Vaccine 37 (2019) 3146–3150

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

An adjuvanted adenovirus 5-based vaccine elicits neutralizing antibodies and protects mice against chikungunya virus-induced footpad swelling

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ARTICLE INFO

Article history:
Received 13 October 2018
Received in revised form 9 April 2019
Accepted 24 April 2019
Available online 29 April 2019

Keywords:
Chikungunya
Oral
Vaccine
Tablet
Adenovirus

ABSTRACT

Over the past decade, chikungunya virus (CHIKV) has emerged as a major cause of mosquito-borne disease with transmission reported in over 100 countries worldwide. Although several strategies have been pursued for the development of a CHIKV vaccine, none has been approved yet. In this study, we describe the development of several vaccine vectors that express the structural proteins of the La Réunion CHIKV strain LR2006-OPY1. Protection from virus-induced pathologic changes was observed in vaccinated C57BL/6 mice, an important model for CHIKV vaccine development because of their ability to recapitulate several signs shown in infected humans. This study uniquely demonstrates the capacity of a mucosally-administered adenovirus vaccine to induce serum antibody responses and confer protective efficacy in a pre-clinical model. Our data provide further evidence in support of the clinical development of this oral Ad-CHIKV vaccine strategy in populations at high risk of contracting the disease.

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1. Introduction

Large epidemics of chikungunya virus (CHIKV) disease occur unpredictably and tend to be explosive. Reports of new infections are estimated at one million per year. Although the acute disease is generally self-limiting, many patients develop chronic polyarthritis that can last years after the initial infection [1,2]. Recently, significant outbreaks have occurred in Mombasa County (Kenya) (2018, 2017) , India (2016), Pakistan (2016), Bangladesh (2017) and, to a lesser extent, on the Mediterranean coast (Italy and France in 2017) [3].

Human protection from CHIKV infection is considered primarily mediated by humoral memory responses and the presence of neutralizing antibodies targeting the highly conserved E1 and E2 envelope glycoproteins. Four distinct lineages of CHIKV currently circulate throughout the world, but because they comprise a single serotype, a single vaccine may provide long-lasting cross-protection against all CHIKV strains [4]. Some vaccine candidates are currently being evaluated in preclinical and early phases of clinical trials but none has been approved yet [4]. We are developing an oral vaccine platform, based on a replication-incompetent adenovirus 5 (rAd) that expresses an antigen and a dsRNA adjuvant [5–9]. The ability to use different antigen genes with the same Ad5 vector-adjuvant gene cassette allows for a modular platform for making new vaccines, which greatly facilitates scalability and downstream production. Additionally, formulation of the rAd vaccine in tablets allows for non-sterile fill and finish processing, in contrast to injectables, further reducing costs and timelines. In humans, tablets of rAd expressing either capsid protein VP1 (for a norovirus vaccine) or hemagglutinin (for an influenza vaccine) have shown that substantial antibody responses are generated after a single administration [6,7]. Here, we describe the immunogenicity and efficacy of several vaccine vectors that express the structural proteins C-E3-E2-6K/TF-E1 of La Réunion CHIKV strain LR2006-OPY1 (accession number ABD95938). Proof-of-concept studies were performed intranasally in C57BL/6 mice, as they recapitulate some signs of CHIKV-infected humans including self-limiting arthritis, myositis, and tenosynovitis [10]. To our knowledge, this is the first report of a vaccine for CHIKV with the potential for an oral delivery that can induce serum antibody responses and confer protective efficacy in a pre-clinical model.

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https://doi.org/10.1016/j.vaccine.2019.04.069
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2. Results

2.1. Ad-CHIKV-SG, Ad-CHIKV-E3/E2/6K and Ad-CHIKV-E3/E2/E1 constructs are immunogenic following intranasal administration in BALB/c mice

BALB/c mice of 6–8 weeks of age were intranasally immunized with 10^7 infectious units (IU) of one of the following three constructs: (1) Ad-CHIKV-SG expressing the CHIKV C-E3-E2-6K/TF-E1 structural proteins of La Réunion strain LR2006-OPY1, (2) Ad-CHIKV-E3/E2/6K expressing proteins E3-E2-6 K/TF, or (3) Ad-CHIKV-E3/E2/E1 expressing proteins E3-E2-6K/TF-E1 (Fig. 1A). Four weeks after the first immunization, animals were boosted with an equal dose of the same construct. Serum was collected 3 and 7 weeks after the initial immunization and tested for the presence of CHIKV-specific IgG antibodies. Constructs Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 gave rise to higher IgG titers than Ad-CHIKV-E3/E2/6K at week 3, with average median endpoint titers of 4.4×10^4 and 7.2×10^4, respectively (Fig. 1B). All three constructs induced high anti-CHIKV serum IgG titers at week 7, directed against virus-like particles (VLP) as measured in an ELISA, although Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 induced significantly higher titers than Ad-CHIKV-E3/E2/6K (Fig. 1C).

