ORIGINAL ARTICLE

Venezuelan Equine Encephalitis Virus V3526 Vaccine RNA-Dependent RNA Polymerase Mutants Increase Vaccine Safety Through Restricted Tissue Tropism in a Mouse Model

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Abstract

Background: Venezuelan equine encephalitis virus (VEEV) is an arbovirus endemic to the Americas, for which no vaccines or antiviral agents have been approved. TC-83 and V3526 are the best-characterized vaccine candidates for VEEV. Both are live-attenuated vaccines and have been associated with safety concerns, although fewer concerns exist for V3526. A previous attempt to improve the TC-83 vaccine focused on further attenuating the vaccine by adding mutations that alter the error-incorporation rate of the RNA-dependent RNA polymerase (RdRp).

Methods: The research herein examined the effects of these RdRp mutations in V3526 by cloning the 3X and 4X strains, assessing vaccine efficacy against challenge in adult female CD-1 mice, examining neutralizing-antibody titers, investigating vaccine tissue tropism, and testing the stability of the mutant strains.

Results: The V3526 RdRp mutants exhibited less tissue tropism in the spleen and kidney than the wild-type V3526, while maintaining vaccine efficacy. Illumina sequencing indicated that the RdRp mutations reverted to wild-type V3526 after five passages in murine pup brains.

Conclusions: The observed genotypic reversion is likely to be of limited concern, because wild-type V3526 remains an effective vaccine capable of providing protection. Our results indicate that the V3526 RdRp mutants may be a safer vaccine design than the original V3526.

Key words: Venezuelan equine encephalitis virus, TC-83, V3526, vaccine, fidelity

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a member of the *Alphavirus* genus in the family *Togaviridae* with an 11.5 kb positive-sense single-stranded RNA genome [1,2]. Venezuelan equine encephalitis (VEE) commonly causes mild flu-like symptoms, but severe symptoms can occur in approximately 4–14% of cases in humans [3–6]. Severe symptoms include encephalitis,

confusion, coma, photophobia, and seizures [3]. Death as a result of VEE is rare but occurs in approximately 1% of cases, depending on the patient age and the outbreak [7,8].

Multiple VEEV outbreaks have occurred across Central and South America; the most recent outbreak occurred in Peru in 2006 [9–15]. A Central American outbreak between 1969 and 1972 resulted in the deaths of 50,000 equines and 93 humans, and hundreds of additional cases of human disease were reported [13]. The outbreak reached southern Texas in 1971, resulting in 1,500 equine deaths and 110 cases of human disease [12]. Another major outbreak in Venezuela between 1992 and 1995 resulted in 4,000 equine deaths and between 75,000 and 100,000 cases of human disease [14]. The virus tends to recede and emerge in epizootic (outbreak) cycles every 14–20 years and to cause disease in humans and equids [9,14]. Nonetheless, VEEV is believed to cause tens of thousands of human infections every year. VEEV is considered a biological risk agent, owing to its high aerosol infectivity, numerous documented infections through laboratory exposure, and potential for weaponization [16,17]. Because of the potential for re-emergence coupled with the biological risk factors, a publicly available vaccine for VEE is needed to protect at-risk populations, healthcare providers, biomedical researchers, and emergency responders in the event of an outbreak.

Numerous vaccine candidates for VEE exist, but TC-83 and V3526 are currently the best-characterized candidates [18,19]. TC-83 was previously approved for limited use to protect military personnel and researchers, but V3526 has demonstrated improved protection, safety, and a lower likelihood of reversion or pseudo-reversion to wild type (WT) [19–21]. TC-83 was made by serial passaging of the Trinidad donkey (TRD) strain of VEEV in fetal guinea pig heart cells 83 times. A single nucleotide polymorphism (SNP) at the 3rd position of the 5´UTR and a substitution at the 120th position of the E2 protein confer the most attenuation [18,22]. The development of V3526 involved site-directed mutagenesis of a TRD cDNA clone to remove the PE2 furin-cleavage site and create an E1 protein F253S point mutation [23]. However, safety concerns exist for both vaccine constructs. Vaccination with TC-83 can lead to clinical symptoms in 23% of human recipients and lack of neutralizing antibodies in 18% of recipients, and the vaccine can potentially be transmitted by a mosquito vector [20,24]. The safety concerns regarding V3526 include fever and flu-like symptoms in human recipients; the presence of viral antigen in the brains of vaccinated animals, thus indicating possible neurovirulence; and the potential for transmission of the virus by a mosquito vector [24–26].

