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Directed molecular evolution improves the immunogenicity and protective efficacy of a Venezuelan equine encephalitis virus DNA vaccine

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ABSTRACT

We employed directed molecular evolution to improve the cross-reactivity and immunogenicity of the Venezuelan equine encephalitis virus (VEEV) envelope glycoproteins. The DNA encoding the E1 and E2 proteins from VEEV subtypes IA/B and IE, Mucambo virus (MUCV), and eastern and western equine encephalitis viruses (EEEV and WEEV) were recombined *in vitro* to create libraries of chimeric genes expressing variant envelope proteins. ELISAs specific for all five parent viruses were used in high-throughput screening to identify those recombinant DNAs that demonstrated cross-reactivity to VEEV, MUCV, EEEV, and WEEV after administration as plasmid vaccines in mice. Selected variants were then used to vaccinate larger cohorts of mice and their sera were assayed by both ELISA and by plaque reduction neutralization test (PRNT). Representative variants from a library in which the E1 gene from VEEV IA/B was held constant and only the E2 genes of the five parent viruses were recombined elicited significantly increased neutralizing antibody titers to VEEV IA/B compared to the parent DNA vaccine and provided improved protection against aerosol VEEV IA/B challenge. Our results indicate that it is possible to improve the immunogenicity and protective efficacy of alphavirus DNA vaccines using directed molecular evolution.

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1. Introduction

Venezuelan, eastern, and western equine encephalitis viruses (VEEV, EEEV, and WEEV) are arthropod-borne alphaviruses that cause periodic epizootics in the Americas [1]. These New World alphaviruses cause diseases in humans characterized by fever, headache, and occasionally encephalitis. VEEV, EEEV, and WEEV are recognized as potential agents of biowarfare or bioterrorism, due largely to their considerable stability and high infectivity in aerosols [2]. The case-fatality rates for VEEV and WEEV are relatively low (1–15%), while EEEV has a case-fatality rate estimated to be as high as 70% [3,4]. No licensed human vaccines currently exist for these pathogens, and the experimental vaccines used under Investigational New Drug (IND) status suffer from high reactogenicity or poor immunogenicity. A live-attenuated VEEV vaccine, TC-83, was developed by serial passage of the virulent Trinidad donkey strain in cultures of guinea pig heart cells [5]. Although TC-83 is generally safe and elicits long-lasting protective immunity, it causes fever, headache, and malaise in ~25% of vaccinated individuals while ~20% of vaccine recipients fail to develop neutralizing antibodies [6]. Formalin-inactivated virus vaccines for VEEV, EEEV, and WEEV are safe and well tolerated, but they require frequent boosting to elicit detectable immune responses and provide only poor protection against aerosol challenge in some rodent models [7]. The reactogenicity of the live vaccine and the poor immunogenicity of the inactivated vaccines have prompted the development of improved vaccines for VEEV, EEEV, and WEEV.

Vaccination with plasmid DNA represents an alternative to conventional methods of developing viral vaccines. Some of the advantages of DNA vaccines include a lack of pre-existing host immunity to the plasmid vectors, low biological containment requirements for production, availability of well-established Good Manufacturing Practices (GMP), and ease of combining antigens to form multiagent vaccines. A DNA vaccine that expresses the VEEV subtype IA/B capsid protein (C) and envelope glycoproteins (E1 and E2) from the cloned structural gene regions (corresponding to the 26S mRNA region of the genome) under the control of the

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14. ABSTRACT

We employed a directed molecular evolution approach consisting of in vitro multigene DNA recombination and screening technologies in an attempt to improve the cross-reactivity and immunogenicity of Venezuelan equine encephalitis virus (VEEV) envelope glycoproteins E1 and E2. DNA encoding E1 and E2 from three VEEV subtypes (IA/B, IIIA, IE) and one strain each of eastern (PE-6) and western (CBA87) equine encephalitis viruses (EEEV and WEEV) were used to create libraries of recombined variants. Five different libraries were generated by recombining one or both parent glycoprotein genes. Several hundred of the recombined variants were used as naked plasmid DNA to vaccinate mice, and their serum samples were screened for the presence of antibodies in a virus-specific ELISA. Selected sera were further assayed for the presence of neutralizing antibodies by plaque reduction neutralization tests (PRNT). We found that approximately 10% of the recombined variants elicited greater levels of cross-reactive antibodies against all five viral antigens as compared to DNA vaccines expressing the parental genes. In addition, representative variants from a library in which only E2 genes were recombined elicited significantly increased neutralizing VEEV antibody titers as compared to the parent DNA vaccine and provided improved protection against aerosol VEEV challenge. These data demonstrate that in vitro, multigene DNA recombination and associated screening can lead to the selection of antigens that exhibit improved cross-reactivity, immunogenicity, and protective efficacy as compared to the wild-type proteins and suggest a broad utility for these technologies in optimizing vaccine antigens.

15. SUBJECT TERMS
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cytomegalovirus (CMV) promoter has been previously developed and evaluated by our laboratory. When delivered by gene gun inoculation, this DNA vaccine elicited high levels of total anti-VEEV IA/B antibodies in mice, but only low levels of VEEV IA/B neutralizing antibodies were observed [8]. In contrast, mice receiving the TC-83 vaccine typically developed high levels of neutralizing antibodies. Some 80% of the DNA-vaccinated mice were protected after a lethal aerosol VEEV IA/B challenge (~1000 LD_{50}), while 90% of the TC-83-vaccinated mice were protected. Although the data from this study demonstrated the feasibility of using a DNA vaccine to protect against aerosolized VEEV, further improvement is required to develop a completely protective VEEV IA/B DNA vaccine.

Directed molecular evolution can be used for developing and identifying novel protein variants with new or improved functionality [9–11]. Large libraries of functional chimeric genes are created by using *in vitro* multi-gene DNA recombination coupled with high-throughput screening of expressed chimeric variants with a relevant assay [12–14]. One application of this technology is the generation of improved vaccine immunogens [15,16]. DNA vaccines encoding the hepatitis B surface (HBs) antigen were readily improved after *in vitro* DNA recombination of the antigen-coding sequences from hepatitis B virus and related hepadnaviruses and screening of the resultant chimeric variants [17]. Two rounds of *in vitro* multi-gene DNA recombination resulted in up to 25-fold improvement in the immunogenicity of particular variants in mice compared to the wild-type sequence. This method has also successfully been used to generate a single chimeric dengue virus envelope protein that induces cross-reactive neutralizing antibodies against all four dengue serotypes in mice vaccinated with plasmid DNA [18]. In addition, improvements in the immunogenicity and cross-reactivity of DNA vaccine candidates for HIV-1 and the malaria parasite *Plasmodium falciparum* have also been made [19]. Here we report the application of directed molecular evolution to generate VEEV envelope glycoprotein variants with improved immunogenicity and protective efficacy against aerosolized VEEV IA/B challenge when administered as DNA vaccines in mice.