2.2. Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 constructs are immunogenic and induce neutralizing antibodies following intranasal administration in C57BL/6 mice

To evaluate the potential of the two most immunogenic constructs, Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1, to induce CHIKV-specific antibodies in the C57BL/6 mouse strain, each construct was tested at different dose regimens in 4-week old mice. One group of animals was intranasally immunized with a single dose of 10^8 IUs of Ad-CHIKV-SG on day 0. In separate groups, construct Ad-CHIKV-E3/E2/E1 was intranasally administered following two different schedules: one group received a single dose of 10^8 IUs on day 0 whereas a second group received 10^8 IUs on day 0 as well as a boost on day 28 (labeled 'single admin' or 'double admin', respectively, on Fig. 2). For all groups, serum was harvested at week 3 and week 8 and CHIKV-specific IgG titers were determined for each time point. At week 3, all constructs gave rise to similar IgG titers (Fig. 2A). By week 8, construct Ad-CHIKVSG and Ad-CHIKV-E3/E2/E1 administered at two doses produced titers that were significantly higher than Ad-CHIKV-E3/E2/E1 at a single dose (Fig. 2B).

The levels of neutralizing antibodies induced by vaccination were also evaluated in PRNT assays with week-8 serum samples following two doses of Ad-CHIKV-SG vs Ad-CHIKV-E3/E2/E1. Five out of six animals immunized with Ad-CHIKV-SG developed $PRNT_{50}$ titers higher than 640. Remarkably, all six animals vaccinated with Ad-CHIKV-E3/E2/E1 in a prime-boost fashion showed higher $PRNT_{50}$ titers than 640. As expected, naïve animals did not show any detectable levels of neutralizing antibodies (Fig. 2C).

2.3. Immunization with Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 induces neutralizing antibodies and prevents footpad swelling and viremia in C57BL/6 mice

To test whether Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 could protect against CHIKV disease, C57BL/6 mice were intranasally immunized with a single dose of 10^8 IUs per animal of either construct, and challenged with $6x10^4$ PFU of the *wt* CHIKV strain 99659 by intradermal footpad injection on day 28 post-immunization [11]. To assess protection from disease signs after challenge, footpad swelling was measured over a 12-day period. Swelling of the footpad commenced immediately after challenge in all control animals treated with PBS and it peaked between days 6 and 7 post-infection. Animals vaccinated with the live-attenuated 181/25 vaccine [12], which was highly immunogenic in Phase 2 clinical trials, only showed reduced footpad swelling 6 days post-infection. Notably, animals vaccinated with either Ad-CHIKV-SG or Ad-CHIKV-E3/E2/E1 showed significantly reduced levels of footpad swelling starting at day 2 and daily after day 5 when compared to control animals (Fig. 3A). As a second measure of vaccine protection, levels of infectious virus in the blood were measured. Viremia was readily observed in PBS-treated animals one day after challenge and it increased ~300-fold from baseline by day 2 post-infection. In contrast, no detectable viremia was observed in any of the vaccinated animals (Fig. 3B). In summary, the adenovirus-based vaccine outperformed the live-attenuated 181/25 vaccine in preventing footpad swelling and equally protected mice against viremia.

To test whether humoral responses elicited by the vaccine were responsible for the protective phenotype, the levels of neutralizing antibodies were quantitated in serum samples collected one day prior to viral challenge. Naïve animals were used as controls.