A previous attempt to improve the TC-83 vaccine candidate focused on the use of mutations altering the error-incorporation rate of the RdRp [27–31]. The mutants included TC-83 3X, which contains the G8R, E31G, and A90T mutations in the RdRp gene nsP4, and TC-83 4X, which contains these nsP4 mutations in addition to C482Y, which was originally discovered in the related alphavirus chikungunya virus (CHIKV) [27,29]. The C482Y

substitution has been shown to decrease the RdRp error frequency in CHIKV and thus has been hypothesized to be a high-fidelity variant [29]. Research on TC-83 has suggested that the 3X and 4X mutants have lower virulence than the WT. Our research examined the effects of these 3X and 4X mutations in the V3526 platform, because V3526 has shown better protection and safety than TC-83 in animal studies. Additionally, the furin-cleavage-site deletion should hinder reversion to WT TRD [19,23,32,33]. Our study indicated that V3526 3X and 4X have less tissue tropism than WT V3526, thereby indicating improved vaccine safety without sacrificing immunogenicity.

METHODS
Animals and viruses
The V3526, V3526 3X, and V3526 4X virus strains were cloned, *in vitro* transcribed, and electroporated to generate viral stocks as previously described for the TC-83 RdRp mutants [27]. Viral stocks were quantified with Vero cell plaque assays before each experiment to ensure consistent inoculation doses. All work with VEEV 3908 was performed in either BSL3 or ABSL3 laboratories, whereas all work with V3526 was performed in BSL2 facilities in accordance with University of Texas Medical Branch (UTMB) regulations and all relevant state and federal laws.

All mice were ordered at 6 weeks of age or as timed-pregnant dams from Charles River, and were housed in the Galveston National Laboratory ABSL2 facilities. CD-1 mice were chosen because they are frequently used in testing for vaccine safety and efficacy, thus facilitating comparisons between this study and previous research on vaccines for VEE [27,28,34,35]. Work was performed after IACUC approval and followed all UTMB regulations, and state and federal laws.

Plaque assays
Plaque assays were performed by seeding Vero (ATCC® CCL-81™) cells at a density of 2×10^5 cells/well in a 12-well plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin and streptomycin, and 10% fetal bovine serum (FBS). The cells were incubated overnight at 37°C with 5% CO_2. Viral samples were serially diluted 10-fold in DMEM supplemented with 1% penicillin and streptomycin, and 2% FBS. The plated cells were infected with the diluted viruses and incubated at 37°C with 5% CO_2 for 1 hour with gentle rocking every 15 minutes. The cells were then overlaid with 0.4% agarose in DMEM supplemented with 1% penicillin and streptomycin, and 2% FBS, then incubated at 37°C with 5% CO_2 for 36–48 hours. The cells were then fixed with formalin and stained with 0.25% crystal violet. Plaques were counted and multiplied by the dilution factor to determine the final concentration.

Vaccine efficacy
Adult female CD-1 mice were vaccinated subcutaneously with 10^4 or 10^5 plaque-forming units (PFU) of V3526

parent, V3526 3X, or V3526 4X. Phosphate-buffered saline was used as a mock control. Weight and clinical signs of infection were monitored daily for the first 14 days after infection. Serum was collected on day 42 after vaccination to assess neutralizing antibodies. The mice were challenged subcutaneously with 10^5 PFU of VEEV 3908 at 60 days after vaccination. Weight and clinical signs of infection were observed for 11 days after the challenge. Survival statistics were assessed with a log-rank (Mantel-Cox) test. Weight statistics were assessed with Tukey's multiple comparison test. All statistics were analyzed in PRISM 8 software.

Plaque reduction neutralization test (PRNT)

The $PRNT_{50}$ and $PRNT_{80}$ assays in this study were performed with the standard method against VEEV V3526, V3526 3X, and V3526 4X [36]. Mouse serum samples were incubated for 1 hour at 56°C for inactivation before a 10-fold dilution in DMEM supplemented with 2% FBS and 1% penicillin and streptomycin. The samples were then diluted 2-fold in the supplemented DMEM from a range of 1:20 to 1:640. The diluted samples were mixed with a known concentration of the virus and incubated for 1 hour. The virus-mixed serum samples were then added to a monolayer of Vero (ATCC® CCL-81™) cells that had been seeded on a 12-well plate the previous day at a density of 2×10^5 cells/well. All plates were treated as standard Vero cell plaque assays after infection. The limit of detection (LOD) was 1:20. Any sample below the LOD was set at 1:10 or half the LOD. The highest dilution was 1:640. Any sample above the highest dilution was set at 1:1280 or twice the highest dilution to differentiate samples at the limits from samples past the limits.

Tissue tropism

Adult female CD-1 mice were infected at 6 weeks of age with 10^4 PFU of V3526 parent, V3526 3X, or V3526 4X. Blood and tissue samples were collected at 1, 2, and 7 days after vaccination. Full-body perfusions were performed on the first two collection days to mitigate the effects of high viremia on tissue titers. Mice were verified daily for weight and clinical signs of infection. Statistical analysis of weight change was performed in PRISM 8 software with Tukey's multiple comparison tests.