2. Materials and methods

2.1. Construction of libraries of recombined variants

The parental 26S structural genes of VEEV subtypes IA/B and IE, MUCV strain BeAn8 (formerly VEEV subtype IIIA), EEEV strain PE-6, and WEEV strain CBA87 having Genbank Accession numbers L01442, U34999, AF075253, AY722102, and DQ432026, respectively, were previously cloned [8, unpublished data]. The structural genes minus the capsid gene (E3-E2-6K-E1) were cloned into the eukaryotic expression vector pES, which contains a Col E1 bacterial origin of replication, a kanamycin resistance marker, a human cytomegalovirus immediate-early (CMV IE) promoter with its associated intron A, and a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA). This plasmid contains AscI and NotI cloning sites between the promoter and terminator sequences and the NotI site is fused in-frame to a DNA sequence encoding the FLAG tag (DYKDDDDK) followed by a stop codon. The envelope genes (E3-E2-6K-E1) of the various parents were amplified by polymerase chain reaction (PCR) using primers that introduced a 5′ AscI and a 3′ NotI site. The 5′ primer also introduced an initiator methionine and an alanine residue before the initial E3 residue for optimal translation initiation. At this time, mutations were also introduced into the 6K regions of the parent clones to create an SfiI restriction site to allow the E2 region to be independently shuttled as an AscI-SfiI fragment and the E1 region to be independently shuttled as an SfiI-NotI fragment. The PCR fragments were digested with AscI and NotI and ligated into the same sites of plasmid pES. All parent clones were tested for correct processing of the E1 protein by western blotting using a monoclonal antibody (mAb) to the C-terminal FLAG tag described previously (data not shown) [20].

Libraries of chimeric variant glycoprotein genes were generated after *in vitro* DNA recombination of the parental E2 and/or E1 genes of the five alphaviruses by using previously described methods [12,13]. A total of five different libraries were prepared from various combinations of the recombined E1 and E2 glycoprotein genes (Table 1). A crossover approach was required to recombine the VEEV IA/B, EEEV, and WEEV parental E2 and E1 genes due to the low homology between these parent sequences. Oligonucleotides were designed to force recombination at 8 crossover points in E2 (positions S90, C151, C184, P231, G207, C263, and F320 relative to E3 Met1) and at 6 crossover points in E1 (positions Y586, A659, Y727, P772, I801, and S897 relative to E3 Met1). Pairs of oligonucleotides consisting of 30 nucleotides from one parent at the 5′ end and 30 nucleotides of a second parent at the 3′ end of the first oligonucleotide and of 30 nucleotides from the second parent at the 5′ end and 30 nucleotides of the first parent at the 3′ end of the second oligonucleotide were required for each crossover point for each pair of parents. The crossover oligonucleotides were used as primers to generate PCR fragments from the VEEV IA/B, EEEV, and WEEV parents that would hybridize to adjacent fragments from the heterologous parents. The fragments were assembled and full-length recombined genes were rescued by PCR using flanking primers containing AscI and SfiI sites for E2 and SfiI and NotI sites for E1. The full-length recombined E2 and E1 genes were then cloned between the AscI and SfiI sites or the SfiI and NotI sites of the VEEV IA/B parent plasmid to generate libraries AG3 and AG5, respectively. The presence of the VEEV IA/B fragments in the recombined E2 gene regions in library AG3 and in the recombined E1 gene regions in library AG5 provided sufficient homology to permit a classical approach to generating libraries containing recombined E2 and E1 genes from all five parents. Fragments 100–200 nucleotides in length were generated from the E2 regions from library AG3 and the VEEV IA/B, IE, and MUCV parent clones, and the fragments were mixed and assembled and full-length recombined E2 genes were rescued by PCR using flanking primers containing AscI and SfiI sites. The full-length recombined E2 gene regions were then cloned into these same sites of the VEEV IA/B parent plasmid to generate the AG4 library. This same approach was used to generate full-length recombined E1 gene regions from the AG5 library and the VEEV IA/B, IE, and MUCV parents and these recombinant E1 gene regions were cloned into the SfiI and NotI sites of the AG3 library to generate library AG1 and of the AG4 library to generate library AG2. The various library ligations were then used to transfect TOP10 cells

Table 1
Alphavirus genes library development and outcome.

Library	E1 gene	E2 gene	Outcome
AG1	VEEV IA/B, IE; MUCV; EEEV; WEEV	VEEV IA/B; EEEV; WEEV	Poorly immunogenic; no cross-reactive antibodies
AG2	VEEV IA/B, IE; MUCV; EEEV; WEEV	VEEV IA/B, IE; MUCV; EEEV; WEEV	Immunogenic; cross-reactive antibodies
AG3	VEEV IA/B Only	VEEV IA/B; EEEV; WEEV	No cross-reactive antibodies
AG4	VEEV IA/B Only	VEEV IA/B, IE; MUCV; EEEV; WEEV	Immunogenic; cross-reactive antibodies
AG5	VEEV IA/B; EEEV; WEEV	VEEV IA/B only	Poorly immunogenic; no cross-reactive antibodies

(Invitrogen, Carlsbad, CA) to generate libraries containing at least 10^7 independent clones. Individual colonies were then used to inoculate cultures in Terrific Broth (Invitrogen) containing 40 μg/ml of kanamycin and individual clone plasmid DNA preparations were purified using the Biorobot (Qiagen, Valencia, CA).