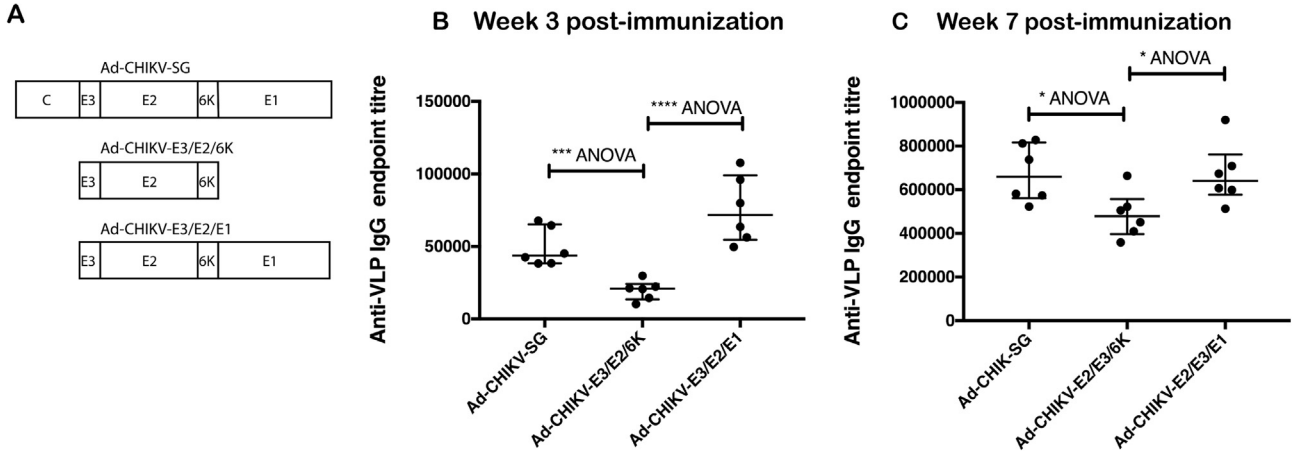

Fig. 1. Ad-CHIKVSG, Ad-CHIKVE3/E2/6K and Ad-CHIKVE3/E2/E1 constructs are immunogenic following intranasal administration in BALB/c mice. (A) Schematic representation of constructs Ad-CHIKV-SG, Ad-CHIKV-E3/E2/6K and Ad-CHIKV-E3/E2/E1, expressing structural proteins of the CHIKV La Réunion strain LR2006-OPY1. (B and C) BALB/c mice were intranasally immunized with 10^7 IU/animal of Ad-CHIKV-SG (N = 6), Ad-CHIKV-E3/E2/6K (N = 6) or Ad-CHIKV-E3/E2/E1 (N = 6) at week 0 and week 4. Serum was collected at week 3 (B) and week 7 (C) and tested for viral-specific IgG antibodies using CHIKV VLPs as an immobilized antigen. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ significantly different to the other vaccinated groups, using one-way ANOVA. Lines represent the median and interquartile range.