Microcentrifuge tubes containing 0.5 mL DMEM supplemented with 1% penicillin and streptomycin, and 2% FBS, in addition to a ball bearing were used to collect the spleen, liver, brain, and kidney samples. A QIAGEN TissueLyser was used to homogenize samples before clarification via centrifugation. Vero cell plaque assays were used to titrate samples. Statistical analysis was performed in PRISM 8 software with Sidak's multiple comparison tests.

Illumina sequencing

V3526 parent, V3526 3X, and V3526 4X were used to intracranially infect 6-day-old mouse pups with 10^4 PFU of virus in biological duplicate. The number of pups in each group varied slightly depending on the litter size, and all pups were randomized. Pup brains were harvested 36–48 hours after vaccination, and a QIAGEN TissueLyser was used to homogenize the samples. Clarification via centrifugation was performed to remove excess tissue and collect virus. Titrations were performed with a Vero cell plaque assay before intracranial injection of the next round of pups until a total of five passages.

RNA isolation was performed on the passage 5 brain homogenate supernatants for V3526, V3526 3X, and V3526 4X with TRIzol™ LS reagent according to the manufacturer's instructions. The UTMB Next Generation Sequencing Core performed the Illumina sequencing with a NextSeq 550 system. RT-PCR conversion of RNA into double-stranded cDNA was used to prepare the sequencing library. Fragmentation of the cDNA was performed before ligation of each fragment to an adapter. A glass flow cell was then populated by the adapter-ligated fragments, and template clusters were amplified. Reversible adapter-ligated terminator nucleotides were used to sequence each cluster. A previously described pipeline was used to assemble reads [37]. The starting plasmid sequences for each vaccine were compared against the sequencing results for the identification of major nucleotide variants.

The Illumina sequencing data were analyzed by examination of common SNPs that occurred in at least three of the four RdRp mutants. Only mutations present at ≥0.5% of the sequenced population were considered in the analyses. A list of 22 SNPs was created according to these criteria. Each mutation was assessed for the percentage frequency in the population for each biological replicate tested, the gene where the mutation occurred, and the amino acid change encoded.

RESULTS
Vaccine efficacy

Vaccine efficacy was examined for the V3526 3X and 4X mutants with adult CD-1 outbred mice. CD-1 mice were vaccinated with 10^5 PFU of the V3526 vaccine parent, 3X, or 4X. The post-vaccination survival analysis for each group (Fig 1A) indicated that no animals succumbed after vaccination, as expected. The post-vaccination weights (Fig 1B) indicated that all animals gained weight over the first 14 days, and no significant differences were observed between mutants and the parent strain.

