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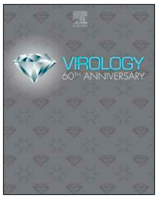

Lack of nsP2-specific nuclear functions attenuates chikungunya virus replication both *in vitro* and *in vivo*

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ABSTRACT

Chikungunya virus (CHIKV) is an important arthritogenic human pathogen that is already circulating in both hemispheres. In the present study, we substituted VLoop, located on the surface of nsP2, by other amino acid sequences. These modifications had deleterious effects on viral nuclear functions and made CHIKV incapable of interfering with the induction of type I interferon and the antiviral response in both mouse and human cells. Importantly, the identified mutations have no significant effects on the synthesis of virus-specific RNAs and viral structural proteins. The designed mutants induced a few orders of magnitude lower viremia but remained highly immunogenic in mice. Thus, the proposed modifications of nsP2 can additionally improve the safety of the attenuated strain CHIKV 181/25. Furthermore, defined mutations in the macro domain of another nonstructural protein, nsP3, additionally reduce cytopathogenicity of nsP2 mutants in human cells, and can be potentially applied for CHIKV attenuation.

1. Introduction

Alphaviruses are a group of small, enveloped viruses, which are broadly distributed over all continents including Antarctic areas (Strauss and Strauss, 1994). Some of them, such as Eilat virus (EILV), replicate only in mosquitoes (Nasar et al., 2012), but most of the alphaviruses circulate between mosquito vectors and vertebrate hosts. Based on geographical distribution, alphaviruses are divided into the New World (NW) and the Old World (OW) alphaviruses. Many NW representatives, exemplified by Venezuelan (VEEV), eastern (EEEV) and western (WEEV) equine encephalitis viruses induce severe meningoencephalitis (Weaver and Service, 2001a, 2001b). Infections caused by natural isolates of VEEV, EEEV, or WEEV have been shown to result in high mortality rates in humans and neurological sequelae among survivors. The OW alphaviruses, such as Sindbis (SINV) and Semliki Forest (SFV) viruses, are generally less pathogenic, and in humans, the major symptoms of the induced diseases are fever, rash, and arthritis (Griffin and Schlesinger, 1986; Griffin, 1989). Within recent years, one of the representatives of the OW alphaviruses, chikungunya virus (CHIKV), has spread widely in both hemispheres (McSweegan et al., 2015; Weaver and Lecuit, 2015). This spread has led to epidemics of polyarthritis characterized by severe joint pain that can continue for months. Moreover, it is also neuroinvasive for newborns and causes meningitis and cognitive disabilities. Despite the significant threat to public health, to date, CHIKV pathogenesis is insufficiently understood on molecular, cellular and systemic levels, and no licensed vaccines have been developed.

CHIKV genome (G RNA) is a ~12 kb single stranded RNA of positive polarity (Tsetsarkin et al., 2007). It mimics the structure of cellular mRNAs in that it is capped at the 5′ terminus and has a poly(A) tail at the 3’ terminus. This G RNA serves as a template for translation of a handful of viral nonstructural proteins, nsP1-to-4, which mediate RNA replication and synthesis of subgenomic RNA (SG RNA) (Lemm et al., 1994). The latter RNA functions as an mRNA for the synthesis of viral structural proteins that ultimately form infectious virions.

Alphavirus nsP2 protein exhibits an exceptionally wide range of activities in viral replication: i) mediates processing of ns polyprotein precursors P123 and P1234 (Das et al., 2014; Shirako and Strauss, 1990, 1994), ii) functions as a helicase in viral RNA synthesis (Das et al., 2014; Karpe et al., 2011; Rikkonen et al., 1994), iii) has NTPase activity (Rikkonen et al., 1994) and iv) serves as RNA phosphatase of viral G and SG RNAs in the cascade of capping reactions (Ahola and Kaariainen, 1995; Vasiljeva et al., 2000). Mutations in the alphavirus nsP2-coding sequence may decrease and increase replication of viral

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RNAs (Frolov et al., 1999; Kim et al., 2013). Additionally, while CHIKV nsP2 is an important structural and functional component of viral replication complexes, RCs, within infected vertebrate cells, a large fraction of this protein is distributed in the cytoplasm and nuclei suggesting additional functions in viral replication. As does the nsP2 protein of other OW alphaviruses, CHIKV nsP2 employs the mechanism similar to cellular transcription-coupled repair (Hanawalt and Spivak, 2008) to rapidly degrade the main catalytic subunit (RPB1) of cellular DNA-dependent RNA polymerase II (Akhrymuk et al., 2012). During the OW alphavirus infections, degradation of RBP1 ultimately results in global inhibition of cellular transcription. It serves as a very efficient means of CHIKV interference with the induction of cell signaling and activation of antiviral genes (Akhrymuk et al., 2018, 2019). Thus, CHIKV nsP2 is an important player in downregulation of the innate immune response, and this makes nsP2 an important target for modifications that may lead to the development of attenuated viral variants.

