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CHIMERIC ALPHAVIRUS VACCINE CANDIDATES FOR CHIKUNGUNYA

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Abstract

Chikungunya virus (CHIKV) is an emerging alphavirus that has caused major epidemics in India and islands off the east coast of Africa since 2005. Importations into Europe and the Americas, including one that led to epidemic transmission in Italy during 2007, underscore the risk of endemic establishment elsewhere. Because there is no licensed human vaccine, and an attenuated Investigational New Drug product developed by the U.S. Army causes mild arthritis in some vaccinees, we developed chimeric alphavirus vaccine candidates using either Venezuelan equine encephalitis attenuated vaccine strain TC-83, a naturally attenuated strain of eastern equine encephalitis virus (EEEV), or Sindbis virus as a backbone and the structural protein genes of CHIKV. All vaccine candidates replicated efficiently in cell cultures, and were highly attenuated in mice. All of the chimeras also produced robust neutralizing antibody responses, although the TC-83 and EEEV backbones appeared to offer greater immunogenicity. Vaccinated mice were fully protected against disease and viremia after CHIKV challenge.

Keywords

chikungunya; arthritis; vaccine; alphavirus

Introduction

Chikungunya virus (CHIKV; *Togaviridae: Alphavirus*), first isolated from febrile human in Tanzania in 1953 [1], derives its name from Swahili, meaning "that which bends up," and refers to the characteristic posture assumed by patients typically suffering severe joint pains. CHIKV has probably caused epidemics in India and Southeast Asia for at least 200 years, and may have caused epidemics in the southern U.S. and Caribbean [2]. Its distribution currently includes most of sub-Saharan Africa, India, Southeast Asia, Indonesia, and the Philippines.

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Since 1953, CHIKV has caused epidemics in both Africa and Southeast Asia, many involving hundreds-of-thousands of people [3–5]. Typically, CHIKV causes a severely incapacitating, self-limited disease characterized by fever, rash and severe joint pains; the latter can persist for months. Most naturally infected humans are symptomatic, and pediatric cases tend to be milder than those in adults. Fatalities have historically been considered rare and are associated with young patients in India and Southeast Asia who develop a hemorrhagic disease leading to shock [6–9]. Because chikungunya fever (CHIK) signs and symptoms often mimic those of dengue fever (DEN) and because CHIKV circulates in DEN virus (DENV)-endemic regions, many DEN cases are probably misdiagnosed and that the incidence of CHIK infection is actually much higher than reported [2]. A case in point is a recent CHIKV epidemic diagnosed in Gabon, the first ever recognized there; this outbreak was likely only diagnosed due to the recent attention on CHIKV resulting from the major outbreaks in the Indian Ocean and in India, attracting diagnostic involvement from the French Armed Forces Medical Service [10].

There are 2 distinct CHIKV transmission cycles: 1) a sylvatic African cycle between wild primates and arboreal *Aedes* mosquitoes [11,12], similar to that of yellow fever virus in the same region, and; 2) urban CHIK outbreaks usually associated with *Ae. aegypti* transmission in a human-mosquitohuman cycle [11,13]. Urban outbreaks are sporadic in occurrence but explosive. During the past 40 years, extensive epidemics have occurred in many large cities of India and Southeast Asia, sometimes affecting hundreds-of-thousands of people [9,14]. However, unlike dengue, which has become endemic in many urban centers in tropical Asia, CHIKV disappears and reappears at irregular intervals [11]. A vertebrate reservoir or sylvan transmission cycle has not been identified outside of Africa, supporting the historical evidence [2] that CHIKV originated in Africa and was subsequently introduced into Asia.

Recently, CHIKV caused epidemics involving millions of people on islands off the eastern coast of Africa that are popular destinations for European tourists [15], as well as in the Indian Subcontinent [16,17]. Phylogenetic studies indicate that the Indian Ocean outbreaks resulted from the recent introduction of a Central/East African CHIKV strain [15] that also caused epidemics in East Africa [18]. Unlike past epidemics that were usually associated with *Ae. aegypti* transmission, *Ae. albopictus* was implicated as the principal mosquito vector in the recent Indian Ocean and some of the Indian outbreaks. Thousands of excess deaths during these epidemics, including neurologic disease [19–21], suggest that CHIKV may have become more virulent. Importations into the Western hemisphere including the United States, via viremic travelers, combined with a 2007 Italian epidemic initiated by a traveler from India [22,23] underscore the severe threat that CHIKV poses to the Americas; the risk for endemic establishment includes both neotropical/subtropical regions inhabited by *Ae. aegypti*, and temperate areas of the U.S. that are populated by the other efficient vector, *Ae. albopictus*. Because humans develop high titer viremia and the urban cycle is identical to that of DENV, a single viremic traveler could initiate endemic or epidemic CHIKV transmission. The introduction of infected mosquitoes carried from epidemic sites in shipping containers (the same mechanism that brought *Ae. albopictus* into the Americas 21 years ago [24,25]) is another potential mechanism of importation. The dramatic spread since 1980 of dengue viruses (DENV) throughout tropical America, via the same vectors and human hosts, underscores the risk to public health in the Americas. Accordingly, CHIKV was recently added to the NIAID priority pathogen biodefense list as a Category C pathogen (<http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/CatA.htm>).

Despite their importance as emerging viruses and potential biological weapons, there are no licensed vaccines or therapeutics for alphaviruses. Development of such products for CHIKV is hampered by the lack of an inexpensive animal model for human disease. In infant mice, CHIKV causes fatal encephalitis, myocarditis and myositis [26,27]. In various nonhuman primates, CHIKV produces viremia of up to 6 days without signs of disease, followed by

seroconversion [11]. In the bonnet macaque (*Macaca radiata*), viremia titers of up to 10^7 LD₅₀/ml are observed, and *Ae. aegypti* and *Ae. albopictus* feeding on these animals become infected [28]. Rhesus macaques (*Macaca mulatta*), which were used for testing of the attenuated CHIKV vaccine strain developed at the U.S. Army Medical Research Institute for Infectious Disease, Frederick, Maryland, develop viremia on days 1–3 after infection, with titers from 3.6–5.3 log₁₀ plaque forming units (PFU)/ml, and seroconvert with neutralizing antibodies within 14 days of intramuscular infection [29].

