A VLP vaccine for epidemic Chikungunya virus protects non-human primates against infection

Wataru Akahata[1], **Zhi-yong Yang**[1], **Hanne Andersen**[2], **Siyang Sun**[3], **Heather A. Holdaway**[3], **Wing-Pui Kong**[1], **Mark G. Lewis**[2], **Stephen Higgs**[4], **Michael G. Rossmann**[3], **Srinivas Rao**[1], and **Gary J. Nabel**[1,*]

[1] Vaccine Research Center NIAID, National Institutes of Health Bethesda, MD 20892-3005

[2] Bioqual, Inc. Rockville, MD 20850-3336

[3] Department of Biological Sciences Purdue University West Lafayette, IN 47907-2054

[4] Department of Pathology University of Texas Medical Branch Galveston, TX 77555-0609

Abstract

Chikungunya virus (CHIKV) has infected millions of people in Africa, Europe, and Asia[1,2] since its re-emergence in Kenya in 2004. The severity of disease and spread of this epidemic virus present a serious public health threat in the absence of vaccines or anti-viral therapies. Here, we describe a novel vaccine that protects against emerging CHIKV infection of non-human primates (NHP). We show that selective expression of viral structural proteins gives rise to virus-like particles (VLPs) *in vitro* that resemble replication-competent alphaviruses. Immunization with these VLPs elicited neutralizing antibodies against envelope proteins from different CHIKV strains. Monkeys immunized with VLPs produced high titer neutralizing antibodies that protected against viremia after high dose challenge. We transferred these antibodies into immunodeficient mice, where they protected against subsequent lethal CHIKV challenge, establishing a humoral mechanism of protection. Immunization with alphavirus VLP vaccines represents a strategy to contain the spread of CHIKV and related pathogenic viruses in humans.

Chikungunya virus (CHIKV), a mosquito-borne *alphavirus* in the family *Togaviridae*, was first isolated in Tanzania in 1952[3]. Infection by this virus in humans is characterized by rash, high fever and, its hallmark feature, severe arthritis that can persist for years[1,2]. An epidemic strain of CHIKV appeared on Reunion Island in 2005 and has since spread to more than 18 countries[4] (see also http://www.cdc.gov/ncidod/dvbid/chikungunya/CH_GlobalMap.html). Dissemination of this epidemic virus is associated with genetic mutations that facilitate adaptation to a new insect vector, the Asian tiger mosquito, *Aedes*

*albopictus*5, that survives in temperate climates and is widely distributed6. CHIKV continues to cause substantial morbidity resulting in significant economic losses, and vaccine development remains a high priority.

CHIKV is composed of a positive single strand genomic RNA of 12,000 nucleotides encoding four nonstructural and five structural polyproteins7. The nonstructural proteins, nsP1, nsP2, nsP3 and nsP4, are required for virus replication; the structural proteins, which consist of capsid (C) and envelope proteins (E; E1, E2, E3 and 6K), are synthesized as polyproteins and are cleaved by capsid autoproteinase and signalases7 (Fig. 1a, upper panel). Based on the similarity of CHIKV to other alphaviruses, CHIKV has spikes on the virion surface that are each formed by three E1-E2 heterodimers8,9, where the E1 glycoproteins mediate fusion and E2 glycoproteins interact with the host receptor7. Phylogenetic analysis of CHIKV has revealed three genotypes: Asian, East/Central/South African and West African. All strains are highly related, with 95.2–99.8% amino acid similarity10. Although CHIKV strains vary, individual strains are antigenically related, so it is potentially possible to design a vaccine that works against heterologous strains11.

We first analyzed the immunogenicity and cross-reactivity of two disparate strains, 37997 from the older West Africa lineage and LR2006 OPY-1, the more recent strain responsible for the current epidemic. To examine the specificity of viral cell entry and their sensitivity to neutralizing antibodies, we developed lentiviral vector reporters pseudotyped with glycoproteins from these different CHIKV strains. Such vectors allow analysis of the mechanism and the specificity of entry and serve to quantitate antibody neutralization more readily than wild type viruses that pose significant biohazard concerns for routine laboratory analyses. They therefore provide a useful tool for the study of CHIKV entry as well as for vaccine development12-14. We inserted CHIKV E genes expressing the native polypeptide, E3-E2-6K-E1 (Fig. 1a, lower panel), for the 37997 and LR2006 OPY-1 strains into an expression vector (E_{37997} and E_{OPY-1}). Incorporation of the two CHIKV Es into the pseudotyped lentiviral vectors was verified by buoyant density gradient sedimentation of the virus. Both CHIKV E and HIV-1 Gag had the same buoyant density as lentivirus particles (Supplementary Fig. 1). The 37997 and LR2006 OPY-1 CHIKV pseudotyped lentiviral vectors infected several cell lines permissive for viral replication15 (Fig. 1b, left). We measured their ability to express a luciferase reporter in 293A human renal epithelial cells and found that entry was dose-dependent (Fig. 1b, right), while a control devoid of CHIKV envelope proteins did not infect these cell lines. To determine whether entry occurred through the same mechanism as native virus, we analyzed pH and endosome dependence of entry 13. CHIKV infects through pH-dependent cell fusion15; thus, addition of ammonium chloride or chloroquine, which prevents acidification of the endosome, caused a dose-dependent reduction in CHIKV pseudotyped vector entry (Fig. 1c). We observed similar inhibition of entry with VSV-G, known to enter in this fashion, but not with amphotropic murine leukemia virus (MuLV) glycoprotein 70, which enters by a pH-independent mechanism13,16. These findings demonstrated that lentiviral vectors pseudotyped with CHIKV envelope mediated entry through the same mechanism as wild type virus. Next, we examined a commercially available immune serum from mice infected with an established CHIKV strain (ATCC). Incubation of immune sera with the CHIKV pseudotyped lentiviral