2.2. Pre-screening of recombined variant clones

The variant clone plasmid DNA was used to transfect COS-7 cells seeded into 96-well plates (2×10^4 cells/well) using FuGENE 6 reagent (Roche Diagnostics, Basel, CH) according to the manufacturer's recommendations. At 24 h post-transfection, the cells were fixed and permeabilized with −20 °C methanol:acetone (1:1) for 10 min and whole-cell enzyme-linked immunosorbent assay (ELISA) was performed using the mAb directed against the C-terminal FLAG tag. Clones with high reactivity to the FLAG tag antibody were then used to transfect cells as described above, and at 24 h post-transfection, these cells were fixed with 2% paraformaldehyde for 15 min. Whole-cell ELISA was then performed using the E1-specific and VEEV, EEEV, and WEEV cross-reactive mAb 2A2C-3 that was obtained from John Roehrig, Centers for Disease Control, Fort Collins, CO and described previously [21] or with polyclonal hyperimmune mouse ascitic fluids (HMAF) against VEEV IA/B, EEEV, or WEEV.

2.3. Vaccination of mice and screening of variants by ELISA

The parent clones and all variant clones selected after pre-screening for expression were used to vaccinate individual 6–8 week-old female BALB/c mice. Each mouse received 10 μg of endotoxin-free plasmid DNA by intradermal injection at days 0, 21, and 42 and serum samples were collected at day 63. Initial antibody characterization was performed by obtaining optical density values only for duplicate 1:100 dilutions of serum samples tested against 250 ng per well of sucrose-purified, irradiated whole VEEV IA/B, IE; MUCV (BeAn8); EEEV (PE-6); or WEEV (CBA87) antigen by using screening ELISAs in 96-well format using methods previously described for VEEV IA/B [22]. Total IgG anti-virus antibody titers were then determined for clones found to generate cross-reactive responses in the screening ELISAs by performing endpoint ELISAs using duplicate serial dilutions of mouse serum. The results from individual mice were then verified by vaccinating groups of 4 mice with the parent clones and selected variant clones and determining endpoint total IgG anti-virus antibody geometric mean titers (GMT) by using standard ELISAs as described above. All ELISA results were normalized using values determined for negative control serum samples obtained from mice vaccinated with empty vector DNA.

2.4. Sequence analysis of variant clones

Nucleotide sequences of clones tested for improved immunogenicity were determined by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI Prism 3130xl genetic analyzer (Applied Biosystems). Translation of the nucleotide sequences into predicted amino acid sequences and nucleotide and amino acid sequence alignments were performed with Lasergene v7.0 software (DNASTAR, Madison, WI).

2.5. VEEV challenge of vaccinated mice

We vaccinated groups of 10 6–8 week-old female BALB/c mice with 4 μg of endotoxin-free negative control empty vector, VEEV IA/B parent, AG4-1C7 variant, AG4-1G2 variant, AG2-5A7 variant, or AG2-5A10 variant plasmid DNA per mouse at days 0, 21, and 42 using the XR-1 particle-mediated epidermal delivery device (PowderMed, Oxford, UK) as described previously [23,24]. A group consisting of 10 positive control mice was vaccinated with each animal receiving a single intramuscular injection of 0.2 ml of the TC-83 live-attenuated VEEV human IND vaccine. At day 70 after the initial dose, the mice were challenged by aerosol exposure with $\sim 10^4$ PFU ($\geq$1000 LD_{50}) of VEEV strain IA/B (strain Trinidad donkey) as described previously [25]. Samples collected from the all-glass impinger attached to the aerosol chamber were analyzed by plaque assay to confirm the dose of the inhaled virus. The mice were monitored daily for signs of illness and death for 28 days post-challenge and any moribund animals were euthanized. Serum samples were collected from all animals before challenge at days 21, 42, and 63 and at day 98 (28 days post-challenge) from challenge survivors. Endpoint total IgG anti-VEEV IA/B antibody titers were obtained for pre-challenge bleed samples by ELISA using whole inactivated VEEV IA/B antigen as described above. Neutralizing antibody titers required for an 80% reduction in the number of plaques were obtained for the final pre-challenge and post-challenge bleed samples by performing plaque reduction neutralization test (PRNT) as described previously [22].

2.6. Statistical analysis

Log_{10} transformations were applied to whole virus ELISA titers, F2 fragment ELISA titers, and $PRNT_{80}$ titers. Mixed model analysis of variance (ANOVA) with Tukey's post hoc tests was used to compare titers between groups at each time point. Survival analysis was performed with the Kaplan Meier method, with log rank tests used for comparison of survival curves. The effects of whole virus ELISA titers, F2 fragment ELISA titers, and $PRNT_{80}$ titers on the probability of survival were assessed using a backwards-selection logistic regression model. Analyses were conducted using SAS Version 9.1.3 (SAS Institute, Cary, NC).

2.7. Radiolabeling and immune precipitation assays

Transient transfection, radiolabeling, and immune precipitation were performed as described previously [24]. Briefly, COS-7 cells grown in 25-cm^2 cell culture flasks were transfected with 5 μg of the appropriate plasmid DNA with FuGENE 6 reagent (Roche Diagnostics) according to the manufacturer's recommendations for each assay. After 24 h, the cells were starved for 30 min in methionine- and cysteine-free medium and then radiolabeled for 4 h in medium containing 200 μCi of Promix ([^{35}S]methionine and [^{35}S]cysteine; Amersham Biosciences, Piscataway, NJ) per 25-cm^2 flask. Cell lysates were made using RIPA lysis buffer and the lysates were normalized for protein concentration by BCA protein assay (Pierce Biotechnology, Rockford, IL). Immune precipitation of 200 μl of labeled lysate was performed with 10 μl of the appropriate mouse vaccination serum sample or hyperimmune mouse ascitic fluids (HMAF). Reduced samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) on 4–12% Bis-Tris gradient gels with MOPS running buffer at 200 V of constant voltage. The gels were fixed, dried, and analyzed by using a Cyclone phosphorimager and OptiQuant software (PerkinElmer, Waltham, MA).

2.8. VEEV E2 fragment ELISA

The region of the VEEV IA/B E2 protein containing known linear neutralizing epitopes (N_{160}-A_{249}) [26] was expressed in *E. coli* using the pMALc vector system (New England Biolabs, Ipswitch, MA). The expressed VEEV IA/B E2 fragment was purified using affinity chromatography as previously described [19,27]. Antibody titers were determined for the final pre-challenge bleed samples against 100 ng per well of the purified protein fragment antigen by ELISA in 96-well format using methods described above.