A live CHIKV vaccine candidate was developed and evaluated by the U.S. Army over 20 years ago by plaque-to-plaque MRC-5 cell culture passage of a wild-type strain [29]. This vaccine strain, called 181/clone 25 (181/25), is attenuated and immunogenic in mice and rhesus macaques, and is highly immunogenic in humans. However, 5 of 59 human vaccinees developed transient arthralgia during phase II safety studies [30]. Although the mechanism of strain 181/25 reactogenicity has not been determined, it is likely that reversions of attenuating point mutations, which typically accompany cell culture passaged, attenuated alphaviruses, can occur. For example, the TC-83 vaccine strain of Venezuelan equine encephalitis virus (VEEV), developed by 83 serial cell culture passages, relies on only 2 attenuating mutations [31]. The CHIKV vaccine strain 181/25, which was developed with only 18 passages, probably has a similar if not lower number of attenuating mutations. The inherent instability of alphavirus RNA genomes indicates that reliance on small numbers of point mutations will always pose the risk of reactogenicity.

To overcome the reversion potential of traditionally attenuated alphavirus vaccines and to address the growing need to prevent CHIKV infections, we used a chimeric approach described previously to develop vaccines against VEEV [32] and eastern equine encephalitis viruses (EEEV) [33], and evaluated 3 new recombinant CHIKV vaccine candidates for attenuation and efficacy in laboratory mice.

Materials and Methods

Cells

Baby hamster kidney (BHK-21) and Vero African green monkey kidney cells were purchased from the American Type Culture Collection (Bethesda, MD) and grown at 37°C in Eagles minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 0.05 mg/ml of gentamycin sulfate (Invitrogen, Carlsbad, CA). The *Aedes albopictus* mosquito cell line C7/10 (a gift from H. Huang, Washington Univ.) was maintained in MEM at 32°C with 10% FBS and 10% tryptose phosphate broth.

Viruses

Chikungunya virus strains La Réunion (LR) and Ross were used for cDNA production and challenge experiments. Strain LR, isolated from a human during the 2006 La Réunion outbreak, was passaged five times in Vero cell culture and once in infant mice before RNA extraction and cDNA cloning. Virus was rescued from this infectious clone by electroporating viral RNA into C7/10 cells as described previously [34]. The Ross strain, isolated from a human during the 1953 Tanzania epidemic, was passaged 175 times in newborn mice, twice in Vero cells, and once in C7/10 cells.

Construction of recombinant alphavirus/CHIKV plasmids

Chimeric alphavirus/CHIKV vaccine viruses were created using recombinant DNA methods as described previously [33]. The alphavirus backbones used included Sindbis virus (SINV) strain AR339, the attenuated VEEV vaccine strain TC-83, and a South American strain of EEEV, BeAr436087, whose distinguishing feature is the inability to cause disease in adult

mice [35] or marmosets [36] despite the induction of viremia. To design the recombinant cDNAs, we followed our previous strategy and replaced the VEEV-, EEEV- and SINV-specific structural polyprotein-coding sequence with the corresponding CHIKV LR strain genes. The nsPs and cis-acting RNA elements (that include 5' and 3' termini and subgenomic promoters) remained backbone-specific.

***In vitro* transcription, transfection and production of chimeric viruses**

Plasmids were purified by centrifugation in CsCl gradients. Before the transcription reaction, the viral and replicon genome-coding plasmids were linearized by *Xho*I digestion. RNAs were synthesized by SP6 RNA polymerase in the presence of cap analog. The yield and integrity of transcripts were analyzed by gel electrophoresis under non-denaturing conditions. Aliquots of transcription reactions were used for electroporation without additional purification.

Electroporation of BHK-21 cells was performed under previously described conditions [32]. To rescue the viruses, 1 µg of *in vitro*-synthesized viral genome RNA was electroporated into cells, which were then seeded into 100-mm dishes and incubated until cytopathic effects (CPE) were observed. Virus titers were determined using a standard plaque assay on BHK-21 cells. To assess the RNA infectivity, 10-fold dilutions of electroporated BHK-21 cells were seeded in 6-well plates. After 1 h incubation at 37°C in a 5% CO₂ incubator, cells were overlaid with 2 ml 0.5% agarose supplemented with MEM and 3% FBS. Plaques were stained with crystal violet after 2 days incubation at 37°C, and infectivity was determined as PFU per µg of transfected RNA.

Passaging of recombinant viruses in Vero cells was performed by infecting cells in 35-mm dishes with 10 µl of viral stocks, harvested on the previous passage. Viruses were harvested after developing profound CPE and titers were determined by plaque assay on BHK-21 and/or Vero cells.

Analysis of viral structural protein synthesis

BHK-21 cells were seeded into 6-well plates and infected with different viruses at a multiplicity of infection (MOI) of 20 PFU/cell. At 16 h post infection, the cells were incubated for 30 min in 0.8 ml of DMEM medium lacking methionine and supplemented with 0.1% FBS and 20 µCi/ml of [³⁵S]methionine. After this incubation, they were scraped into the media, collected by pelleting at 1500 rpm, and dissolved in 100 µl of standard protein loading buffer. Equal amounts of proteins were loaded onto each lane of the sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. After electrophoresis, the gels were dried, autoradiographed and analyzed on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mouse infections

NIH Swiss and C57BL/6 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained under specific-pathogen-free conditions. To monitor body temperature, 3-week-old or older mice were anaesthetized with isofluorane 3 days before infection and implanted subcutaneously (SC) with a pre-programmed telemetry chip according to the manufacturer's instructions (IPTT-300; Bio Medical Data Systems, Inc., Seaford, DE). Body temperatures and weights were recorded daily/weekly without anesthesia. Three-week or older mice were infected with CHIKV SC in the medial thigh or intraperitoneally (IP) in a total volume of 100 µl. Intranasal infections (IN) used a dose of 6.5 log₁₀ PFU in a total volume of 25 µl. Blood samples were collected from the retroorbital sinus and virus titers were determined by plaque assay on Vero cells [37]. Infant mice were infected intracerebrally (IC) or SC in the dorsal shoulder region in a volume of 20 or 50 µl, respectively.

Immunizations and challenges with CHIKV

Three week-old mice were vaccinated SC in the medial thigh. Controls were sham-infected with phosphate buffered saline (PBS). Blood samples were collected from the retroorbital sinus to detect viremia for 3 days after vaccination. Animals were checked daily to observe clinical signs of infection, and body weights were monitored on days 1–7, 14 and 21 after vaccination. Sera were collected on day 21 after vaccination and a plaque reduction neutralization test (PRNT) was performed to examine antibody (Ab) responses. Mice were then challenged with the Ross CHIKV strain at a dose of $6.5 \log_{10}$ PFU by the IN route.