vector, but not a VSV-G pseudotyped vector, inhibited entry (Fig. 1d). The specificity and potency of neutralizing antibodies could therefore be quantified without exposure to infectious virus.

We analyzed eukaryotic expression vectors encoding C-E3-E2-6K-E1 from strains 37997 and LR2006 OPY-1 (C-E$_{37997}$ and C-E$_{OPY-1}$) for their ability to give rise to VLPs. We transfected 293T human kidney cells with plasmids C-E$_{37997}$ or C-E$_{OPY-1}$ or the E expression vectors described above, E$_{37997}$ or E$_{OPY-1}$ (Fig. 2a, upper panel), and confirmed expression in the cell lysates by Western blotting (Fig. 2a, lower panel). We also detected C and E1/E2 proteins in the supernatant by Western blotting after transfection of the C-E$_{37997}$ or C-E$_{OPY-1}$ vector, suggesting that CHIKV VLPs had been generated. We purified VLPs by buoyant density gradient sedimentation. The yield of VLPs from strain 37997 was 10–20 mg L^{-1} – approximately 100 times higher than that from strain LR2006 OPY-1. For this reason and because of the high degree of amino acid similarity among CHIKV strains, we used the 37997 strain to produce VLPs. Fractionation of clarified supernatant showed greatest incorporation of E1/E2 into the VLPs at a density of 1.2 g mL^{-1} (Fig. 2b, left), comparable to the density of wild type CHIKV17. Examination of the purified fraction from strain 37997 by electron microscopy revealed VLPs with the same morphologic appearance as wild type virus (Fig. 2b, right).

Cryoelectron microscopy and three dimensional image reconstruction, assuming icosahedral symmetry, showed that the VLPs had an external diameter of 65 nm and a core diameter of 40 nm (Fig. 2c). The potent immunogenic E1/E2 glycoproteins are organized into 240 heterodimers, assembled into 80 glycoprotein spikes arranged with T=4 quasi symmetry on the surface of the VLPs (Fig. 2c), closely similar to the structure of Sindbis virus (Supplementary Fig. 2; see also refs. 8,9,18).

Because of the ease of DNA vaccine preparation and its broad applicability, we compared the relative immunogenicity of plasmid DNA to VLP vaccines in mice by immunizing with DNA vaccines encoding C-E or E (strains 37997 and LR2006 OPY-1) or VLPs from strain 37997 (VLP$_{37997}$) in the presence or absence of Ribi adjuvant. BALB/c mice injected twice intramuscularly with VLPs with adjuvant generated the highest titer neutralizing responses against both the homologous strain 37997 (Fig. 3a, right panel; IC$_{50}$, 1:10,703) and the heterologous strain LR2006 OPY-1 (Fig. 3b, right panel; IC$_{50}$, 1:54,600). While three immunizations with the plasmids encoding C-E and E from both strains elicited neutralizing responses, these titers were less than five percent those of VLP-immunized mice (Fig. 3a, b; left panel; IC$_{50}$ titer of 319 for DNA C-E$_{37997}$ against strain 37997; 525 for DNA C-E$_{37997}$ against strain LR2006 OPY-1). These results suggested that VLPs elicited a more potent neutralizing antibody response than DNA vaccines.