The CD-1 mice were challenged 60 days after vaccination with 10^5 PFU of the epizootic VEEV 3908. All vaccinated mice survived the challenge, whereas unvaccinated (mock) mice all succumbed by day 5 after the challenge (Fig 1C). Furthermore, all vaccinated animals maintained their weights after the challenge, whereas the mock-vaccinated animals steadily lost weight until they met the criteria for euthanasia (Fig 1D). The average weight of the mock-vaccinated animals was significantly lower than that in each of the vaccine test groups on days 2–5 after the challenge, as measured by Tukey's multiple comparison test. We observed no significant differences in animal survival
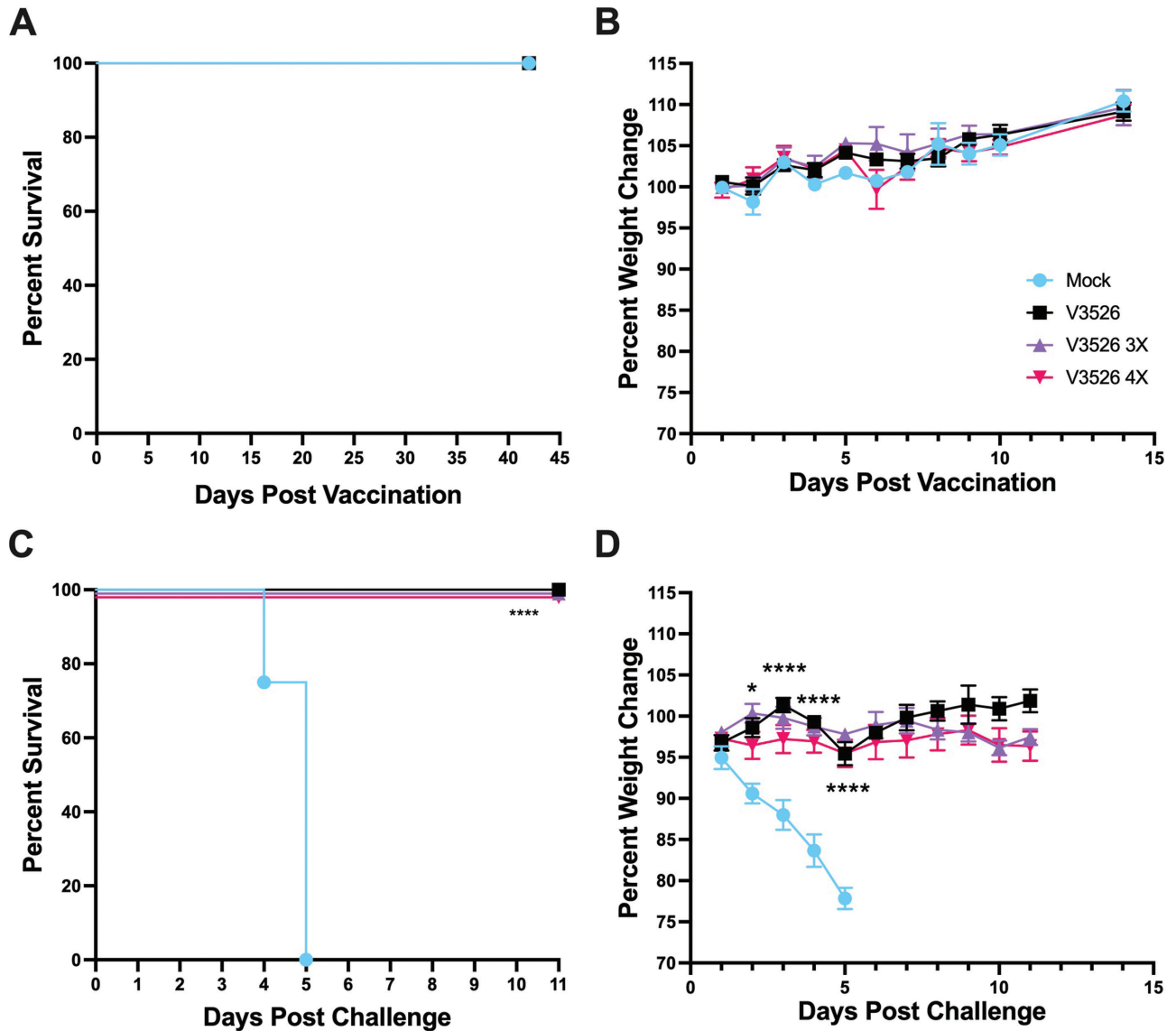
FIGURE 1 | Percentage survival and percentage weight change after vaccination and challenge of CD-1 mice. Adult female CD-1 mice received 10 PFU of V3526 parent, V3526 3X or V3526 4X for the vaccination, and 10% PFU of VEEV 3908 for the challenge. Mice were monitored for A) post-vaccination survival and C) post-challenge survival. Weight charts are represented as B) post-vaccination and D) post-challenge. In the post-challenge survival graph, **** represents a P-value <0.0001 for each test group vs. mock. V3526 3X and 4X are offset from 100% for improved data visualization. In the post-challenge weight graph, * represents a P-value <0.05, and **** represents a P-value <0.0001 vs. mock at each indicated timepoint (2–5 DPC). A log-rank Mantel-Cox test was used for survival analysis, and Tukey's multiple comparison test was used for weight analysis. Weight-chart error bars represent SEM.