Histopathology and immunohistochemical staining

On days 3 and 7 post-infection, 3 animals per group of 5-week-old female C57BL/6 mice (14–16 g) infected with the Ross CHIKV strain and age-matched, sham infected mice were bled and sacrificed, and tissues (brain, heart, lung, liver, spleen, kidney, and skeletal muscle) were collected and titered for virus. Brain tissues were also fixed in 10% buffered formalin solution for 24 h, then stored in 70% ethanol prior to embedding, sectioning, and staining using hematoxylin and eosin (H&E) and immunohistochemistry. For immunohistochemistry, the formalin-fixed, paraffin-embedded tissue sections (3–4 μ m thick) were deparaffinized and immersed in 3% H_2O_2 for 30 min to block endogenous peroxidase activity. This was followed by an antigen retrieval step with 10% target retrieval solution (DAKO, Carpinteria, CA) at 90°C for 30 min. CHIKV mouse hyperimmune ascitic fluid was used as the primary Ab at a dilution of 1:150, and bound primary Ab was directly labeled and detected by use of a commercially available mouse-on-mouse Iso-IHC AEC kit (InnoGenex, San Ramon, CA). Primary Ab against a flavivirus, Modoc virus (MODV), was used as a negative control.

Results

Design of chimeric viruses

Our previous studies indicated that chimeric viruses, with the replicative enzymes of one alphavirus in combination with the structural proteins derived from another, demonstrate highly attenuated phenotypes *in vivo* using small animal models [32,33,38]. However, these chimeric alphaviruses elicited efficient immune responses against the viruses whose structural genes were used in the chimeric design. Therefore, in the present work to develop new vaccine candidates against CHIKV, we developed 3 different recombinant alphaviruses expressing CHIKV structural protein genes. The nonstructural protein genes, both 5'- and 3'-specific cis-acting promoter elements, and the subgenomic promoters in the recombinant genomes were derived either from VEEV vaccine strain TC-83, EEEV strain BeAr436087, or SINV strain AR339; the structural genes and 5'UTR of the subgenomic RNA were derived from the LR strain of CHIKV. This design was aimed at promoting i) the most efficient replication of viral genomes, ii) transcription of the subgenomic RNA (which were achieved by using nonstructural protein gene promoter elements from the same virus), and iii) efficient translation of the structural protein genes by using the 5'UTR from the same virus as the structural protein genes. The rationale for using the EEEV and TC-83 backbones is supported by recent studies that identified VEEV- and EEEV-specific capsid proteins as strong molecular determinants of host cell gene expression shutoff and pathogenesis [39,40], while capsids of the Old World alphaviruses that include CHIKV apparently do not function in the transcriptional shutoff. In the latter viruses, nonstructural protein 2 (nsP2) appears to serve this function. Therefore, combination of VEEV and EEEV nsPs and CHIKV structural protein genes (including the capsid) in chimeric genomes was expected to result in attenuation. The rationale for the SIN/CHIKV chimera design was also based on inefficient replication of SINV *in vivo* in mammals. Thus, the use of SINV-specific replicative enzymes in combination with heterologous, CHIKV-specific structural proteins was expected to generate a virus with attenuated phenotype that results from its inefficient replication *in vivo*.

All of the recombinant viral genomes were synthesized *in vitro* by using SP6 DNA-dependent RNA polymerase and transfected into BHK-21 cells by electroporation. Fractions of electroporated cells were used for an infectious center assay and the rest of the cells were used to generate viral stocks. The VEE/CHIKV and EEE/CHIKV constructs demonstrated high specific infectivities in the infectious center assay, ca. 1×10^6 and 5×10^5 PFU/ μ g of RNA, respectively, indicating that these viruses were capable of replication and causing CPE without accumulating additional, adaptive mutations in their genomes. In contrast, the SIN/CHIKV chimera did not form detectable plaques in the infectious center assay, but ultimately caused CPE under liquid medium. This indicated that the original SIN/CHIKV chimeric genome was capable of replication, but did not develop productive, spreading infection to produce plaques; most likely, accumulation of additional adaptive mutations was required for more efficient virus replication.

Adaptation of the chimeric viruses to replication in cell culture

The CHIKV structural protein genes were originally derived from a virus isolate that was passaged only 5 times in cell culture. However, alphaviruses are notorious for their ability to accumulate the adaptive mutations in the envelope proteins that increase their positive charge for enhanced binding to heparan sulphate, leading to more efficient replication [41,42]. Therefore, we analyzed the adaptation of not only of the SIN/CHIKV chimera, but of all of the recombinants, to replication in Vero cells, which are approved vaccine substrates. The SIN/CHIKV (original, very low titer stock), VEE/CHIKV and EEE/CHIKV chimeric viruses were passaged 3 times in Vero cells as described in the Materials and Methods, and titers of stocks, harvested after the third passage at 24 and 48 h post infection, are presented in Table 1.

It should be noted that all of the chimeras continued to develop heterogeneous plaque sizes even after 3 passages, indicating the presence of different genetic variants in the viral populations. Therefore, to generate more homogeneous and genetically stable vaccine stocks for testing, we randomly selected 2 plaques for each chimera and used them to generate new stocks and to sequence their viral genomes; large plaques were preferentially selected to maximize yields. Titers of virus stocks, prepared from Vero cells 24 h after infection with individual plaque clones, are presented in Table 2. The plaque-purified variant of VEE/CHIKV with the higher titer (plaque 1) had two mutations, N72→Y and S159→R, in the E2 glycoprotein, and the variant with the lower titer had no mutations in the structural genes. Both isolates of EEE/CHIKV had the same W64R mutation in the E2 glycoprotein, and the SIN/CHIKV isolates had mutation E2 substitution E150→K and a deletion of nt 9, located in the 5'UTR of the subgenomic RNA. This and all other E2 mutations found in the recombinant viruses increased the positive charge of this glycoprotein and most likely adapted them for more efficient binding to and replication in Vero cells. Such variants are therefore probably more suitable for large-scale vaccine production.

The nt 9 mutation was interesting because it could explain the ability of this variant to spread more efficiently. Therefore, the modified 5'UTR was transferred into the original SIN/CHIKV genome, and the *in vitro*-synthesized RNA of this variant, SIN/CHIKV Δ 9, indeed demonstrated in the infectious center assay an infectivity of $2\text{--}5 \times 10^6$ PFU/ μ g of RNA that was comparable to infectivities of other recombinant genomes (see Fig. 1 for details). This was an indication that this mutation had a dramatically positive effect on the ability of virus to productively replicate in cell culture.