To characterize VLP-induced immune responses in a model with stronger predictive value for humans, we immunized rhesus macaques with VLPs. Monkeys received intramuscular injections of VLP$_{37997}$ or PBS alone as a control. We tested sera from immunized and control monkeys against CHIKV strain 37997 and LR2006 OPY-1 pseudotyped lentiviral vectors. All non-human primates (NHP) immunized with VLPs developed substantial neutralizing activity to both homologous and heterologous strains after primary

immunization that increased after boosting (Fig. 3c; left panel: IC_{50} titer of 10,219 against strain 37997, right panel: IC_{50} titer of 15,072 against strain LR2006 OPY-1). Interestingly, there was slightly increased neutralization of LR2006 OPY-1 compared to 37997 in both mice and monkeys, although these effects were not substantial, suggesting that LR2006 OPY-1 may present a common epitope to the immune system somewhat better than 37997. To confirm that these antibodies neutralized infectious virus, we performed a plaque reduction neutralization test (PRNT) against the CHIKV LR2006 OPY-1. The antisera from the immunized monkeys elicited neutralizing antibody responses against LR2006 OPY-1 at $PRNT_{50}$ titers of 40,133 (Fig. 3d). These data suggested that neutralizing antibodies using pseudotyped lentiviral vectors correlated with the PRNT assay, and that all VLP-immunized monkeys generated potent neutralizing antibody responses against CHIKV.

To establish the CHIKV challenge model in monkeys, we injected two naïve rhesus monkeys intravenously with a high titer LR2006 OPY-1 virus stock. Viremia commenced at 6 h and lasted until at least 72 h after challenge in these monkeys, with the peak viremia at 24 h after infection (Supplementary Fig. 3a). Similar to humans, infection in NHPs resulted in viremia and was not lethal. In addition, we observed a similar transient acute lymphopenia and a pro-inflammatory response as measured by transient neutrophilia and an increase in monocyte counts (Supplementary Fig. 3b). We assessed the ability of the VLP vaccine to protect against infection by intravenous challenge of monkeys immunized with VLPs or controls using the same high titer LR2006 OPY-1 virus stock 15 weeks after the final immunization. In this model, all immunized monkeys controlled the challenge virus completely (Fig. 4a). In contrast, we observed viremia in all the control monkeys injected with CHIKV, as in the pilot experiment. Similarly, monocyte counts were markedly increased in control monkeys 4 days after challenge, while they remained unchanged in vaccinated monkeys (Fig. 4b, Control, at day 0 vs. 7, $P = 0.0015$; VLPs at day 0 vs. 7, $P = 0.38$). These data suggest that immunization protected against both viremia and the inflammatory consequences of infection.

To define the mechanism of protection in these animals, we investigated whether or not immune IgG could protect against lethal challenge using an adoptive transfer model. Previous studies have shown that immunodeficient mice with defective type-I interferon signaling are susceptible to lethal CHIKV infection, displaying pathologic manifestations of infection[19], and providing a model to evaluate immune mechanisms of protection. For example, Couderc *et. al*[20] showed that antibodies play an important role in protecting against CHIKV infection by passive transfer of IgG from infected humans in this model. Here, we intravenously transferred purified total IgG from immunized or control monkeys into these mice, then challenged the recipient mice intradermally with a lethal dose of LR2006 OPY-1 24 h later. Recipients of purified IgG from CHIKV-immunized monkeys demonstrated no detectable viremia after challenge and were completely protected from lethality (Fig. 4c,d). In contrast, all mice that received purified IgG from control monkeys showed severe infection and viremia, and all died. These results indicate that humoral immune responses induced by CHIKV VLPs conferred protection against CHIKV infection.

To date, there has been limited success in developing a safe and effective CHIKV vaccine. A live attenuated CHIKV vaccine candidate[21,22] caused transient arthralgia in

volunteers23. Other efforts include a formalin-killed vaccine24,25, a Venezuelan equine encephalitis/CHIKV chimeric live attenuated vaccine26 and a consensus-based DNA vaccine27 that have not yet proven both safe and effective. Development of a safe and effective CHIKV vaccine will require additional evaluation in humans. However, the safety and efficacy of VLP vaccines in general make them promising candidates for further study.

VLPs are known to be highly immunogenic and elicit higher titer neutralizing antibody responses than subunit vaccines based on individual proteins28-30. Such VLPs authentically present viral spikes and other surface components in a repetitive array that effectively elicits recognition by B-cells to stimulate antibody secretion28,29. This recognition leads to B cell signaling and MHC class II up-regulation that facilitates the generation of high titer specific antibodies. VLPs from other viruses, including hepatitis B virus and human papillomavirus, elicit high titer neutralizing antibody responses that contribute to protective immunity in humans30,31. The vaccines described here represent the first use of recombinant VLPs to prevent infection by *alphaviruses*. At a time when there are no commercially available vaccines and CHIKV is emerging, it has the potential to significantly impact the spread of this disease. Changes in trade, travel and global climate have aided the spread of mosquito species worldwide, which may potentially cause other *alphavirus* outbreaks. This approach to vaccine development may prove useful for other *alphaviruses* of increasing concern, including Western, Eastern, and Venezuelan equine encephalitis viruses, o'nyong-nyong virus and Ross River virus.