or weight between the mutants and their respective parent strains after the challenge.
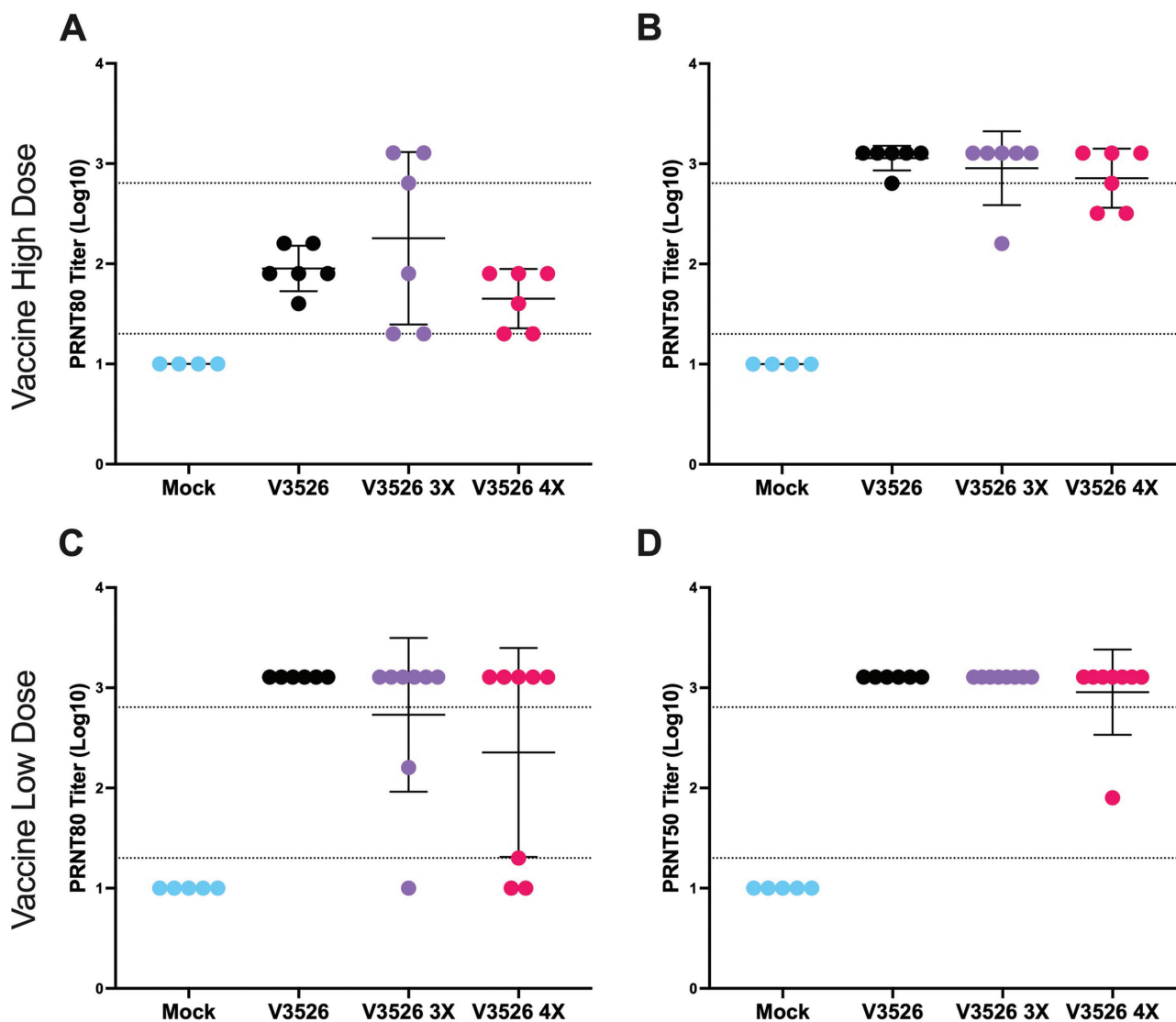
Neutralizing-antibody titers

Neutralizing-antibody levels were measured with PRNTs with serum from mice vaccinated with either 10^5 PFU (Fig 2A, B) or 10^4 PFU (Fig 2C, D). PRNT levels in mice vaccinated with 10^5 PFU of V3526 3X or 4X mutants produced neutralizing-antibody titers similar to those with the parental V3526, and no significant differences were detected, as measured by Sidak's multiple comparison test. For the 10^4 PFU dose, no significant differences were observed between the RdRp mutants and the V3526 parent strain.

Interestingly, more mice vaccinated with 10^4 PFU had titers outside the assay's detectable range (>1:640), thus indicating a potential dose effect. Given that the lower dose might elicit higher neutralizing-antibody titers, this dose was used in subsequent studies.

Vaccine tissue tropism

Vaccine tissue tropism was examined because previous TC-83 RdRp mutant studies indicated that the 3X and 4X mutants have a diminished ability to overcome the host barriers to infection present during tissue dissemination and the establishment of neurovirulence [27]. CD-1 mice were vaccinated with 10^4 PFU of V3526 parent, 3X, or 4X.

FIGURE 2 | Neutralizing-antibody titers in CD-1 mice after vaccination with 10^5 or 10^4 PFU. Neutralizing-antibody titers were observed in adult female CD-1 mice after vaccination with 10% PFU of V3526 parent, V3526 3X, or V3526 4X for A) 80% plaque reduction and B) 50% plaque reduction. Neutralizing-antibody titers were observed after vaccination with 10^4 PFU of V3526 parent, V3526 3X, or V3526 4X for C) 80% plaque reduction and D) 50% plaque reduction. The highest dilution was set at $\log_{10} = 2.8$ (1:640), and the LOD was set at $\log_{10} = 1.3$ (1:20). No statistical differences were observed via Sidak's multiple comparison test.

Serum, brain, spleen, kidney, and liver samples were collected on days 1, 2, and 7 after vaccination to measure viral load. Mice were perfused with phosphate-buffered saline on the first 2 days to eliminate potential viremia contamination. The weights for all infected animals (Fig S1A) averaged between 95% and 105% of the initial weight over the course of the experiment, similarly to the post–vaccination weights observed for the 10^5 PFU dose, without signs of disease.

Viral loads were undetectable in the serum (Fig S1B), brain (Fig 3A), or liver (Fig 3D) at all timepoints for each of the V3526 vaccines tested. The viral load in the spleen was detectable for the V3526 parent strain on days 1 and 2 after infection, but the V3526 mutants did not show a detectable viral load at any timepoint (Fig 3B). The V3526 parent strain had a significantly higher viral load than the mutants on the first 2 days after infection, as measured by Sidak's multiple comparison test. The viral load in the kidney was detectable in one mouse for the V3526 parent strain on the first day after infection, but the viral load in the spleen was undetectable at each timepoint for the V3526 mutants (Fig 3C).