In additional tests, we demonstrated that recombinants produced similar levels of CHIKV structural proteins. BHK-21 cells were infected with VEE/CHIKV, EEE/CHIKV and SIN/CHIKV Δ 9 at the same MOI, and at 16 h post infection, cells were metabolically labeled with [^{35} S]methionine. Cell lysates were analyzed by electrophoresis in SDS-10% polyacrylamide

gels. All of the viruses produced similar levels of the virus-specific structural proteins (Fig. 2), indicating that all of them were suitable for further development as candidate vaccines.

Mouse models for CHIKV

Because little research has been done on animal models for CHIKV, we conducted pilot studies to develop a murine challenge model to test vaccine candidates. Initially, cohorts of three 10-week-old (to allow for vaccination at an age of 6 weeks and challenge at 10 weeks) NIH Swiss mice were infected with the LR and Ross strains by the SC, IP and IN routes. The LR isolate was used because it represents CHIKV strains circulating recently in the Indian Ocean and India, and the Ross strain was selected because of its extensive mouse passage history, which may have increased virulence. Animal body temperatures and weights were monitored daily, and blood was collected daily up to 4 days postinfection. Viremia was detected on day 1 after IN infection in all 3 animals infected with the LR strain (mean titer $\log_{10}$ 2.8 PFU/ml $\pm$ 0.2), but none was detected thereafter. No viremia was detected in the Ross group 24 hr after IN infection, and one mouse developed viremia on day 2 after IN infection ($\log_{10}$ 3.2 PFU/ml). No detectable viremia occurred on days 3–4 after infection in any of the cohorts (data not shown). No febrile response was detected, nor weight loss or other signs of disease.

In an attempt to produce more disease for comparative attenuation evaluations, cohorts of 5-week-old (rather than 10-week-old) inbred C57BL/6 or outbred NIH Swiss mice were infected using the IP and IN routes. As shown in Table 3, both CHIKV strains delivered IN produced viremia lasting 2–3 days with a peak titer of about 3 $\log_{10}$ PFU/ml, while IP infection yielded less consistent results. Viremia following IN infection lasted. Only the Ross strain produced clinical signs of disease after IN infection; all animals exhibited ruffled fur, a hunched posture, and inactivity by day 6 and died by day 7 or 8 after infection. NIH Swiss mice had similar viremia titers after IN infection (data not shown), but no signs of infection. All other groups remained apparently healthy.

To assess pathologic changes, a separate experiment was conducted in which, in addition to viremia measurements, 3 C57BL/6 mice were euthanized 3 or 7 days after IN infection with the Ross CHIKV strain. Virus titers in the brain were high on both days 3 and 7 post infection, with the highest titers over 7 $\log_{10}$ PFU/g. On day 3, all animals also had detectable virus in the heart, and one mouse had infectious virus in the kidney and skeletal muscle of the leg (Table 4). No viremia was detected after day 2 after infection, and on day 7 after infection, no virus was detected in any organ except for the brain.

Signs of disease in the 5-week-old C57BL/6 mice infected with the Ross strain included a reduction in body weight gain beginning by day 5 post-infection ($p < 0.05$, ANOVA with Tukey-Kramer multiple comparisons test) and continuing until death ($p < 0.001$). No significant febrile response was detected; however, some mice had slight elevations in body temperature (38–38.5°C) on 1 or 2 days after infection.

Histopathologic evaluation and immunohistochemical detection of CHIKV antigen

Upon histopathological analysis, the CHIKV (Ross strain)-infected brains of the 5-week-old C57BL/6 mice on day 3 after infection showed minimal perivascular mononuclear cellular infiltration that was located in focal areas of the cerebral cortex. Throughout the deep cerebral cortex, there were mild increases in the number of microglial cells as well as diffuse areas of neuronal degeneration and apoptosis. Nuclear fragmentation of neurons, expressed as nuclear dust, was also found in focal areas of the deep cerebral cortex, and in the cerebellum, some Purkinje neuron cells showed condensation and degeneration.

On day 7 after infection, the CHIKV-infected brains showed severe, multifocal inflammation and liquefactive necrosis in the cerebral cortex (Fig. 3). In regions of the superficial cerebral cortex, severe spongiform changes and a large number of apoptotic bodies and microglial cells were evident. Composed primarily of lymphocytic cells, perivascular cuffs were located diffusely throughout the cerebral cortex. Similar to the lesions in the cerebral cortex, focal areas of severe spongiform changes, liquefactive necrosis, and neuronal degeneration were found in the hippocampus, and there was multifocal lymphocytic leptomeningitis.

Based on immunohistochemical staining, CHIKV antigen was detected in the brain of the C57BL/6 mice on days 3 and 7 after IN infection. On day 3 post-infection, clusters or individual neurons with antigen-positive cytoplasmic staining were observed in multifocal areas of the cerebral cortex. Other regions of the brain were negative. By day 7 after infection, the number of positive staining neurons had increased that were primarily located in the necrotic areas of the cerebral cortex (Fig. 3) and hippocampus.

Based on these murine model results, our vaccine attenuation studies focused on SC infection of 3-week-old mice as well as 2 more sensitive models of disease developed previously: IC infection of 6-day-old mice as a measure of neurovirulence [33], as well as SC infection of 3–4-day-old mice as a measure of viremia induction, peripheral replication in muscle and joint tissue, and neuroinvasion [43].

Attenuation and immunogenicity of vaccine candidates

To assess attenuation of the chimeric CHIKV vaccines, 3 week-old female C57BL/6 mice (5 per cohort) and NIH Swiss mice (10 per cohort) were inoculated SC with the TC-83/CHIKV, EEE/CHIKV and SIN/CHIKV chimeras at doses of 5.8, 5.3 and 5.8 log₁₀ PFU respectively. No infectious virus was detected in the serum of any vaccinated mice by plaque assay 1–3 days after infection (limit of detection=8 PFU/ml). None of the vaccinated animals exhibited any signs of infection, and continued to gain weight after vaccination (Fig. 4). Three weeks after immunization, sera were collected and Ab responses were assessed by PRNT. All animals had positive (≥ 20) titers ranging from 20–320 when tested against the CHIKV LR strain (Table 5).

The chimeric vaccine candidates were also evaluated using the more sensitive newborn mouse IC and SC infection models. IC infection of 6-day-old mice yielded no mortality, while the wild-type (wt) CHIKV LR strain killed approximately half of mice by day 6 (Fig. 5). The survival curves for the chimeras were significantly different from that of the wt LR strain (Logrank test, $p=0.001$). Comparison of other alphavirus vaccines including VEEV strains TC-83 [44], V3526 [45] and the recently described SIN/VEEV [38] and SIN/EEEV [33] chimeras all yielded higher mortality rates than the CHIKV chimeras.