METHODS

Vector construction

We synthesized plasmids encoding structural polyproteins C, E1, E2, E3 and 6K (strains 37997 and LR2006 OPY-1, GenBank EU224270 and EU224268, respectively) as previously described14 (GeneArt). We amplified plasmids encoding the polyproteins E3, E2, 6K, and E1 by PCR using sense primer 5′-GCTCTAGACACCATGAGCCTCGCCCTCCCGGTCTTG-3′ and antisense primer 5′-TGGATCCTCATTAGTGCCTGCTAAACGACA-3′ (37997) and sense primer 5′-GCTCTAGACACCATGAGTCTTGCCATCCCAGTTATG-3′ and antisense primer 5′-TGGATCCTCATTAGTGCCTGCTGAACGACA-3′ (LR2006 OPY-1). We inserted XbaI and BamHI sites for cloning. We digested each fragment with XbaI/BamHI and inserted it into a eukaryotic expression vector, CMV/R14 (C-E$_{37997}$, C-E$_{OPY-1}$, E$_{37997}$ and E$_{OPY-1}$). The CMV/R vector comprises the human CMV IE enhancer/promoter, an HTLV-1 R region containing a splicing donor, a CMV IE splicing acceptor and bovine growth hormone poly A signal.

Production of pseudotyped lentiviral vectors

We created lentiviral vectors expressing glycoproteins from different CHIKV strains. The method for producing recombinant lentiviral vectors expressing a luciferase reporter gene has been previously described12,14. Briefly, we cotransfected 293T cells with 500 ng CHIKV E plasmid from either strain (E$_{37997}$ or E$_{OPY-1}$), 7 μg of a transducing vector encoding a luciferase reporter gene under the control of a CMV promoter (pHR'CMV-

luciferase plasmid), and 7 μg of a packaging plasmid that expresses all human immunodeficiency virus type 1 (HIV-1) structural proteins except envelope (pCMV R8.2) (Supplementary Fig. 1a). Additional methods and neutralization assay with CHIKV E pseudotyped lentiviral vectors are described in the Supplementary Methods.

Buoyant density gradient sedimentation analysis and purification of VLPs

We transfected 293F cells (2.5×10^8) (Invitrogen) with 293fectin transfection reagent (Invitrogen) and 125 μg of C-E$_{37997}$ plasmid following the manufacturer's recommendations. Detailed methods for buoyant density gradient analysis and purification of VLPs have been described in a previous publication[32] and in the Supplementary Methods.

Cryo-electron microscopy and image analysis

We flash-froze Chikungunya VLPs on holey grids in liquid ethane, and recorded images at 47K magnification with a CM300 FEG microscope with electron dose levels of approximately 20 e$^-$/Å2. We digitized all micrographs at 6.35 μm per pixel using a Nikon scanner, and boxed individual particle images using the program e2boxer in the EMAN2 package[33]. We used the CTFIT program from the EMAN package[34] to determine CTF parameters and flip phases. We constructed an initial model in EMAN using assigned 2-, 3-, and 5-fold views and refined it in EMAN assuming icosahedral symmetry. The number of particles incorporated into the final reconstruction was 1489, giving a final resolution of 18 Å based on a 0.5 Fourier shell correlation threshold.

Immunizations and challenge of mice and monkeys

We mixed 19 μg of VLPs (equivalent to approximately 10 μg of E1/E2) in 60 μl normal saline with 60 μl of Ribi solution (Sigma Adjuvant system, Sigma-Aldrich) per mouse. We injected female 6- to 8-week-old BALB/c mice in the right and left quadriceps muscles with VLPs in normal saline or Ribi in 120 μl total volume, two times at weeks 2 and 6. For DNA vaccination, we injected mice in the right and left quadriceps with a total of 15 μg of purified plasmid C-E$_{37997}$, E$_{37997}$, C-E$_{OPY-1}$ or E$_{OPY-1}$ suspended in 100 μl of normal saline three times at weeks 0, 3 and 6. Each group contained five mice. We collected sera and spleen 10 days after the last injection. In monkey experiments, we injected 3–4 year old rhesus macaques weighing 3–4 kg intramuscularly in the anterior quadriceps with either 20 μg of VLPs in 1 mL PBS (VLP group) or 1 mL PBS alone (control group) at weeks 0, 4 and 24. Each group contained six monkeys. To measure antibody titers, we collected blood on days –14, 0, 10, 28, 38, 56, 70, 161 and 178. We challenged the monkeys (n=3 per group, randomly selected from each group) with 10^{10} PFU of CHIKV (strain LR2006 OPY-1) 15 weeks after the final immunization by intravenous injection. We collected blood to measure viremia at 0, 6, 24, 48, 72, 96, 120 and 168 hours, and sacrificed the monkeys 168 h after challenge. All animal experiments were reviewed and approved by the Animal Care and Use Committee, VRC, National Institute of Allergy and Infectious Diseases (http://www.niaid.nih.gov/vrc) in accordance with relevant federal guidelines and regulations.