3. Results

3.1. In vitro multi-gene DNA recombination of alphavirus envelope genes

The gene sequences encoding the E1 and E2 envelope glycoproteins from VEEV subtypes IA/B and IE, MUCV strain BeAn8 (formerly VEEV subtype IIIA), EEEV strain PE-6, and WEEV strain CBA87 were aligned to determine regions of homology and divergence. Using proprietary software, we were able to predict the sites where homologous recombination was most likely to occur, and the optimal conditions for recombining the parent sequences were determined. Libraries of genes expressing envelope glycoprotein variants were then created after *in vitro* recombination of the parental sequences using both classical and crossover approaches as described under Section 2 [12,13]. A total of five different libraries were created using various combinations of the parental E1 and E2 genes (Table 1).

3.2. In vitro pre-screening of recombined variant clones

A two-tiered *in vitro* pre-screening approach was used to select functional recombined variants for further analysis. The genes from the various recombinant libraries were first evaluated for high levels of protein expression based on the presence of a C-terminal FLAG epitope tag [20]. Transfected cells expressing the variant envelope glycoproteins were fixed, permeabilized, and stained with a monoclonal antibody (mAb) directed against the FLAG epitope (data not shown). Since the FLAG epitope sequence was present at the 3′ end of the gene encoding the envelope polypeptide, it was expected to be present on the cytosolic end of the processed E1 protein. Thus, a high anti-FLAG signal in this assay was most likely indicative of high-level expression of full-length or processed envelope polypeptide. The clones producing high levels of anti-FLAG signal from the first tier of pre-screening were then used to transfect cells, which were then fixed and tested for reactivity to E1-specific and VEEV, EEEV, and WEEV cross-reactive mAb 2A2C-3 that was described previously [21] or with polyclonal hyperimmune mouse ascitic fluids (HMAF) against VEEV IA/B, EEEV, or WEEV (data not shown). Since this second assay was performed in the absence of cell permeabilization, clones displaying high reactivity most likely expressed mature, processed envelope proteins on the cell surface. Using this pre-screening approach, we selected 50–100 clones that expressed high levels of processed variant envelope glycoproteins from each of the five recombinant libraries for immunogenicity studies in mice.

3.3. In vivo screening of recombined variants in mice

High-throughput screening of sera from approximately 300 individual mice vaccinated with single recombined variant constructs by intradermal injection of plasmid DNA was performed using virus-specific ELISAs. Control samples consisted of sera from mice vaccinated with empty vector DNA plasmid or with the wild-type parental VEEV, MUCV, EEEV, or WEEV envelope glycoprotein DNA vaccines. Approximately 10% of the sera from the recombined variants displayed average optical density (OD) values of 0.1 or greater against all five viral antigens in screening ELISAs (data not shown). In order to perform a more comprehensive analysis, we also determined endpoint ELISA titers for all cross-reactive sera. We found that the selected recombined variant DNA vaccines elicited substantial antibody titers against all five viral antigens (Table 2). At this time, we also observed that vaccination with certain recombined variants from the AG4 library resulted in higher anti-VEEV IA/B titers than those seen for the VEEV IA/B parental DNA (Table 2). All of the identified variants found to induce cross-reactive antibodies were members of either the AG2 library, in which the E1 and E2 genes from all five parent clones were recombined, or the AG4 library, in which the VEEV IA/B E1 gene was constant and the E2 genes from all five parent clones were recombined (Table 1). Consequently, no further studies were performed with recombined variants from the AG1, AG3, and AG5 libraries.

Table 2
Alphavirus parent and selected chimeric variant ELISA cross-reactivity.

Construct	VEEV IA/B	VEEV IE	MUCV	EEEV	WEEV
VEEV IA/B parent	4.19[a]	3.04	2.46	0.00	1.55
VEEV IE parent	2.61	3.97	1.83	1.16	0.00
MUCV parent	2.87	2.06	4.05	1.11	0.00
EEEV parent	1.92	1.89	1.36	2.77	1.70
WEEV parent	2.21	1.65	1.61	1.43	3.99
AG2-1B6	2.10	2.80	2.78	2.39	2.58
AG2-2H6	2.20	2.69	2.66	2.45	2.47
AG2-5A7	2.39	2.69	2.65	2.36	2.51
AG2-5A10	2.36	2.31	2.21	2.06	2.14
AG4-1C7	4.71	3.62	3.43	1.97	2.68
AG4-1G2	4.61	3.89	3.66	2.74	2.19
AG4-1G9	4.45	3.65	3.27	2.70	1.86
AG4-1H2	4.53	3.77	3.32	2.41	2.32

[a] ELISA results are presented as $\log_{10}$ titers and homologous titers are underlined.

To verify our findings on DNAs injected into individual mice, we vaccinated groups of four mice with each of the recombined variants selected from our initial high-throughput screening. We then measured cross-reactive antibodies in the resulting serum samples by comparing the GMT against VEEV IA/B, EEEV, and WEEV antigens (Fig. 1). These results confirmed that the selected recombined variants from the AG2 and AG4 libraries elicited high levels of cross-reactive antibodies against VEEV, EEEV, and WEEV glycoproteins. The AG4 library provided the largest number of clones inducing cross-reactive antibody responses, and once again the best variants from this library displayed increased antibody titers to VEEV IA/B compared to the VEEV IA/B parental clone. However, none of the sera from the recombined variants displayed greater reactivity to VEEV IE, MUCV, EEEV or WEEV antigens than did sera from mice vaccinated with the respective parental clones. Therefore, although our initial screening focused on the identification of recombined variants with high cross-reactivity, the two variant clones from each of the AG2 and AG4 libraries that induced the highest levels of anti-VEEV IA/B antibodies (AG2-5A7 and AG2-5A10, AG4-1C7 and AG4-1G2) were selected for more extensive analysis.