Evaluation of virulence using the 3–4-day SC murine model also yielded evidence of attenuation of the CHIKV chimeras. All produced lower titers and shorter duration of viremia than the wt LR CHIKV or the 181/25 vaccine strain, and there was no evidence of chimera replication in the femorotibial (knee) joints or in the brains 2–10 days after infection (Fig. 6).

To assess the immune responses to different vaccine doses, the TC-83/CHIKV and EEE/CHIKV candidates, which appeared to be the most immunogenic (Table 5), were inoculated SC into 3-week-old C57BL/6 mice at doses from 3.8–5.9 log₁₀ PFU. Controls consisted of strain 181/25 (positive) at a dose of 5.5 log₁₀ PFU, and diluent (negative). Mice vaccinated with the chimeric strains all seroconverted, with the highest Ab titers occurring after the highest vaccine doses (Table 6). However, even the lower chimeric virus doses produced robust mean neutralizing Ab titers >100 , and all mice were protected from IN challenge with the neurovirulent Ross CHIKV strain. Survival rates in vaccinated mice were significantly higher than in sham-vaccinated mice (Fisher's exact test, $p=0.008$). The 181/25 vaccine strain also

yielded seroconversion and protection in all mice, but mean Ab titers were lower than those produced by the chimeras at most doses.

Protection against CHIKV challenge

The vaccinated C57BL/6 mice described above were challenged IN with $6.5 \log_{10}$ PFU of the Ross CHIKV strain 21 days after immunization. Vaccinated and sham-vaccinated NIH Swiss mice were divided into 2 groups; each group (5 per cohort) was challenged either IN with the Ross strain as described above, or IN with the LR strain at dose of $6.5 \log_{10}$ PFU. All sham-vaccinated C57BL/6 mice produced viremia on days 1–2 after challenge (Table 7) and showed clinical signs by day 6, with ruffled hair, a hunched posture, inactivity and neurologic signs, as well as significant body weight losses (ANOVA, $p < 0.001$) (Fig 7). In contrast, all mice vaccinated with the chimeras were active and showed no signs of disease. Sham-vaccinated NIH Swiss mice exhibited some signs of disease after challenge with the Ross strain, including weight loss on days 7 and 8 (ANOVA, $p < 0.05$ as compared to TC-83/CHIKV or EEE/CHIKV-vaccinated animals, see Fig. 7), and viremia during the first two days post-challenge (Table 7). Vaccinated NIH Swiss mice challenged with the LR strain also showed no significant body weight alterations, in contrast to sham-vaccinated animals.

Discussion

Recent, massive epidemics of highly debilitating, chronic arthralgia in India and islands off the east coast of Africa have underscored the importance CHIKV as an emerging tropical arbovirus [46]. Furthermore, the introduction of CHIKV into a temperate region of northern Italy, followed by autochthonous transmission there by *Ae. albopictus* [22], indicates the potential for CHIKV to become endemic in temperate areas of the Europe, North and South America, and Asia where this mosquito vector is established.

Currently, no effective therapy exists for treating patients suffering from CHIK, and prevention relies on mosquito control, which is largely ineffective for other tropical arboviruses like DENV that are transmitted by peridomestic vector mosquitoes in urban settings. A live CHIKV vaccine candidate developed by the U.S. Army, strain 181/25 [29], is immunogenic in mice, rhesus macaques, and humans [30], but proved to be mildly reactogenic during phase II safety studies. Like other traditionally derived RNA virus vaccines that depend on small numbers of attenuating point mutations, attenuation of this vaccine is probably unstable and prone to reversion during replication in vaccinees.

We therefore developed an alternative approach to live alphavirus vaccines: chimeric viruses that are uniformly attenuated with no evidence of reactogenicity in murine models [32,33, 38]. The live virus vaccine approach was selected because it is the most practical for diseases of developing nations due to its low manufacturing cost and the rapid, long-term immunity that is usually generated. For example, the live-attenuated 17-D yellow fever virus vaccine is administered at a cost of only US\$0.65 per person, and boosters are recommended only 10 years after the single initial dose [47].

Attenuation and Safety

Like previous chimeras that were developed as vaccines against VEE and EEE [32,33,38], 3 different CHIKV vaccine strains were attenuated in various mouse models compared to the parent viruses and other alphavirus vaccine strains. The chimeras also induced robust humoral immunity in vaccinated mice, with no detectable reactogenicity, even after relatively high doses of 5.3 – $5.8 \log_{10}$ PFU. None of the adult or subadult mice we infected exhibited any signs of neurologic disease, febrile responses, or growth delays as indicated by continued weight gain. Attenuation of the chimeric vaccine candidates appeared to be better than that of vaccine strain

181/25, which produced higher levels of viremia and replication in the joints of newborn mice. The joints are important sites of pathology in CHIKV-induced arthralgic disease, and strain 181/25 produced arthralgia in some vaccinees [30]. Therefore, the finding that our chimeric vaccine candidates replicate less efficiently in and/or around the joints of mice suggests that they may be less reactogenic in humans than strain 181/25.

Although the murine model for CHIK reproduced disease only in younger age groups, vaccination of 3-week-old mice with the chimeras, and IP or IN challenge 3 weeks later, demonstrated significant protection from weight loss as well as challenge virus replication in most organs and serum, measured at 2 different time points.

Immunogenicity

All of the chimeric vaccine candidates produced robust neutralizing Ab titers after a single SC inoculation of 5.3–5.8 log₁₀ PFU. These doses were selected based on the titers of previously reported chimeric alphavirus vaccine candidates required to achieve uniformly robust immunity and protection against severe challenge [32,33,38]. In the present study, the CHIKV chimera versions that used the attenuated VEEV strain TC-83 or naturally attenuated EEEV backbones were consistently more immunogenic in outbred or inbred mice (Table 5) than the chimera with a SINV backbone, as measured by mean neutralizing Ab titers, yet were not detectably more virulent. Both of these chimeras also appeared to be more immunogenic than vaccine strain 181/25 (Table 6) even though they produced lower viremia and replication in the joints of newborn mice (Fig. 6).