Sequencing vaccine reversion during neurovirulence testing

V3526, and the 3X and 4X RdRp mutants were passaged five times in duplicate in neonatal mouse pup brains to assess the safety and stability of the mutant constructs. Mouse pup brains were chosen for this experiment because they are a highly permissive model that allows the virus to replicate to high titers and mutate rapidly in the process [38,39]. Illumina sequencing of passage 5 virus was used to examine changes in the mutant repertoire of V3526 and the RdRp mutants over the course of passaging.
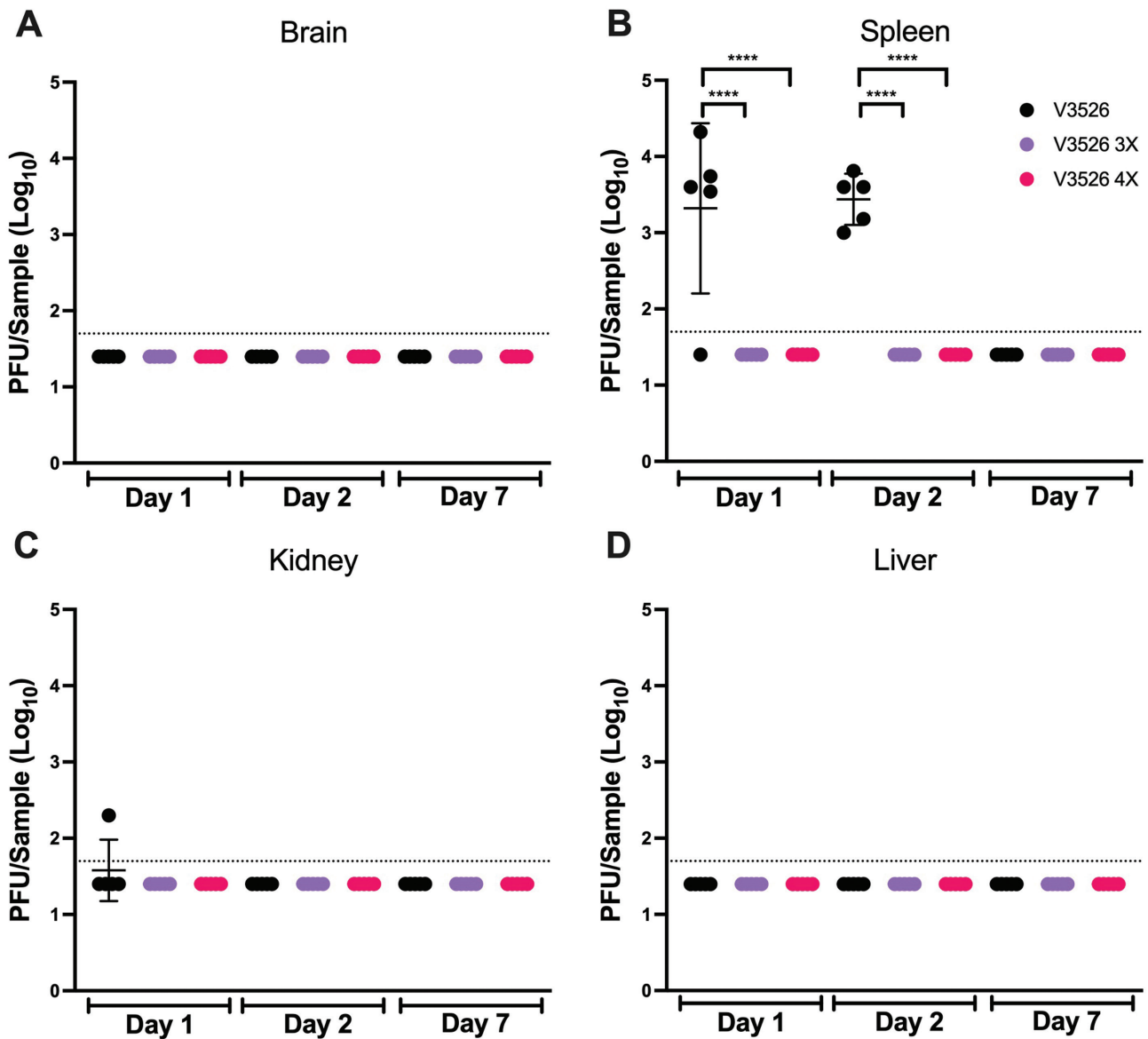
FIGURE 3 | Viral load in tissues of adult female CD-1 mice vaccinated with 10^4 PFU V3526 parent, 3X, or 4X. Vaccinated mice were monitored for viral load in the A) brain, B) spleen, C) kidney, and D) liver at 1, 2, and 7 days post-vaccination. The LOD was set at 50 PFU or PFU/sample (Logo) of 1.7. **** depicts a P-value <0.0001, as measured by Sidak's multiple comparison test vs. V3526 parent. Error bars represent standard deviation.