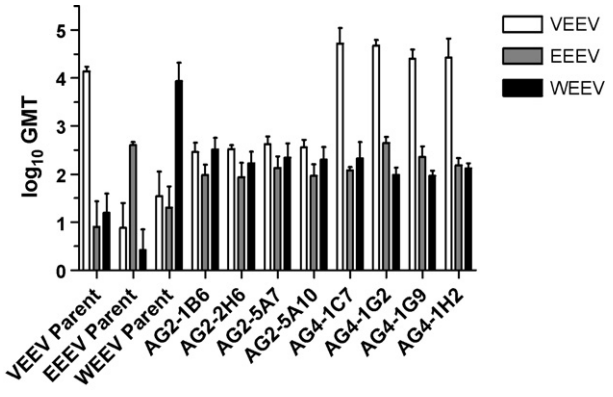

Fig. 1. Cross-reactivity of antibodies elicited to the variant alphavirus envelope glycoprotein antigens. Groups of four BALB/c mice were vaccinated intradermally on days 0, 21, and 42 with 10 μg of the VEEV IA/B parent, EEEV parent, WEEV parent, or recombined variant DNA. On day 63, serum samples were collected and endpoint total IgG anti-virus ELISA titers against purified whole, inactivated VEEV IA/B, EEEV, or WEEV antigen preparations were determined. The $\log_{10}$ group geometric mean titers (GMT) with the standard error of the mean (SEM) are shown for the parent constructs and representative clones from the AG2 and AG4 libraries.

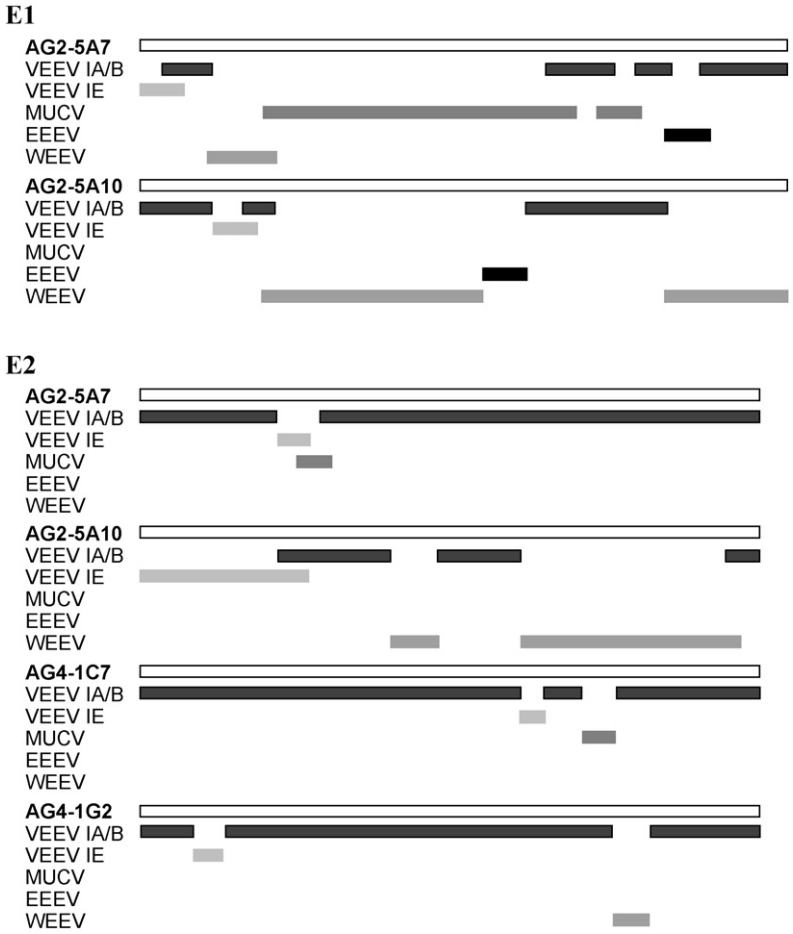

Fig. 2. Representations of the sequences of selected variant envelope glycoproteins. The protein sequences of the two variant clones from each of the AG2 and AG4 libraries displaying the highest levels of cross-reactive antibody responses in mice were aligned with the parental protein sequences. For each clone, blocks of protein sequence originating from the various VEEV, MUCV, EEEV, or WEEV parents are shown. Overlapping areas represent regions of complete homology between two or more parent sequences present in the variant clones.

3.4. Sequence analysis of selected recombined variants

We compared the nucleotide and predicted amino acid sequences of the E1 and E2 genes of the selected variants with those of the five parental viral genes. Examination of the predicted amino acid sequences of the selected AG2 clones showed that they were generally well recombined within E1 with sequences from four or more of the parents present (Fig. 2). The AG2-5A10 clone also exhibited significant recombination in E2, while more limited changes in E2 were observed for AG2-5A7. The selected clones from the AG4 library exhibited limited changes isolated exclusively in E2, VEEV IA/B E1 having been held constant in this library.

3.5. VEEV challenge studies in mice

We next evaluated the protective efficacy of the selected AG2 and AG4 recombined variant clones against VEEV IA/B aerosol challenge after they were administered as DNA vaccines in mice. Groups of 10 mice were vaccinated three times at three-week intervals with negative control empty vector, VEEV IA/B parent, AG2-5A7 variant, AG2-5A10 variant, AG4-1C7 variant, or AG4-1G2 variant DNA by particle-mediated epidermal delivery. A group of 10 positive control mice received single intramuscular injections of TC-83 live-attenuated VEEV human IND vaccine. Serum samples were obtained from all mice before each vaccination and 3 weeks after the final vaccination, and endpoint antibody titers against VEEV IA/B were determined by using ELISA (Fig. 3). As seen during the screening process, the AG4 variants elicited geometric mean antibody titers that were higher than those of the VEEV IA/B parent and the AG2 variants. The AG4 DNAs also induced anti-VEEV IA/B antibody levels similar to those observed for the TC-83 vaccine. The

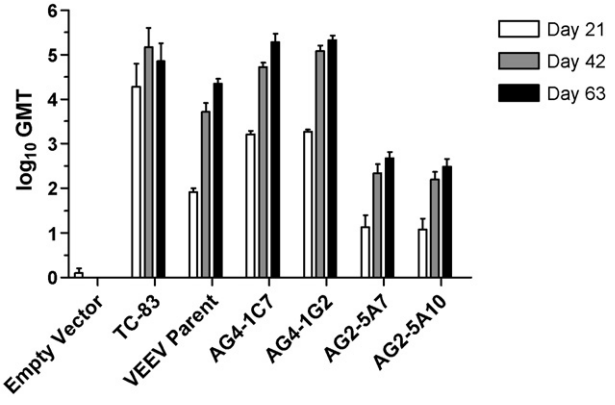

Fig. 3. Mouse anti-VEEV IA/B antibody responses. Groups of 10 BALB/c mice were vaccinated at days 0, 21, and 42 with 4 μg of empty vector, VEEV IA/B parent, or selected AG4 and AG2 variant DNA by particle-mediated epidermal delivery. A group of 10 positive control mice each received 0.2 ml of the TC-83 live-attenuated VEEV human IND vaccine by intramuscular injection. Serum samples obtained from all mice on days 21, 42, and 63 after the initial vaccination were assayed for total IgG anti-VEEV IA/B antibodies by using ELISA. The $\log_{10}$ GMT and SEM are shown for all groups.