Considering the natural attenuation of the SA EEEV strain BeAr436087 in mice [33], hamsters, guinea pigs (SCW, unpublished) and marmosets [36], the documented role of the nsPs of this strain in natural attenuation [35], and the epidemiological evidence that all South American EEEV strains are attenuated for humans [48], the EEE/CHIKV may be the most promising of the 2 vaccine candidates. Additional studies to determine if strain BeAr436087 is attenuated in other rodent models and equids would be useful to support the rationale for further development of this vaccine candidate.

Future studies should focus on determining the lowest vaccine doses needed to achieve adequate immunity and protection against challenge, and the use of longer-lived nonhuman primate models to determine the duration of immunity.

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References

1. Karabatsos, N. International Catalog of Arboviruses Including Certain Other Viruses of Vertebrates. 4th ed.. San Antonio: American Society of Tropical Medicine and Hygiene; 1985.
2. Carey DE. Chikungunya and dengue: a case of mistaken identity? J Hist Med Allied Sci 1971;26(3): 243–262. [PubMed: 4938938]
3. Halstead SB, Udomsakdi S, Scanlon JE, Rohitayodhin S. Dengue and chikungunya virus infection in man in Thailand, 1962–1964. V. Epidemiologic observations outside Bangkok. American Journal of Tropical Medicine & Hygiene 1969;18(6):1022–1033. [PubMed: 4390976]
4. Halstead SB, Scanlon JE, Umpaivit P, Udomsakdi S. Dengue and chikungunya virus infection in man in Thailand, 1962–1964. IV. Epidemiologic studies in the Bangkok metropolitan area. American Journal of Tropical Medicine & Hygiene 1969;18(6):997–1021. [PubMed: 4390977]

5. Rao TR. Recent epidemics caused by chikungunya virus in India, 1963–1965. *Scientific Culture* 1966;32:215.
6. Nimmannitya S, Halstead SB, Cohen SN, Margiotta MR. Dengue and chikungunya virus infection in man in Thailand, 1962–1964. I. Observations on hospitalized patients with hemorrhagic fever. *Am J Trop Med Hyg* 1969;18(6):954–971. [PubMed: 5355242]
7. Carey DE, Myers RM, DeRanitz CM, Jadhav M, Reuben R. The 1964 chikungunya epidemic at Vellore, South India, including observations on concurrent dengue. *Transactions of the Royal Society of Tropical Medicine & Hygiene* 1969;63(4):434–445. [PubMed: 5821266]
8. Sarkar JK, Chatterjee SN, Chakravarti SK, Mitra AC. Chikungunya virus infection with haemorrhagic manifestations. *Indian J Med Res* 1965;53(10):921–925. [PubMed: 5849867]
9. Laras K, Sukri NC, Larasati RP, Bangs MJ, Kosim R, Djauzi, et al. Tracking the re-emergence of epidemic chikungunya virus in Indonesia. *Trans R Soc Trop Med Hyg* 2005 Feb;99(2):128–141. [PubMed: 15693148]
10. Peyrefitte CN, Bessaud M, Pastorino BA, Gravier P, Plumet S, Merle OL, et al. Circulation of Chikungunya virus in Gabon, 2006–2007. *Journal of medical virology* 2008 Mar;80(3):430–433. [PubMed: 18205212]
11. Jupp, PG.; McIntosh, BM. Chikungunya virus disease. In: Monath, TP., editor. *The Arbovirus: Epidemiology and Ecology*. Vol II. Boca Raton, Florida: CRC Press; 1988. p. 137–157.
12. Diallo M, Thonnon J, Traore-Lamizana M, Fontenille D. Vectors of chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg* 1999;60(2):281–286. [PubMed: 10072152]
13. Tesh RB. Arthritides caused by mosquito-borne viruses. *Ann Rev Med* 1982;33:31–40. [PubMed: 6123291]
14. AbuBakar S, Sam IC, Wong PF, MatRahim N, Hooi PS, Roslan N. Reemergence of endemic Chikungunya, Malaysia. *Emerg Infect Dis* 2007 Jan;13(1):147–149. [PubMed: 17370532]
15. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med* 2006 Jul;3(7):e263. [PubMed: 16700631]
16. Charrel RN, de Lamballerie X, Raoult D. Chikungunya outbreaks--the globalization of vectorborne diseases. *N Engl J Med* 2007 Feb 22;356(8):769–771. [PubMed: 17314335]
17. Kalantri SP, Joshi R, Riley LW. Chikungunya epidemic: an Indian perspective. *Natl Med J India* 2006 Nov–Dec;19(6):315–322. [PubMed: 17343016]
18. Chretien JP, Anyamba A, Bedno SA, Breiman RF, Sang R, Sergon K, et al. Drought-associated chikungunya emergence along coastal East Africa. *Am J Trop Med Hyg* 2007 Mar;76(3):405–407. [PubMed: 17360859]
19. Robin S, Ramful D, Le Seach F, Jaffar-Bandjee MC, Rigou G, Alessandri JL. Neurologic Manifestations of Pediatric Chikungunya Infection. *J Child Neurol*. 2008 Feb 20;
20. Jossieran L, Paquet C, Zehgnoun A, Caillere N, Le Tertre A, Solet JL, et al. Chikungunya disease outbreak, Reunion Island. *Emerg Infect Dis* 2006 Dec;12(12):1994–1995. [PubMed: 17354339]
21. Mavalankar D, Shastri P, Bandyopadhyay T, Parmar J, Ramani KV. Increased mortality rate associated with chikungunya epidemic, ahmedabad, India. *Emerg Infect Dis* 2008 Mar;14(3):412–415. [PubMed: 18325255]
22. Beltrame A, Angheben A, Bisoffi Z, Monteiro G, Marocco S, Calleri G, et al. Imported Chikungunya Infection, Italy. *Emerg Infect Dis* 2007 Aug;13(8):1264–1266. [PubMed: 17953112]
23. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, Panning M, et al. Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* 2007 Dec 1;370(9602):1840–1846. [PubMed: 18061059]
24. Sprenger D, Wuithiranyagool T. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. *J Am Mosq Control Assoc* 1986 Jun;2(2):217–219. [PubMed: 3507493]
25. Forattini OP. Identification of *Aedes* (*Stegomyia*) *albopictus* (Skuse) in Brazil. *Rev Saude Publica* 1986 Jun;20(3):244–245. [PubMed: 3809982]
26. Weinbren MP. The occurrence of Chikungunya virus in Uganda. II. In man on the Entebbe peninsula. *Trans R Soc Trop Med Hyg* 1958 May;52(3):258–259. [PubMed: 13556870]