Passive transfer of immunoglobulin and challenge in IFN-α/βR^{-/-} mice

We used a HiTrap™ Protein G HP column (GE Healthcare) and a Melon Gel IgG Purification Kit (Pierce) for antibody purification. We dialyzed purified IgG three times against PBS. We administered 2 mg of purified IgG (from approximately 200 μl of serum) into each recipient IFN-α/βR$^{-/-}$ mouse by tail vein injection 24 h before challenge. The challenge consisted of 30 PFU of CHIKV (strain LR2006 OPY-1), administered by intradermal injection.

Statistical analysis

All results are expressed as means ± s.e.m. We analyzed the data with unpaired two-tailed *t* tests (Prism 5).

Additional methods

Additional methods are described in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

REFERENCES

1. Simon F, Savini H, Parola P. Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. Med. Clin North Am. 2008; 92:1323–1343. [PubMed: 19061754]

2. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen. Virol. 2007; 88:2363–2377. [PubMed: 17698645]

3. Ross RW. The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. J. Hyg. (Lond.). 1956; 54:177–191. [PubMed: 13346078]

4. Staples JE, Breiman RF, Powers AM. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clin Infect. Dis. 2009; 49:942–948. [PubMed: 19663604]

5. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS. Pathog. 2007; 3:e201. [PubMed: 18069894]

6. Enserink M, Entomology. A mosquito goes global. Science. 2008; 320:864–866. [PubMed: 18487167]

7. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. 1994; 58:491–562. [PubMed: 7968923]

8. Cheng RH, et al. Nucleocapsid and glycoprotein organization in an enveloped virus. Cell. 1995; 80:621–630. [PubMed: 7867069]

9. Zhang W, et al. Placement of the structural proteins in Sindbis virus. J Virol. 2002; 76:11645–11658. [PubMed: 12388725]

10. Arankalle VA, et al. Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. J Gen. Virol. 2007; 88:1967–1976. [PubMed: 17554030]

11. Harrison VR, Binn LN, Randall R. Comparative immunogenicities of chikungunya vaccines prepared in avian and mammalian tissues. Am. J Trop. Med. Hyg. 1967; 16:786–791. [PubMed: 4965219]

12. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. USA. 1996; 93:11382–11388. [PubMed: 8876144]

13. Yang Z-Y, et al. pH-dependent entry of Severe Acute Respiratory Syndrome coronavirus is mediated by the Spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. J. Virol. 2004; 78:5642–5650. [PubMed: 15140961]

14. Yang Z-Y, et al. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science. 2007; 317:825–828. [PubMed: 17690300]

15. Sourisseau M, et al. Characterization of reemerging chikungunya virus. PLoS. Pathog. 2007; 3:e89. [PubMed: 17604450]

16. McClure MO, Sommerfelt MA, Marsh M, Weiss RA. The pH independence of mammalian retrovirus infection. J Gen. Virol. 1990; 71(Pt 4):767–773. [PubMed: 2157795]

17. Eckels KH, Harrison VR, Hetrick FM. Chikungunya virus vaccine prepared by Tween-ether extraction. Appl. Microbiol. 1970; 19:321–325. [PubMed: 4985431]

18. Pletnev SV, et al. Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. Cell. 2001; 105:127–136. [PubMed: 11301008]

19. Couderc T, et al. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. PLoS. Pathog. 2008; 4:e29. [PubMed: 18282093]

20. Couderc T, et al. Prophylaxis and therapy for Chikungunya virus infection. J Infect. Dis. 2009; 200:516–523. [PubMed: 19572805]

21. Levitt NH, et al. Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine. 1986; 4:157–162. [PubMed: 3020820]

22. McClain DJ, et al. Immunologic interference from sequential administration of live attenuated alphavirus vaccines. J Infect. Dis. 1998; 177:634–641. [PubMed: 9498442]

23. Edelman R, et al. Phase II safety and immunogenicity study of live chikungunya virus vaccine TSI-GSD-218. Am. J Trop. Med. Hyg. 2000; 62:681–685. [PubMed: 11304054]

24. Harrison VR, Eckels KH, Bartelloni PJ, Hampton C. Production and evaluation of a formalin-killed Chikungunya vaccine. J Immunol. 1971; 107:643–647. [PubMed: 4999088]

25. Tiwari M, et al. Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. Vaccine. 2009; 27:2513–2522. [PubMed: 19368794]