The mutational frequencies for each major nucleotide variant (i.e., >0.5% prevalence) were compiled (Tables S1–S3) and then narrowed (Table 1) to identify SNPs occurring in at least three of the four RdRp mutant samples. Common mutations were found in all genetic regions except for the 5´UTR, capsid, and 6K genes. The mutations C5724G, G5794A, and A5970G correspond to the R8G, G31E, and T90A genetic reversions to the V3526 parent strain genotype in the nsP4 region, respectively. The T7208C mutation corresponds to a silent substitution in the nsP4 protein coding region. The mutation A7147G was present in both replicates of the V3526 4X strain encoding a Y482C genetic reversion in the nsP4 coding region. Each of these substitutions, including the silent mutation, represents a genotypic reversion to the V3526 parent strain from the fidelity variant strains after serial passage in mouse pup brains. All other major nucleotide variants in Table 1 were present in at least one of the V3526 parent strain replicates.

DISCUSSION

V3526 and TC-83 are among the best–characterized vaccine candidates for VEE [18,19]. TC-83 is currently the only vaccine candidate approved for limited military use, but V3526 has shown similar or greater protection to that of TC-83 in previous animal studies [19,32,33]. Owing to the presence of a furin–cleavage–site deletion in V3526, reversion to WT TRD should be more difficult than that for the two SNPs associated with the attenuation observed in TC-83 [23]. Neither vaccine candidate has been approved for public use, and researchers and military personnel have limited

TABLE 1 | Common mutations in the V3526 3X and 4X RdRp mutants compared with mutations in parental V3526. The criterion for including a major mutation in this table was presence in at least three of the four sequenced fidelity variants. The nucleotide positions of SNPs are represented as "Position." Nucleotides present in the starting plasmid are represented as "Reference." Nucleotides present in Illumina sequencing data are represented as "Mutation." The percentages of mutations present in each Illumina sequenced population are depicted as "Freq." followed by the name of the sample. Genes in which mutations occurred are depicted as "Gene." Substitutions that occurred as a result of the mutation are depicted as "Amino Acid Change." * in the Position column denotes a mutation that was present in the RdRp mutants but absent in the V3526 parent strain. * in the Amino Acid Change column represents a genotypic reversion to resemble the V3526 parent strain.

Commonly observed mutations									
Position	Reference	Mutation	Freq. parent (A)	Freq. parent (B)	Freq. 3X (A)	Freq. 3X (B)	Freq. 4X (A)	Freq. 4X (B)	Gene	Amino acid change
1545	C	T	98.02%	0.00%	99.93%	99.61%	97.82%	0.00%	nsP1	R501C
1696	C	A	99.72%	0.00%	99.91%	99.80%	98.10%	0.00%	nsP2	A16D
5275	A	G	99.35%	0.00%	99.94%	99.78%	98.00%	0.00%	nsP3	H415R
5724*	C	G	0.00%	0.00%	100.00%	99.81%	98.30%	0.00%	nsP4	R8G*
5794*	G	A	0.00%	0.00%	99.98%	99.85%	98.25%	0.00%	nsP4	G31E*
5970*	A	G	0.00%	0.00%	99.97%	99.78%	97.97%	1.85%	nsP4	T90A*
7208*	T	C	0.00%	0.00%	99.90%	99.70%	97.95%	0.00%	nsP4	Silent
8549	G	A	18.68%	0.00%	19.27%	19.76%	16.41%	0.00%	E3	G55R
8552	T	A	16.15%	0.00%	15.56%	15.11%	14.43%	0.00%	E2	S1T
8572	G	T	99.51%	1.36%	99.95%	99.62%	97.58%	77.76%	E2	K7N
8804	C	T	99.70%	0.00%	99.88%	99.70%	97.39%	0.00%	E2	H85Y
8910	C	G	99.70%	0.00%	99.95%	99.77%	97.77%	0.00%	E2	T120R
9061	A	G	99.73%	0.00%	99.95%	99.70%	97.54%	0.00%	E2	Silent
9126	T	A	99.77%	0.00%	99.94%	99.71%	97.45%	0.00%	E2	V192D
9267	T	A	99.69%	0.00%	99.94%	99.72%	97.80%	0.00%	E2	I239N
9438	C	T	99.76%	0.00%	99.92%	99.76%	98.03%	0.00%	E2	T296I
9475	T	C	99.80%	0.00%	99.95%	99.75%	97.86%	0.00%	E2	Silent
9519	G	A	99.56%	0.00%	99.78%	99.58%	97.30%	0.00%	E2	G323E
10469	T	A	99.71%	0.00%	99.93%	99.71%	97.98%	0.00%	E1	L161I
10621	A	T	99.73%	0.00%	99.94%	99.66%	97.73%	0.00%	E1	Silent
10746	C	T	99.70%	0.00%	99.90%	99.74%	98.01%	0.00%	E1	S253F
11373	C	T	0.95%	0.00%	0.79%	0.78%	1.09%	0.00%	3'UTR	N/A

access to TC-83 vaccination, because the only distributor is the Special Immunizations Program [40]. Therefore, improving V3526 vaccine candidate safety is critical. Herein, we accomplished this improvement by adding the 3X and 4X RdRp mutations previously described in TC-83 and CHIKV to develop a vaccine candidate showing evidence of increased safety by decreasing tissue tropism without sacrificing immunogenicity, as measured by PRNTs [27].