27. Weinbren MP, Haddow AJ, Williams MC. The occurrence of Chikungunya virus in Uganda. I. Isolation from mosquitoes. *Trans R Soc Trop Med Hyg* 1958 May;52(3):253–257. [PubMed: 13556869]
28. Paul SD, Singh KR. Experimental infection of *Macaca radiata* with Chikungunya virus and transmission of virus by mosquitoes. *Indian J Med Res* 1968 Jun;56(6):802–811. [PubMed: 4971384]
29. Levitt NH, Ramsburg HH, Hasty SE, Repik PM, Cole FE, Lupton HW. Development of an attenuated strain of chikungunya virus for use in vaccine production. *Vaccine* 1986;4(3):157–162. [PubMed: 3020820]
30. Edelman R, Tacket CO, Wasserman SS, Bodison SA, Perry JG, Mangiafico JA. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. *Am J Trop Med Hyg* 2000 Jun;62(6):681–685. [PubMed: 11304054]
31. Kinney RM, Chang GJ, Tsuchiya KR, Sneider JM, Roehrig JT, Woodward TM, et al. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. *J Virol* 1993;67(3):1269–1277. [PubMed: 7679745]
32. Paessler S, Fayzulin RZ, Anishchenko M, Greene IP, Weaver SC, Frolov I. Recombinant Sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. *J Virol* 2003 Sep;77(17):9278–9286. [PubMed: 12915543]
33. Wang E, Petrakova O, Adams AP, Aguilar PV, Kang W, Paessler S, et al. Chimeric Sindbis/eastern equine encephalitis vaccine candidates are highly attenuated and immunogenic in mice. *Vaccine* 2007 Oct 23;25(43):7573–7581. [PubMed: 17904699]
34. Tssetsarkin K, Higgs S, McGee CE, De Lamballerie X, Charrel RN, Vanlandingham DL. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. *Vector Borne Zoonotic Dis* 2006 Winter;6(4):325–337. [PubMed: 17187566]
35. Aguilar PV, Adams AP, Wang E, Kang W, Carrara AS, Anishchenko M, et al. Structural and nonstructural protein genome regions of eastern equine encephalitis virus are determinants of interferon sensitivity and murine virulence. *J Virol* 2008 May;82(10):4920–4930. [PubMed: 18353963]
36. Adams AP, Aronson JF, Tardif SD, Patterson JL, Brasky KM, Johnson CE, et al. Common marmosets (*Callithrix jacchus*) as a nonhuman primate model to assess the virulence of eastern equine encephalitis virus strains. *J Virol*. 2008in press
37. Beaty, BJ.; Calisher, CH.; Shope, RE. Arboviruses. In: Schmidt, NJ.; Emmons, RW., editors. *Diagnostic procedures for viral, rickettsial and chlamydial infections*. 6th edition. Washington, D. C.: American Public Health Association; 1989. p. 797-855.
38. Paessler S, Ni H, Petrakova O, Fayzulin RZ, Yun N, Anishchenko M, et al. Replication and clearance of Venezuelan equine encephalitis virus from the brains of animals vaccinated with chimeric SIN/VEE viruses. *J Virol* 2006 Mar;80(6):2784–2796. [PubMed: 16501087]
39. Aguilar PV, Weaver SC, Basler CF. Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. *J Virol* 2007 Apr;81(8):3866–3876. [PubMed: 17267491]
40. Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I. The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* 2007 Mar;81(5):2472–2484. [PubMed: 17108023]
41. Byrnes AP, Griffin DE. Large-plaque mutants of Sindbis virus show reduced binding to heparin sulfate, heightened viremia, and slower clearance from the circulation. *J Virol* 2000;74(2):644–651. [PubMed: 10623725]
42. Bernard KA, Klimstra WB, Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* 2000;276(1):93–103. [PubMed: 11021998]
43. Ziegler SA, Lu L, Travassos da Rosa A, Xiao SY, Tesh RB. An animal model for studying the pathogenesis of chikungunya virus infection. *Am J Trop Med Hyg*. 2008in press
44. Berge TO, Banks IS, Tigertt WD. Attenuation of Venezuelan equine encephalomyelitis virus by *in vitro* cultivation in guinea pig heart cells. *Am J Hyg* 1961;73:209–218.
45. Davis NL, Brown KW, Greenwald GF, Zajac AJ, Zacny VL, Smith JF, et al. Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal

- combined with a second-site suppressor mutation in E1. *Virology* 1995;212(1):102–110. [PubMed: 7676619]
46. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J Gen Virol* 2007 Sep;88(Pt 9):2363–2377. [PubMed: 17698645]
47. Monath TP, Nasidi A. Should yellow fever vaccine be included in the expanded program of immunization in Africa? A cost-effectiveness analysis for Nigeria. *Am J Trop Med Hyg* 1993 Feb; 48(2):274–299. [PubMed: 8447531]
48. Aguilar PV, Robich RM, Turell MJ, O'Guinn ML, Klein TA, Huaman A, et al. Endemic eastern equine encephalitis in the Amazon region of Peru. *Am J Trop Med Hyg* 2007 Feb;76(2):293–298. [PubMed: 17297038]

Fig. 1.

Diagram of the genetic composition of the chimeric CHIKV vaccine candidates and the specific infectivities of transcribed RNAs following electroporation of BHK cells. nsP1–4, nonstructural proteins 1–4; SG, subgenomic promoter; C, capsid; E1 and E2, envelope glycoproteins 1 and 2.

Fig. 2.

Expression of structural proteins by chimeric vaccine candidates. BHK-21 cells were infected with SINV Toto1101, VEEV TC-83, VEE/CHIKV, EEE/CHIKV and SIN/CHIKVD9 at an MOI of 20 PFU/cell. At 16 h post infection, cells were washed three times with PBS and incubated for 1 h at 37°C in DMEM lacking methionine and supplemented with 0.1% FBS and 20 mCi/ml of [³⁵S]methionine. After this incubation, cells were scraped into PBS, pelleted by centrifugation and dissolved in standard gel-loading buffer. Equal amounts of proteins were loaded onto sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Markers indicate p62, E1+E2 (envelope glycoproteins E1 and E2), and capsid.

Fig. 3. Histopathology and immunohistochemistry of 5-week-old C57/BL6 mouse brains following IN infection with the Ross strain of CHIKV. H&E staining of mouse brains at day 7 after IN infection with either PBS (A) or CHIKV (B). CHIKV-infected mice showed severe, multifocal inflammation and liquefactive necrosis in the cerebral cortex. Perivascular cuffs were also located diffusely throughout the cerebral cortex (arrow). Immunohistochemical staining of the cerebral cortex at day 7 after IN infection with CHIKV (C, D). Primary antibodies against Modoc virus (MODV) were used as a negative control (C). Using primary antibodies against CHIKV, there was intense positive staining (dark red) of degenerating neurons located in the necrotic areas of the cerebral cortex (D).