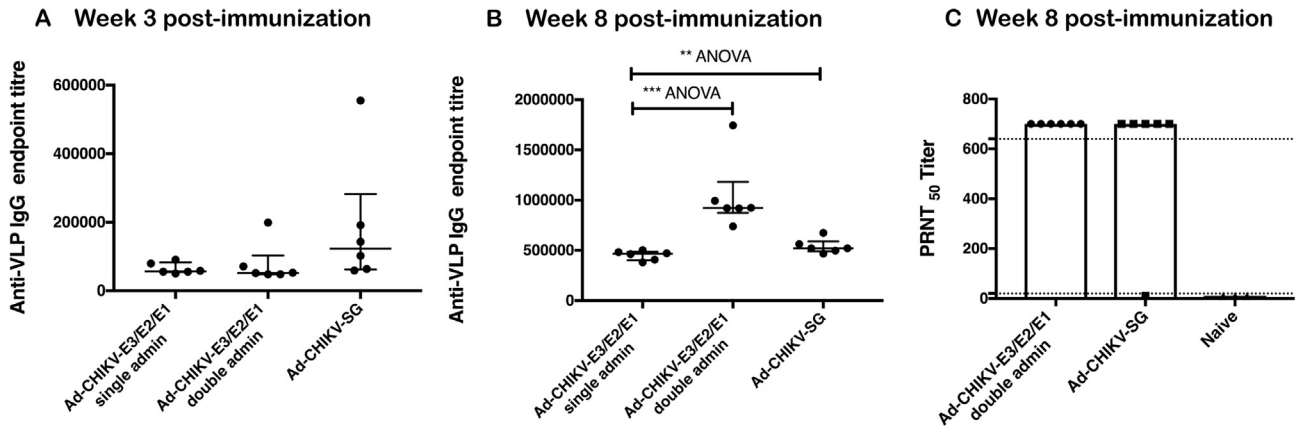

Fig. 2. Ad-CHIKVSG and Ad-CHIKVE3/E2/E1 constructs are immunogenic and generate neutralizing antibodies following intranasal administration in C57BL/6 mice. (A and B) C57BL/6 mice were intranasally immunized with 10^8 IU/animal (N = 6) of Ad-CHIKV-SG at week 0 or either one dose of 10^8 IU/animal (N = 6) of Ad-CHIKV-E3/E2/E1 at week 0 (denoted 'single admin') or two doses of 10^8 IU/animal (N = 6) at week 0 and week 4 (denoted 'double admin'). Serum was collected at week 3 (A) and week 8 (B) and tested for viral-specific IgG antibodies in ELISAs. (C) Week 8 serum samples were tested for the presence of neutralizing antibodies in $PRNT_{50}$ assays. $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different to the other vaccinated groups, using one-way ANOVA. Lines/bars represent the median and interquartile range.

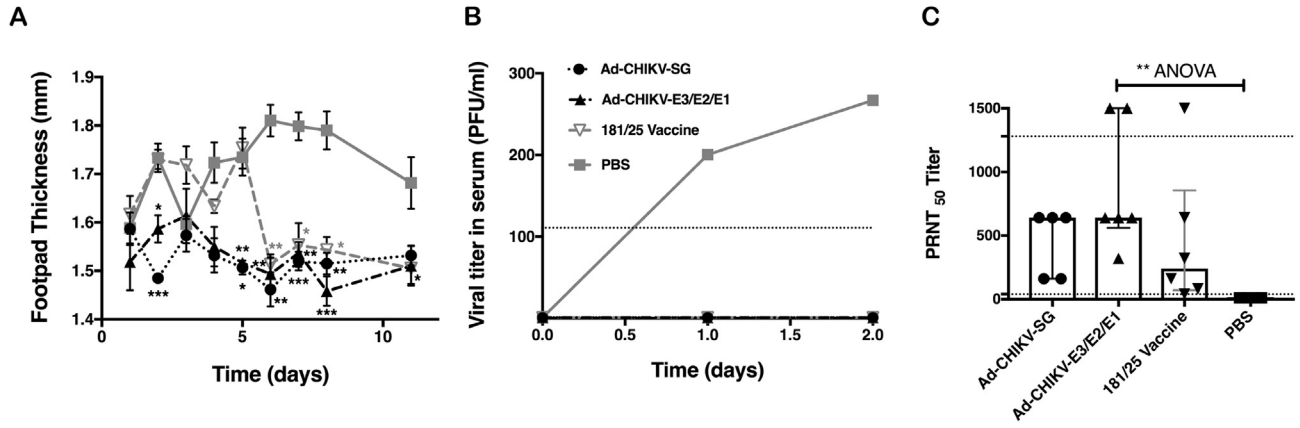

Fig. 3. Immunization with Ad-CHIKV-SG and Ad-CHIKV-E3/E2/E1 induces neutralizing antibodies and prevents footpad swelling and viremia in C57BL/6 mice. Groups of six four-week old C57BL/6 mice were intranasally immunized with 10^8 IUs/animal of Ad-CHIKV-SG or Ad-CHIKV-E3/E2/E1 or PBS, or intramuscularly with the live-attenuated 181/25 vaccine, and subsequently challenged intradermally with 6×10^4 PFUs of *wt* CHIKV 99659 on day 28 post-immunization. (A) Footpad swelling was measured for 12 days post-challenge. (B) Viral titers in serum were measured for up to two days post-challenge for all three vaccines and compared to PBS control. (C) Serum was collected one day prior to CHIKV challenge and analyzed for the presence of neutralizing antibodies using a PRNT assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different to non-vaccinated group, using one-way ANOVA. Bars represent the median and interquartile range.

Animals immunized with Ad-CHIKV-E3/E2/E1 and Ad-CHIKV-SG produced the highest levels of neutralizing antibodies, with a $PRNT_{50}$ median titer of 640. Animals immunized with the 181/25 vaccine produced a $PRNT_{50}$ median titer of 240. As expected, naïve animals did not have detectable neutralizing antibodies. This observation is consistent with serum neutralizing antibodies being responsible for protection against viral disease.