Neutralizing-antibody titers are a widely recognized correlate of protection for evaluating alphavirus vaccines [41–43]. Consequently, $PRNT_{80}$ titers are often discussed in the evaluation of VEE vaccine candidates [17,27,44]. The V3526 RdRp mutants produced titers equivalent to those of the parent strain after inoculation with 10^4 or 10^5 PFU of each construct for both $PRNT_{80}$ and $PRNT_{50}$. However, the

$PRNT_{80}$ results after inoculation with 10^4 PFU indicated that more mice produced titers above the highest dilution of 1:640 than after inoculation with 10^5 PFU. Because samples above 1:640 were not titrated beyond this cutoff, the titers might have been even higher than indicated in this study. Three mice had titers below the LOD for the $PRNT_{80}$ after inoculation with 10^4 PFU, but the $PRNT_{50}$ data demonstrated that all animals seroconverted. The neutralizing-antibody data indicated that vaccination with the V3526 mutants produced neutralizing-antibody titers equivalent to those of the V3526 parent strain.

Antigens for V3526 have been detected in the brain and olfactory mucosa via immunohistochemistry, and undetectable viremia has been described in multiple animal models [19,25,44,45]. In accordance with prior reports, our study

did not detect viremia in mice vaccinated with 10^4 PFU of any of the V3526 vaccines, thus suggesting that the vaccines cannot be transmitted to a mosquito vector [24,46]. Viremia after TC–83 vaccination might have resulted in unintentional vaccine transmission among mosquito populations in the past, thus supporting the use of the V3526 vaccine rather than the TC–83 vaccine in the future [47]. Low viral titers have been reported in the liver in C3H/HeN mice [48]; consequently, the undetectable liver viral load in our study was an expected result. Undetectable viral loads in the brain indicate that the incorporated RdRp mutations do not result in neuroinvasion after subcutaneous injection. Decreased tissue tropism for the 3X and 4X mutants in the spleen and kidney on the first 2 days after vaccination may indicate further organ tropism restriction while still maintaining efficacy, thereby resulting in a safer vaccine design than the V3526 parent strain. Some limitations of this study are the lack of data on viral load in additional lymphoid tissues and the absence of descriptions of the histopathological changes in the organs tested. These aspects, in addition to determination of whether mosquitoes can serve as vectors for these vaccines, should be investigated in future studies.

Serial passaging of viruses in the brains of mouse pups allows the viruses to replicate to high titers quickly while acquiring mutations that may occur during an infection [38,39]. This technique is used to assess vaccine stability and screen for mutations with the potential for reversion or pseudo–reversion to WT VEEV [39]. The Illumina sequencing data from this study indicated a genotypic reversion of the 3X and 4X mutants encoding G8R, E31G, A90T, and C482Y to the V3526 parent strain genotype after five passages in mouse pup brains. However, this genotypic reversion is probably of limited concern, because parental V3526 remains an effective vaccine capable of preventing disease [19]. The genotypic reversions to the parent strain might have occurred because mouse pup brains are a highly permissive model that allows the virus to grow with little selective pressure from a host immune system, and this effect was enhanced by each passage performed before sequencing [38,39]. The decrease in tissue tropism observed after vaccination with the mutants in the adult CD–1 mouse model suggests that the risk of vaccines genotypically reverting in healthy adults is low. This possibility should be interesting to pursue in future studies.

The Illumina sequencing data from this study also revealed a large difference in the mutations present between the biological replicates of V3526 and V3526 4X. Variations in the mutation profiles of the two virus strains may be explained by a founder effect resulting from the high mutation rate associated with alphaviruses [49]. All three viral strains were rescued from cDNA clones, in an effort to minimize founder effects and start the mouse pup brain passaging from a well–defined stock. However, V3526, and the 3X and 4X RdRp mutants were rescued in cell culture before the experiment began and consequently might have led to genetic diversity between replicates.

Because of VEEV's status as an agent of biological concern, coupled with the increased risk of re–emergence and the effects of global warming on vector–borne disease, a publicly available vaccine for VEE is important for improving biosafety and biosecurity [50]. The research presented herein improves on the V3526 vaccine candidate through the inclusion of RdRp mutations, which decreased viral tissue tropism while maintaining the efficacy of the parent strain. Future work will focus on increasing the stability of these mutations. These data provide further evidence that RdRp mutants can be used to create safer vaccines against alphavirus–caused diseases.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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