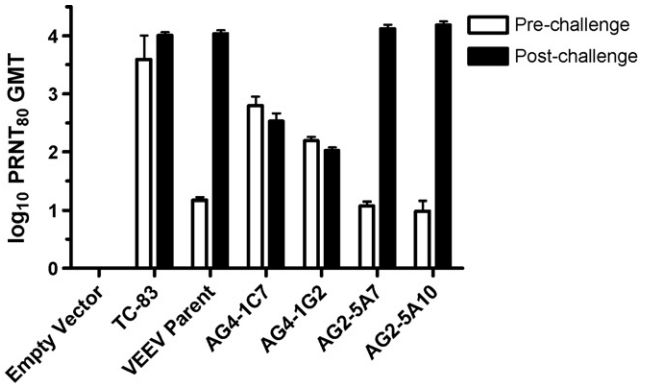

Fig. 4. Mouse anti-VEEV IA/B neutralizing antibody responses. Day 63 pre-challenge serum samples from all mice and post-challenge serum samples from all mice surviving the VEEV IA/B aerosol challenge were assayed for the presence of VEEV IA/B neutralizing antibodies by PRNT. The $\log_{10}$ GMT antibody titers required for 80% reduction in the number of plaques ($PRNT_{80}$) and SEM for the empty vector, TC- 83 positive control, VEEV IA/B parent, and selected AG4 and AG2 variants are shown.

VEEV IA/B parent DNA elicited levels of anti-VEEV IA/B antibodies similar to those reported in a previous study [8]. The levels of VEEV IA/B neutralizing antibodies in the final pre-challenge bleed serum samples were examined by PRNT (Fig. 4). As seen previously, vaccination with TC-83 resulted in very high titers of VEEV IA/B neutralizing antibodies, while mice vaccinated with the VEEV IA/B parent DNA developed low neutralizing antibody responses. The levels of VEEV IA/B neutralizing antibodies induced by the AG4 variants were significantly higher than those obtained by vaccination with the VEEV IA/B parent or the AG2 variant DNAs ($P < 0.001$).

The ability of the envelope variants to elicit protective immune responses against VEEV IA/B infection was evaluated by aerosol challenge of the vaccinated mice with $\sim 10^4$ PFU of VEEV IA/B ($\geq$1000 LD_{50}). The VEEV IA/B parent construct protected 80% of the mice from the lethal challenge (Fig. 5), which is the same level of protection elicited by this DNA vaccine in a previous study [8]. As in the previous study, mice vaccinated with the VEEV IA/B parent displayed signs of illness after challenge including ruffled fur, loss of appetite, and inactivity; however, the surviving mice completely recovered before the end of the 28 day post-challenge observation period. The AG2-5A7 and AG2-5A10 variants protected 80% and 70% of the mice, respectively, and the mice vaccinated with these constructs also displayed similar signs of illness as the VEEV IA/B parent mice although many also ultimately survived the challenge. In contrast, the AG4-1G2 and AG4-1C7 variants protected 100% and 90% of the mice, respectively, and the surviving mice did not exhibit

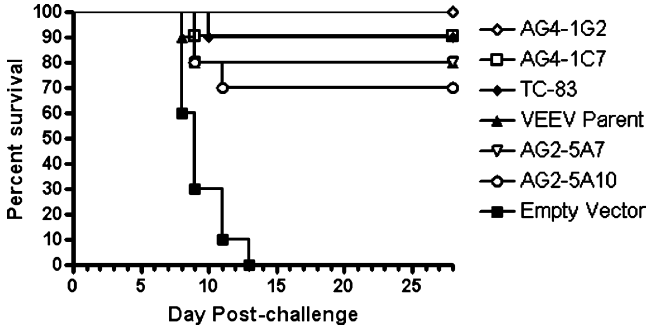

Fig. 5. Survival of vaccinated mice after VEEV IA/B aerosol challenge. Mice vaccinated with negative control empty vector DNA, TC-83 positive control, VEEV IA/B parent DNA, or selected AG4 and AG2 variant DNA were challenged with $\sim 10^4$ PFU ($\geq$1000 LD_{50}) of VEEV IA/B virus by the aerosol route. A Kaplan-Meier survival analysis is shown indicating the percentage of surviving mice at various times after challenge.

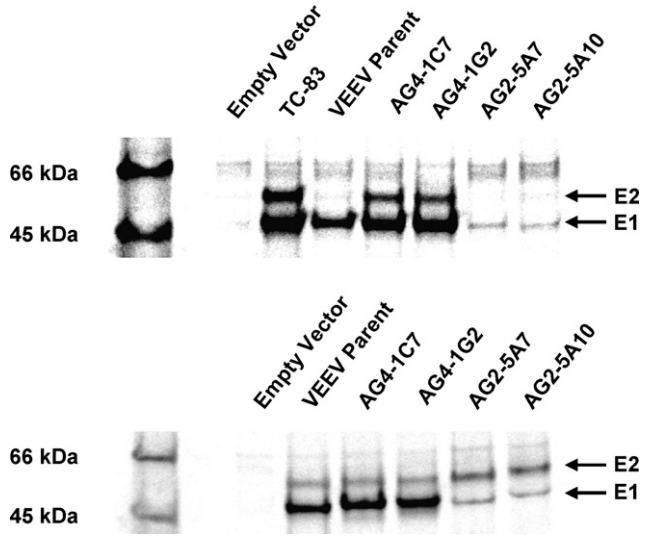

Fig. 6. Immune precipitation analysis of radiolabeled proteins. (A) Pools of serum samples obtained on day 63 following the initial vaccination of mice with the empty vector DNA, TC- 83 positive control, VEEV IA/B parent DNA, and selected AG4 and AG2 variant DNAs were used to immune precipitate radiolabled VEEV E1 and E2 envelope glycoproteins from lysates of COS-7 cells transfected with the parent VEEV IA/B parent DNA. (B) Lysates from radiolabeled COS-7 cells transfected with the empty vector, wild-type VEEV IA/B parent, or selected AG4 and AG2 variant DNAs were immune precipitated with hyperimmune mouse ascitic fluids (HMAF) to VEEV. Immune precipitation products were analyzed by PAGE and autoradiography. The results of phosphor imager analysis are shown, and the locations of 66-kDa and 45-kDa molecular size standards and of the E1 and E2 glycoproteins are indicated.