Fig. 4. Body weights in 3-week-old female C57BL/6 mice after SC vaccination with EEE/CHIKV, TC-83/CHIKV or SIN/CHIKV at doses of 5.8, 5.6 and 5.8 $\log_{10}$ PFU/mouse, respectively, or of 3-week-old female NIH Swiss mice after SC vaccination with EEE/CHIKV, TC-83/CHIKV or SIN/CHIKV at doses of 5.8, 5.6 and 5.8 $\log_{10}$ PFU/mouse, respectively.

Fig. 5. Mortality in 6-day-old NIH Swiss mice after IC infection with ca. 10^6 PFU of chimeric CHIKV vaccine candidates, vaccine strain 181/25, or the wt CHIKV LR strain. Other alphavirus vaccine strains including VEEV strains TC-83, V3526, and chimeric SIN/VEEV and SIN/EEEV are included for comparison.

Fig. 6. Replication of CHIKV chimeric vaccine candidates or the wt LR CHIKV strain after SC infection of 3–4-day-old NIH Swiss mice with ca. 10^5 PFU. The limits of detection are indicated by the dashed lines.

Fig. 7. Body weight of vaccinated and sham-vaccinated mice after CHIKV challenge. Cohorts of five 3-week-old females were vaccinated with EEE/CHIKV, TC-83/CHIKV or SIN/CHIKV at doses of 5.3–5.8 $\log_{10}$ PFU/mouse. Three weeks later, the mice were challenged with the Ross strain of CHIKV IN at a dose 6.5 $\log_{10}$ PFU.

Table 1

Titers of chimeric alphaviruses found in Vero cell passage 3 supernatants after infection at a multiplicity of approximately 1.

Virus	Cells used for plaque assay			
	Vero		BHK-21	
	24h	48h	24h	48h
VEE/CHIKV	2×10^8	1.5×10^8	1.5×10^8	1.25×10^8
EEE/CHIKV	5×10^7	4×10^7	7.5×10^7	1×10^8
SIN/CHIKV	5×10^7	5×10^7	7×10^6	7×10^6

Table 2

Titers in Vero cell cultures 24 hours after infection with plaque-purified chimeric alphaviruses.

	VEE/CHIKV	EEE/CHIKV	SIN/CHIKV
Plaque 1	2.5×10^9	1.15×10^9	1×10^8
Plaque 2	2×10^7	1.1×10^9	1.5×10^8

Table 3
Viremia in 5 week-old, female C57BL/6 mice infected with CHIKV

Virus strain	Number tested	Route of inoculation*	Mean viremia 24h after infection (log ₁₀ PFU/ml ±SD)	Mean viremia 48 h after infection (log ₁₀ PFU/ml ±SD)	Mean viremia 72 h after infection (log ₁₀ PFU/ml ±SD)
LR	3	IN	<0.9	2.6±0.7	2.4 ±0.3
	3	IP	3**	<0.9	2.3**
Ross	3	IN	2.6±0.5	2.5±0.3	<0.9
	4	IP	2.3±0.4***	<0.9	<0.9

* IN, intranasal; IP, intraperitoneal; Doses: 7.8 log₁₀ PFU (IP) or 6.5 log₁₀ PFU (IN)

** One mouse was positive

*** Two mice were positive

Table 4

Ross strain CHIKV titers in 5-week-old C57BL/6 mice after intranasal infection

Organ/tissue	Virus titer ($\log_{10}$ PFU/ml or g $\pm$ SD)	
	Day 3	Day 7
Blood	<0.9	<0.9
Brain	7.1 $\pm$ 0.5	6.2 $\pm$ 1.3
Heart	2.9 $\pm$ 0.7	<0.9
Lung	2.9 $\pm$ 0.9**	<0.9
Liver	<0.9	<0.9
Spleen	2.2*	<0.9
Kidney	<0.9	<0.9
Muscle	2.1*	<0.9

* One mouse was positive

** Two mice were positive

Table 5

Neutralizing antibody titers in mice 3 weeks after immunization with vaccine candidates

Virus strain	Mouse strain	Vaccine dose (log ₁₀ PFU)	Number seropositive [*]	Mean antibody titer ± SD
EEE/CHIKV	NIH Swiss	5.3	10/10	116±88.8
	C57BL/6	5.8	5/5	80±49.0
TC-83/CHIKV	NIH Swiss	5.8	10/10	136±82.6
	C57BL/6	5.6	5/5	72±17.9
SIN/CHIKV	NIH Swiss	5.8	10/10	43±19.7
	C57BL/6	5.8	5/5	40±24.5

* 80% plaque reduction neutralization titer ≥20

Table 6

Dose-response in neutralizing antibodies and protection from challenge* 3 weeks after vaccination of C57BL/6 mice

Vaccine strain	Vaccine dose (log ₁₀ PFU)	Number seropositive**	Mean antibody titer ± SD	Survival after challenge
EEE/CHIKV	5.9	5/5	200 ± 80	5/5
	4.9	5/5	144 ± 36	5/5
	3.9	5/5	112 ± 44	5/5
TC-83/CHIKV	5.8	5/5	260 ± 120	5/5
	4.8	5/5	256 ± 88	5/5
	3.8	5/5	224 ± 88	5/5
181/25	5.5	5/5	144 ± 115	5/5
Sham		0/5		0/5

* IN challenge with 6.5 log₁₀ PFU of the Ross CHIKV strain** PRNT₈₀ ≥ 20

Table 7

Viremia in vaccinated or sham-vaccinated mice after IN challenge with CHIKV*

Mouse strain	Vaccine strain	Virus titer (log ₁₀ PFU/ml) ±SD		
		Day 1	Day 2	Day 3
C57BL/6	Sham	2.5±0.4	2.0±0.0	<0.9
	EEE/CHIKV	<0.9	<0.9	<0.9
	TC-83/CHIKV	<0.9	<0.9	<0.9
	SIN/CHIKV	<0.9	<0.9	<0.9
NIH Swiss	Sham	3.3 ± 0.8	3.2 ± 0.7	<0.9
	EEE/CHIKV	<0.9	<0.9	<0.9
	TC-83/CHIKV	<0.9	<0.9	<0.9
	SIN/CHIK	<0.9	<0.9	<0.9

* IN challenge with 6.5 log₁₀ PFU of the Ross CHIKV strain