26. Wang E, et al. Chimeric alphavirus vaccine candidates for chikungunya. Vaccine. 2008; 26:5030–5039. [PubMed: 18692107]

27. Muthumani K, et al. Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. Vaccine. 2008; 26:5128–5134. [PubMed: 18471943]

28. Chackerian B. Virus-like particles: flexible platforms for vaccine development. Expert. Rev. Vaccines. 2007; 6:381–390. [PubMed: 17542753]

29. Bachmann MF, et al. The influence of antigen organization on B cell responsiveness. Science. 1993; 262:1448–1451. [PubMed: 8248784]

30. Noad R, Roy P. Virus-like particles as immunogens. Trends Microbiol. 2003; 11:438–444. [PubMed: 13678860]

31. Ludwig C, Wagner R. Virus-like particles-universal molecular toolboxes. Curr. Opin. Biotechnol. 2007; 18:537–545. [PubMed: 18083549]

32. Akahata W, Yang ZY, Nabel GJ. Comparative immunogenicity of human immunodeficiency virus particles and corresponding polypeptides in a DNA vaccine. J. Virol. 2005; 79:626–631. [PubMed: 15596858]

33. Tang G, et al. EMAN2: an extensible image processing suite for electron microscopy. J Struct. Biol. 2007; 157:38–46. [PubMed: 16859925]

34. Ludtke SJ, Baldwin PR, Chiu W. EMAN: semiautomated software for high-resolution single-particle reconstructions. J Struct. Biol. 1999; 128:82–97. [PubMed: 10600563]
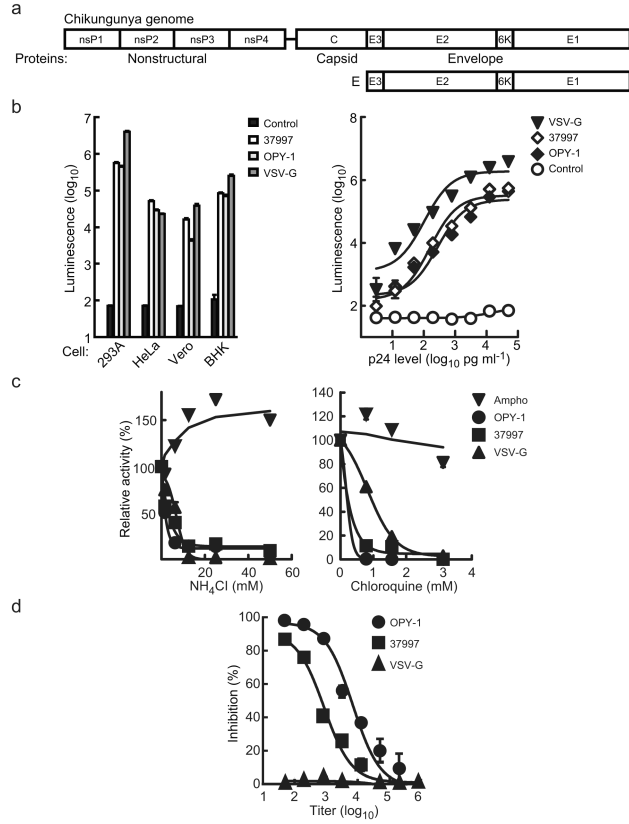
Figure 1. Characterization of CHIKV E pseudotyped lentiviral vectors
(**a**) Schematic representation of the CHIKV genome and CHIKV E expression vector used for incorporation of CHIKV E from strains 37997 and LR2006 OPY-1 into pseudotyped lentiviral vectors. The CHIKV genome consists of nonstructural polyproteins nsP1, nsP2, nsP3 and nsP4 and structural polyproteins capsid (C) and envelope (E: E3, E2, 6K and E1) (top). The polypeptide E genes from strains 37997 and LR2006 OPY-1 were inserted into an expression vector (bottom; schematic representation of vector is in Supplementary Fig. 1a, top). (**b**) Infectivity of the indicated pseudotyped lentiviral vectors in several CHIKV-permissive cell lines including 293A human renal epithelial, HeLa cervical epithelial, Vero renal epithelial and baby hamster kidney (BHK) cells. The pseudotyped vectors were standardized by HIV-1 Gag p24 (left) or the indicated concentration of p24 and used to infect 293A cells (right). After incubation with pseudotyped vectors for 24 h, cells were lysed and luciferase activity was measured. (**c**) pH-dependent entry of CHIKV pseudotyped lentiviral vectors incubated in the presence of the indicated amounts of ammonium chloride (left) and chloroquine (right). Data are presented as the percentage of activity at the indicated dose relative to activity with no treatment. (**d**) Neutralization measured with pseudotyped lentiviral vectors in sera from immune mice infected with CHIKV strain S-27 (ATCC). Sera were incubated at the indicated dilutions with VSV-G, CHIKV strain 37997 or LR2006 OPY-1 E-pseudotyped lentiviral vectors and the mixture infected to 293A cells. No inhibition was observed with control non-immune antisera.
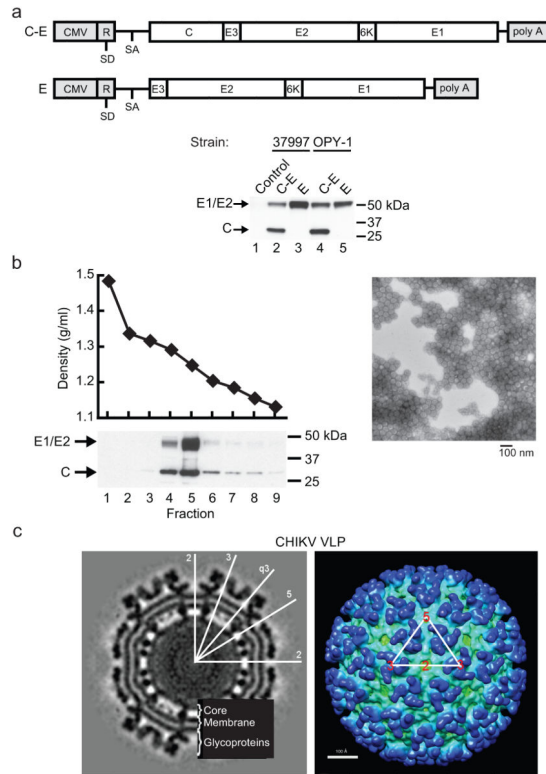
Figure 2. Characterization of CHIKV VLPs