any signs of illness post-challenge. The improved protection of the AG4 variants correlated with the increased level of induced neutralizing antibodies (data not shown). The positive control vaccine group had a protection level of 90%, and the single animal that did not survive the challenge had no detectable neutralizing antibody response after vaccination with the TC-83 vaccine. Other than this one non-responder, the TC-83 mice also showed no signs of illness after the viral challenge.

To further assess vaccine efficacy, we measured the VEEV IA/B neutralizing antibody titers of sera obtained from all challenge survivors. The surviving mice from the groups vaccinated with the VEEV IA/B parent and AG2 library variants all exhibited significant increases in the post-challenge titers of VEEV IA/B neutralizing antibodies as compared to those present pre-challenge ($P < 0.0001$), while the survivors in the TC-83 and AG4 library variant groups did not show a significant increase in post-challenge neutralizing antibodies (Fig. 4).

3.6. Characterization of improved immunogenicity of selected recombined variants

In earlier studies, the majority of the VEEV neutralizing epitopes were shown to reside in the E2 envelope glycoprotein [21,26]. Therefore, we wanted to determine whether the increased induction of neutralizing antibodies by the improved AG4 chimeric variants was due to increased antibodies to the VEEV E2 protein. Consequently, we used pools of the day 63 pre-challenge sera from each vaccination group of the pathogen challenge experiment to immuno-precipitate wild-type VEEV IA/B E1 and E2 envelope glycoproteins (Fig. 6A). As seen previously in our laboratory, the pool of sera from mice vaccinated with the wild-type parental VEEV IA/B DNA vaccine mostly precipitated the E1 protein with only a small amount of E2 protein detectable (unpublished data). Antibodies elicited by the AG2 library variants precipitated less E1 protein than did sera from mice vaccinated with the VEEV parent and also

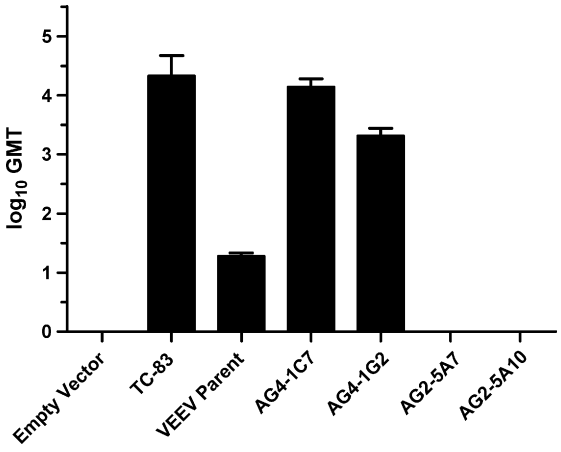

Fig. 7. Antibody responses to VEEV IA/B E2 neutralizing domains. ELISA was performed in 96-well format using 100 ng/well of purified VEEV IA/B E2 protein fragment (N_{160}-A_{249}) containing known VEEV neutralizing epitopes as antigen. The $\log_{10}$ GMT and SEM are displayed for serum samples obtained on day 63 after the initial vaccination of groups of mice vaccinated with negative control empty vector DNA, TC-83 positive control, VEEV IA/B parent DNA, or selected AG4 and AG2 variant DNA.

had only slight reactivity with the E2 protein. In contrast, sera from the AG4 variant vaccinated mice precipitated the E2 protein at levels more similar to that achieved with sera from TC-83 vaccinated mice.

It has further been demonstrated that a number of linear E2 neutralizing epitopes are located within a particular domain of the VEEV E2 protein (N_{160}-A_{249}) [26]. This fragment was expressed in *E. coli* as a MBP fusion protein, purified using affinity chromatography, and used as antigen in ELISA. The VEEV IA/B parent DNA immune sera showed a low level of reactivity to this fragment of VEEV E2, while the AG2 library variant samples did not react (Fig. 7). In contrast, the AG4 library variant samples elicited significantly higher antibody titers against this E2 neutralizing fragment as compared to VEEV IA/B parent samples ($P < 0.001$), and this increased reactivity correlated with the increased protection against VEEV IA/B aerosol challenge (data not shown).

Increased E2 gene expression could explain the increased antibody responses against the VEEV E2 glycoprotein and its neutralizing epitopes elicited by the selected AG4 variants. To examine this possibility, we tested the VEEV IA/B parent and selected AG2 and AG4 variant DNAs in transient expression assays. Immune precipitation with hyperimmune mouse ascitic fluids (HMAF) to VEEV revealed no apparent difference in the levels of E1 and E2 proteins produced by the AG4 variants as compared to the VEEV IA/B parent (Fig. 6B). However, the AG2 variants produced apparently lower levels of E1 protein compared to the VEEV IA/B parent and AG4 variants. We confirmed this lack of a noticeable difference in the levels of envelope glycoprotein production between the AG4 variants and the VEEV IA/B parent DNA by whole-cell ELISA against fixed, permeabilized cells transfected with these constructs and measured using the anti-FLAG epitope tag antibody (data not shown).

4. Discussion

Safe and effective vaccines are needed to protect against the encephalitic alphaviruses VEEV, EEEV, and WEEV, which continue to be recognized for their potential as agents of biological warfare. Due to the significant limitations associated with the available live-attenuated and formalin-inactivated encephalitic alphavirus vaccines, such vaccines are not currently being pursued for licensure. One platform for the development of next-generation vaccines is plasmid DNA. We previously demonstrated that a DNA vaccine expressing the wild-type structural proteins of VEEV IA/B protected mice and guinea pigs against peripheral challenge with VEEV IA/B and provided partial protection to mice against aerosol challenge; however, the vaccine elicited very low levels of neutralizing antibodies [8]. To further the development of DNA vaccines for the encephalitic alphaviruses, we sought to create chimeric alphavirus envelope glycoprotein antigens possessing improved cross-reactivity and immunogenicity by the application of directed molecular evolution.