3. Discussion

Prophylactic and therapeutic treatment of CHIKV disease using neutralizing monoclonal antibodies (mAbs) in animal models, including non-human primates, can induce rapid virus clearance and reduced severity of joint inflammation [13–15]. To date, more than 15 chikungunya vaccine candidates, based on a range of platforms, have been reported, but only two candidates have successfully completed Phase I clinical trials: a recombinant measles virus (MV)-based vaccine and a VLP-based vaccine. The former recently completed a Phase II clinical trial [16]. Both vaccines express the structural proteins of CHIKV and are administered intramuscularly [14,16–18]. While VLP- and MV-based vaccines have high safety profiles, the cost of production and the potential requirement for additional boosters may discourage widespread use in resource-poor countries where CHIKV is endemic, such as India, Bangladesh and southern Indian Ocean islands.

Vaxart is developing an adenovirus-based vaccine that expresses the structural proteins of chikungunya virus and has the potential to be administered orally. Oral delivery provides several advantages over needles for manufacturing, distribution, and administration, particularly during a pandemic when time and efficiency are critical. Vaxart's platform has been tested clinically using room-temperature stable tablets as a dose form. With substantial progress in recombinant adenovirus manufacturing processes, vector yields of $\sim 2 \times 10^4$ viral particles per infected cell can be produced. At cell densities greater than 2×10^7 cells/ml, this translates to 4×10^{14} viral particles per liter of production culture [19]. Further product development optimization will likely result in increased productivity, resulting in reduced vaccine product cost.

In the current study, we show that a single administration of Ad-CHIKV-E3/E2/E1, a replication-incompetent adenovirus 5 vector expressing CHIKV structural proteins, can induce high titers

of anti-CHIKV antibodies in both BALB/c and C57BL/6 strains of mice. Importantly, vaccinated C57BL/6 animals were also protected against viral replication and viral-induced footpad inflammation, a surrogate for CHIKV-induced arthritis in humans, after intradermal challenge with the *wt* CHIKV Caribbean strain 99659 representing most recent exposure in the Americas. Our data provide strong support for continued development of this oral platform especially given its thermostability and potential for ease of administration to populations at high-risk for contracting chikungunya. Clinical trials using this same platform delivery for vaccines against norovirus (clinical trial NCT03125473) and influenza (clinical trial NCT02918006) have shown promising immune profiles. Importantly, the influenza vaccine was able to protect subjects from influenza viral challenge (data not published), attesting to the effectiveness of the novel platform approach described here.

4. Methods

4.1. Ad-CHIKV-SG, Ad-CHIKV-E3/E2/6K and Ad-CHIKV-E3/E2/E1 vaccine construction

The adenovirus vectors Ad-CHIKV-SG, Ad-CHIKV-E3/E2/6K and Ad-CHIKV-E3/E2/E1 express the structural proteins C-E3-E2-6K-E1 of the La Réunion CHIKV strain LR2006-OPY1 (accession number ABD95938) under the CMV promoter. Within the same construct, a dsRNA hairpin is also expressed and driven by a separate CMV promoter. Generation and propagation of this construct was conducted as previously described [8].

4.2. Cell culture

Vero African green monkey kidney cells (ATCC CCL-81; American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and penicillin-streptomycin at 37 °C under 5% CO_2.

4.3. Virus stocks

CHIKV Caribbean strain 99659 was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at UTMB. The virus was amplified twice in Vero cells, twice in C6/36 mosquito cells and a final Vero amplification was done prior to titration on Vero cells. CHIKV-LR was rescued from a cDNA infectious clone as previously described [20]. The titers were confirmed by plaque assay on Vero cells.

4.4. Viral plaque assays

Plaque assays were performed on Vero cells as previously described [21]. Briefly, 10-fold dilutios of virus were made and 0.1 mL was used to infect Vero cell monolayers in a 12-well plate. One hour later, a 0.4% agarose overlay was placed on the monolayers and incubated for 2 days at growth conditions. Plaques were visualized by crystal violet staining of intact monolayers.

