [35–38], and DNA vaccines expressing VEEV subunit antigens [39,40].

The TC83 vaccine is the only vaccine used in the U.S. to protect at-risk medical personnel [41]; however, it requires improvement because of adverse reactions. The long clinical history warrants inclusion of TC83 attenuating mutations into the next generation VEEV vaccines. Previously, we have reported DNA-launched TC83 vaccine [15]. Unlike previous efforts that involved *in vitro* transcription and transfection of the full-length RNA in the cytoplasm of cultured cells to generate virus [42,43], in the iDNA infectious clone, the full-length RNA is transcribed in the nucleus and transported into the cytoplasm. A similar approach of generating live virus by plasmid transfection has been reported for Sindbis alphavirus [44], paramyxovirus [45], poliovirus [46], and Kunjin, a subtype of the West Nile flavivirus [47]. We previously used iDNA infectious clone technology to prepare an experimental vaccine for chikungunya alphavirus [48], as well as for Yellow Fever and Japanese Encephalitis flaviviruses [49,50]. The described V4020 vaccine combined several strategies designed to improve VEEV vaccine safety, including genetic rearrangement and stabilized attenuating mutations. Rearranged genomes lead to the attenuation of many viruses including VEEV and are resistant to reversions because many independent mutations would be required to restore the wild type virus sequence [18–20]. We showed that V4020 is also highly immunogenic and efficacious against challenge with pathogenic VEEV. The V4020 can be either used directly for vaccination or can be delivered *in vivo* using pMG4020 plasmid encoding the functional genome of V4020 downstream from the CMV promoter. Both V4020 virus and pMG4020 plasmid induced strong protective immunity following a single-dose vaccination. The advantage of V4020 vaccine prepared from pMG4020 iDNA is a well-controlled vaccine virus population to improve safety, as well as the ability to induce protection with a single vaccination. The latter can be especially important for containing outbreaks of VEEV. The rearranged genome does not contain mutations in the immunologically important epitopes, yet provides attenuation, which is resistant to reversion. The vaccine also includes clinically validated attenuating mutations derived from the TC83 vaccine. Thus, V4020 vaccine may represent a successful application of rational attenuation and molecular biology methods for preparation of live-attenuated vaccine. In addition, iDNA technology provides alternative way of vaccination via DNA immunization with potential advantages, such as vaccination or vaccine storage in warm climates [51].

The results showed comparable immunogenicity and efficacy of V4020 and TC83 vaccines in a mouse VEEV challenge model, with V4020 vaccine showing safety advantage. Additional research is needed including pMG4020 plasmid and V4020 virus stability studies, in-depth studies of the kinetics of immune response and seroconversion, as well as efficacy experiments using intranasal and/or aerosol challenge with VEEV. Additional experiments are planned to understand possible differences in the mechanism of action of the live-attenuated V4020 virus versus pMG4020 iDNA vaccine. If safety and efficacy is confirmed, the proposed V4020 vaccine can be a viable alternative to the current TC83 IND vaccine.

Declaration

The authors declare that they have no conflicts of interest except IT, AT and PP are the full-time Medigen employees and PP serves as director at Medigen.

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Appendix A. Supplementary material

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