(**a**) Schematic representation of CHIKV C-E or E expression vectors used for DNA vaccine and VLP production. The CHIKV structural polyproteins capsid plus envelope (C-E) or E alone from strains 37997 and LR2006 OPY-1 were inserted into a CMV/R expression vector14 (**Methods**). 293T cells were transfected with the indicated plasmids. Expression was measured 48 h after transfection by Western blotting32 as described (Supplementary Methods) (**b**) VLPs were purified (**Methods**)from the supernatants of 293F cells transfected with C-E expression vector (C-E$_{37997}$) (left) 72 h after transfection. Each fraction was characterized for its buoyant density (left upper panel) and protein content (left lower panel) by Western blot analysis. The fractionated VLPs were observed by transmission electron microscopy with magnification 20,000× (right, bar 100 nm). (**c**) Cryo-EM reconstruction of CHIKV VLP reveals its structural similarity to alphaviruses. Shaded-surface representation of the 3D density map of CHIKV VLP is viewed along an icosahedral 2-fold axis and resembles Sindbis virus (Supplementary Fig. 2; see also refs. 8,9,18). The white triangle marks the boundary of an icosahedral asymmetric unit. The numbers show the positions of the icosahedral 2-, 3-, and 5-fold axes limiting an asymmetric unit. The central cross-section through the cryo-EM maps of CHIKV VLP (left) and Sindbis virus (Supplementary Fig. 2) are similar. The orientations of the icosahedral (2-, 3-, and 5-fold) axes as well as the quasi-threefold (q3) axis are shown with white lines. The map is calculated to 18 Å resolution.
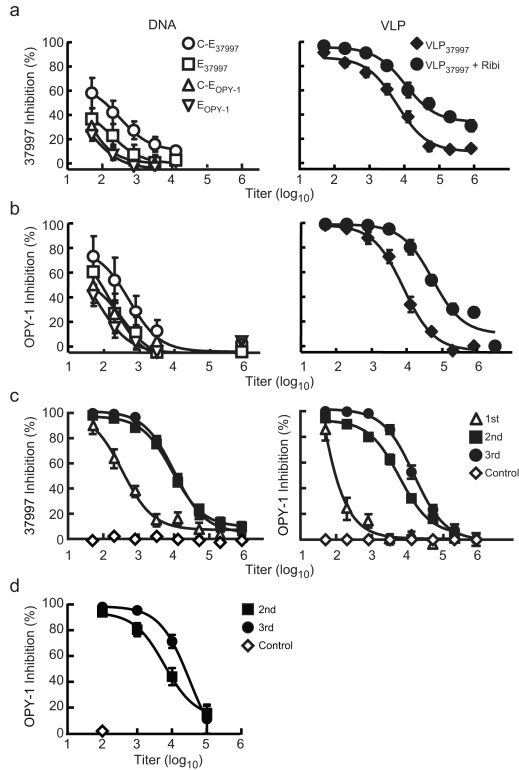
Figure 3. Neutralization of CHIKV strains 37997 and LR2006 OPY-1 after DNA or VLP vaccination in mice and monkeys