4.5. Mouse immunization and challenge

Animal research was approved by the Institutional Animal Care and Use Committees (IACUC) at Vaxart and the University of Texas Medical Branch (Galveston, TX). BALB/c and C57BL/6 mouse strains were used in all studies. BALB/c mice (6–8 week-old, purchased from Charles River Laboratories, Wilmington, MA) were immunized intranasally with 10^7 infectious units (IUs) of Ad-CHIKV-SG, Ad-CHIKV-E3/E2/6K or Ad-CHIKV-E3/E2/E1 and C57BL/6 mice (4-week-old purchased from Jackson Laboratories, Sacramento, CA) were immunized with 10^8 infectious units of Ad-CHIKV-SG or Ad-CHIKV-E3/E2/E1. Serum was collected on week 3 (or 4) and week 7 (or 8).

For the challenge study, 4-week old C57BL/6 mice were intranasally immunized with 10^7 IUs of either Ad-CHIKV-SG or Ad-CHIKV-E3/E2/E1 and challenged intradermally (ID) in the left rear footpad with 6×10^4 PFU of the *wt* CHIKV strain 99659 on day 28 post-immunization. Animals were monitored daily for footpad swelling for a total of 12 days and bled on days 1 and 2 post-infection to quantify virus in the serum clarified brom blood as previously described [20].

4.6. CHIKV serology assays

4.6.1. ELISA assays

Specific antibody titers were measured similarly to those previously described in [22]. Briefly, microtiter plates (MaxiSorp:Nunc) were coated with 1ug/ml CHIKV VLP (The Native Antigen Company, Oxford, UK) in 1X carbonate buffer. Plates were incubated overnight at 4 °C, then washed with PBS + 0.05% Tween 20 (PBST) and blocked with 1% Bovine Serum Albumin (BSA) for 1 h.

Serum or tissue samples were serially diluted in 1% BSA solution and added to the coated plate. After a 2-h incubation, washed plates were incubated with either an anti-cotton rat IgG-HRP (Gallus Immunotech, Cary, NC), or alternatively, goat anti-mouse IgA-HRP (Southern Biotech, Birmingham, AL). Each secondary antibody was used at a 1:5000/1:1000 dilution. After a 1-hour incubation, antigen-specific mouse antibodies were detected with 3,3′,5,5′-tetramethyl-Benzidine (TMB) substrate (Rockland, Limerick, PA). A stop solution (1 M H_2SO_4 concentration) was added and the plates were read at 450 nm on an Emax ELISA plate reader.

4.6.2. Plaque Reduction Neutralization Titer (viral neutralization, PRNT)

To quantify the titer of neutralizing antibody against CHIKV, PRNT assays were done as previously described [23]. Briefly, serum was serially two-fold diluted in DMEM with 2% FBS. An equal volume of CHIKV La Réunion strain LR2006-OPY1, approximately 800 PFU/mL. was added to the diluted serum and the mixture incubated at 37 °C for 1 h. The serum virus mixture was added to Vero cells, previously seeded at a density 1.5×10^5 cells per well the day before and incubated at 37 °C for 1 h. Wells were covered with 0.4% agarose in DMEM with 2% FBS and incubated for 2 days at 37 °C. Plaques were developed and counted as outlined above. The endpoint neutralizing antibody titer was assigned to the concentration of serum that reduced the number of plaques by 50% $PRNT_{50}$), compared to the non-neutralized, serum-free, control.

4.7. Statistical analysis

Where mentioned in the text, one-way ANOVA was used to determine significance between groups or paired *t*-test (Wilcoxon test) to determine significant changes from prime to boost within each group. P values less than 0.05 were considered significant.

Acknowledgements

We would like to thank the Animal Resource Center at UTMB for their support during the animal studies. We are also grateful to Dr. Robert Tesh and the WRCEVA for supplying CHIKV strain 99569. We thank Jonathan Lindbloom and Ciaran Scallan for discussions about cloning strategy and performing vaccine expansion. We thank Katie Hodgson and Christina Joyce for technical assistance with mice and assays. Thanks to Debora Tingley for critical review

of the manuscript. Funding for this work was supported by Vaxart Inc.

Competing interests

RM, EGD, and SNT are current employees and/or own stock in Vaxart, the sponsor of the studies.

Contributions

RM, SNT, SCW, and SLR designed the studies. EGD designed, constructed, and performed experiments. EGD and RM performed experiments and analyzed the data. RM wrote the paper.

Funding

Vaxart, Inc. funded this study, and virus reagents were supported by the WRCEVA through NIH grant R24AI120942.

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