After *in vitro* DNA recombination of parental E1 and E2 glycoprotein genes from VEEV IA/B, IE, MUCV, EEEV, and WEEV, we identified recombined variant genes that could elicit cross-reactive antibody responses when used as DNA vaccines in mice. These particular variants were derived by recombination of both the E1 and E2 envelope glycoprotein genes of VEEV IA/B, IE, MUCV, EEEV, and WEEV or by recombining only the E2 genes, while holding the VEEV IA/B E1 gene constant. The two representative variant clones from each of these two libraries displaying the highest level of anti-VEEV IA/B antibody responses were selected for evaluation of immunogenicity and protective efficacy in aerosol VEEV IA/B pathogen challenge studies in mice. We found that variants with only the E2 genes recombined, but not those with both genes recombined, induced both increased total anti-VEEV IA/B antibody responses and increased neutralizing antibody responses and possessed improved ability to protect against VEEV IA/B aerosol challenge relative to the wild-type VEEV IA/B parental DNA vaccine. Unlike mice given the parental DNA vaccine, or representative clones with both E1 and E2 chimeric genes, mice vaccinated with selected variant clones in which only the E2 gene had been subjected to recombination displayed no signs of illness post-challenge. This striking observation, taken together with the lack of a significant increase in post-challenge neutralizing antibodies, support the possibility that these variants are capable of inducing sterile immunity against VEEV aerosol infection in mice. However, additional analysis would be required to confirm a complete lack of viral replication in the brains of vaccinated mice after aerosol viral challenge.

Although the most widely accepted correlate of protection against VEEV is the presence of neutralizing antibodies directed against the E2 glycoprotein [1,28–31], it was previously shown that non-neutralizing antibodies could also be protective [32]. In agreement with those findings, both in our earlier studies and those reported here, we found that our parental VEEV IA/B DNA vaccine elicited very low levels of neutralizing antibodies in mice and guinea pigs but offered complete to partial protection against peripheral or aerosol challenges with VEEV IA/B [8]. Here we showed that the chimeric E2 vaccines elicited significantly improved neutralizing antibody responses in mice and that this was likely correlated with increased response to the E2 protein, as compared to the parental VEEV IA/B DNA vaccine. Further, our observations that the chimeric E2-containing DNA vaccines did not show increased E2 protein production, but did elicit noticeably increased antibody responses to a fragment of VEEV E2 with known linear neutralizing epitopes, suggest that the likely basis for the improved reactivity was due to improved presentation of the E2 neutralizing epitopes due to structural features of the variant proteins. Although currently there are insufficient data available regarding the structure of the VEEV envelope glycoproteins to completely confirm this possibility, some predictions can be made based on existing structural information for the envelope glycoproteins of the prototype alphavirus Sindbis virus (SINV) [33]. Based on the existing data, it appears that the changes in the chimeric E2 proteins that have the potential to alter structure are primarily localized in the region directly adjacent to the transmembrane spanning domain and not in the epitope-rich N-terminus of the E2 spike. However, recent functional characterization of the SINV

E2 glycoprotein using transposon linker-insertion mutagenesis has demonstrated that in addition to being involved in E1-E2 and E2-E2 interactions, this same region is also important for protein transport [34]. Therefore, potential for the changes in the E2 chimeras to affect protein processing or transport and influence subsequent antigen presentation cannot be completely discounted at this time. In our current studies, we are attempting to model the chimeric and wild type E2 proteins and are performing extensive epitope mapping studies to try to clarify the basis for the improved immunogenicity of the recombined E2 proteins.

Our analyses of the various libraries suggests that at least for VEEV, subtle changes concentrated in the E2 protein were more beneficial than more drastic changes involving both glycoproteins. In addition, the improvements seem to be related to recombination of several similar genes, rather than diverse genes. This hypothesis stems from our finding that the presence of the VEEV IA/B, IIIA, and MUCV E2 genes correlated with improved cross-reactivity. A library that was created by recombining only the VEEV IA/B E2 gene with EEEV and WEEV E2 genes, but still holding the E1 gene of VEEV IA/B constant, did not produce improved variants. Similarly, a library generated by recombining the E1 and E2 genes of VEEV IA/B, EEEV, and WEEV, or a library that had recombination concentrated in E1, also did not produce improved variants. Because we did not try to create a library from only multiple VEEV genes, we do not know if the EEEV and WEEV genes played any role in the improvements in the variants. Also, none of the variant clones exhibited improved immunogenicity against EEEV or WEEV. Together, these results suggest that recombination among several homologous genes that result in small changes in the overall amino acid sequence is likely a better approach to generating improved DNA vaccines than recombination of fewer or more distant genes. It would be interesting to attempt further recombinations using multiple E2 genes from EEEV and WEEV and to perform additional rounds of recombination of the already improved chimeric VEEV DNA vaccines.

In summary, our data suggest that it is possible to improve the immunogenicity and protective efficacy of alphavirus DNA vaccines after *in vitro* multi-gene recombination. We identified E2-chimeric DNA vaccines that, similar to the TC-83 live-attenuated VEEV human IND vaccine, offered protection against VEEV IA/B aerosol challenge in mice that correlated with potent virus-neutralizing ability. To our knowledge, this is the first report of the application of directed molecular evolution to generate recombined variant DNA vaccines that displayed such large improvements in the ability to elicit neutralizing antibodies. To extend and confirm our findings, we are currently examining the ability of the selected AG4 recombined variant DNA vaccines to confer protection against aerosol challenge with VEEV IE and MUCV in mice. We will also evaluate the immunogenicity and protective efficacy of the wild-type VEEV IA/B parent DNA vaccine and the selected AG4 recombined variants in an aerosol model of nonhuman primate infection with VEEV IA/B [35].

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The opinions, interpretations, conclusions, and recommendations contained herein are those of the authors and are not necessarily endorsed by the U.S. Army. All animal research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principles stated in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996. The USAMRIID facility where part of the animal research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

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