(**a**) and (**b**) Sera from immunized BALB/c mice 10 days after the final immunization were tested with CHIKV strain 37997 (**a**) or LR2006 OPY-1 (**b**) E pseudotyped lentiviral vectors. Mice were immunized with the indicated 15 μg of DNA or 19 μg of VLP_{37997}. Each C-E or E (strain 37997 and LR2006 OPY-1, respectively) plasmid was injected intramuscularly three times at 0, 3 and 6 weeks. VLP_{37997} with or without Ribi adjuvant was injected twice at 2 and 6 weeks. The experiment was performed in triplicate. The symbols show the average of the five mice and bars show the standard error of the mean. The curve fit was calculated by Prism software. (**c**) Rhesus monkeys were immunized with 20 μg of VLP_{37997} or PBS (control) intramuscularly three times at 0, 4, and 24 weeks. A neutralizing assay was performed with CHIKV strain 37997 (left panel) or LR2006 OPY-1 (right panel) E pseudotyped lentiviral vectors in sera collected from immunized monkeys at 10 days after each immunization. The symbols show the average of the six monkeys and bars show the standard error of the mean. (**d**) Neutralizing activity against CHIKV LR2006 OPY-1 in immunized monkeys' sera after the 2nd and 3rd immunizations was confirmed by a standard plaque reduction neutralization test (PRNT). The symbols show the average of the six monkeys and bars show the standard error of the mean.
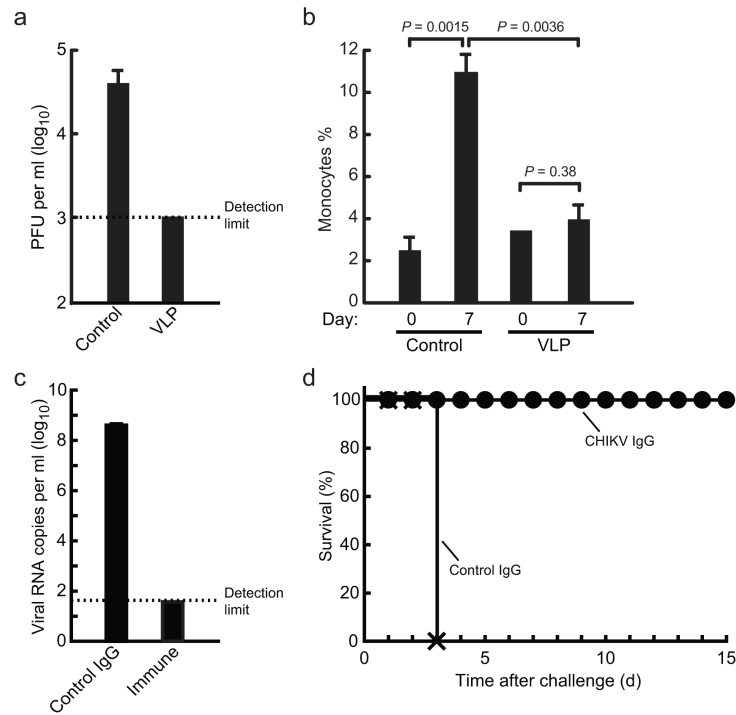
Figure 4. Protection against CHIKV LR2006 OPY-1 challenge in monkeys immunized with VLPs and in a CHIKV mouse model after passive transfer of purified IgG

(**a**) Monkeys injected with PBS (Control) or immunized with VLP_{37997} were challenged intravenously with 10^{10} PFU of the CHIKV strain LR2006 OPY-1 15 weeks after the final boost. The peak viremia at 24 h after challenge was measured by plaque assay. The detection limit was 1000 PFU per mL. Error bars represent the standard error of the mean. (**b**) The percentage of monocytes in the monkeys' white blood cells was measured using a hematology analyzer before and 7 days after challenge with CHIKV. Error bars represent the standard error of the mean. An unpaired two-tailed *t* test was used for statistical analysis (Control at day 0 vs. 7, $P = 0.0015$; VLPs at day 0 vs. 7, $P = 0.38$; Control vs. VLPs at 7 days, $P = 0.0036$). (**c**) Purified IgG from a monkey immunized with VLPs (Immune) or a control monkey (Control IgG) was passively transferred into IFN-α/βR$^{-/-}$ mice intravenously (2 mg of total IgG per mouse, n=5 per group). Recipient mice were challenged 24 h after IgG transfer with a lethal LR2006 OPY-1 challenge (30 PFU) by intradermal injection. The viremia in the mice after challenge was measured by quantitative RT-PCR (limit of detection = 40 RNA copies per mL). Error bars represent the standard error of the mean. (**d**) Survival curve of mice passively transferred with control IgG or CHIKV immunized IgG against lethal LR2006 OPY-1 challenge.