The accumulating data strongly suggest that the very C-terminal S-adenosyl methionine-guanylyl transferase (SAM MTase)-like domain of nsP2 plays a critical role in the protein's transcription inhibition function(s) (Garmashova et al., 2006). Point mutations in this domain can affect the ability of SINV- and SFV-specific G RNAs that lack structural genes (replicons) to induce transcriptional shutoff and cytopathic effect (CPE) (Frolov et al., 1999; Perri et al., 2000). Most of the identified mutations also strongly alter replication rates of viral genomes and transcription of SG RNAs. However, recently, we have identified a small, highly variable loop (VLoop) on the surface of SINV and CHIKV nsP2-specific SAM MTase-like domains that determine the nuclear functions of the protein (Akhrymuk et al., 2019). The designed CHIKV replicons and CHIK viruses encoding mutated VLoop lost the ability to downregulate cellular transcription in rodent cell lines and thus, became either less cytopathic or entirely noncytopathic (Akhrymuk et al., 2019). Most importantly, the introduced mutations did not have negative effects on viral replication rates in rodent cells that demonstrate intact I IFN induction and signaling. Thus, the introduced mutations affected the nuclear functions of CHIKV nsP2 without altering its activity in viral RCs.

In the present study, we continued to investigate the effects of the CHIKV nsP2 mutations on viral replication both *in vitro* and *in vivo*. Our data demonstrate that i) in contrast to parental CHIKV 181/25, the developed viral mutants are very potent type I IFN inducers in human cells, despite retaining efficient replication rates; ii) the mutations strongly attenuate a pathogenic variant of CHIKV in terms of its ability to cause viremia in mice; however, iii) the designed mutants remain immunogenic. Thus, manipulations with CHIKV nsP2 SAM MTase-like domain may be used to improve the safety of the previously designed attenuated CHIKV strain 181/25. The original strain has been shown to be highly immunogenic but demonstrated some residual adverse effects in human trials (Edelman et al., 2000), a major drawback that needs to be eliminated. Lastly, we show that additional mutations can be made within the macro domain of CHIKV nsP3, and they have a negative effect on viral cytopathogenicity in human cells. These mutations in nsP3 macro domain provide an additional possibility for CHIKV attenuation for humans.

2. Materials and methods

Cell cultures. The BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). The NIH 3T3, BJ-5ta, MRC-5, HFF-1, Vero clone 6 and HEK 293 cells were obtained from the American Tissue Culture Collection (Manassas, VA). Huh7 cells were kindly provided by Charles Rice (Rockefeller University, New York, NY). BHK-21, NIH 3T3, Vero and HEK 293 cells were maintained in alpha minimum essential medium supplemented with 10% fetal bovine serum (FBS) and vitamins. BJ-5ta, MRC-5, Huh7 and HFF-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS.

Plasmid constructs. Plasmid encoding infectious cDNA of CHIKV 181/25 was provided by Dr. Scott Weaver (University of Texas Medical Branch at Galveston, TX) (Gorchakov et al., 2012). In the present study, this construct was used for making new modifications in nsP2- and nsP3-coding sequences. The infectious cDNA clone of more pathogenic CHIKV variant AF15561$^{E2K200R:\Delta UTR}$, shown to be more pathogenic in a mouse model of infection, was fully described elsewhere (Hawman et al., 2017). The cDNA of this genome was reproduced on the basis of CHIKV 181/25 by PCR-based mutagenesis. The cDNAs of AF15561$^{E2K200R:\Delta UTR}$ and the designed mutants were cloned into a low-copy number plasmid under the control of CMV promoter. The poly(A) tail of the viral genome was also fused with the hepatitis delta ribozyme (RBZ) sequence. To simplify presentation, the AF15561$^{E2K200R:\Delta UTR}$ variant is referred to as wCHIKV in the text. CHIKV/GFP that encodes GFP gene under control of subgenomic promoter has been described elsewhere (Kim et al., 2016; Meshram et al., 2018). All the mutations introduced into nsP2 and nsP3 are indicated in the figures. They were designed using a PCR-based approach and other standard recombinant DNA techniques. Nucleotide sequences of the plasmids are available from the authors upon request.

RNA transcriptions, RNA infectivity assay, and rescue of recombinant viruses. Plasmids were purified by ultracentrifugation in CsCl gradients. Viral G RNAs were synthesized *in vitro* by using SP6 RNA polymerase (Invitrogen) in the presence of cap analog (New England BioLabs) according to recommendations of the manufacturers. The quality and yields of the transcripts were analyzed by agarose gel electrophoresis under nondenaturing conditions. Aliquots of the reaction mixtures were used without additional purification for electroporation of BHK-21 cells in the previously described conditions (Liljeström et al., 1991). Viruses were harvested at 24 h post electroporation, and titers were determined by plaque assay on BHK-21 cells (Lemm et al., 1990).

RNA infectivities were analyzed by the infectious center assay (ICA). Briefly, the 10-fold dilutions of cells electroporated with the *in vitro*-synthesized RNAs were seeded in 6-well Costar plates with monolayers of naïve BHK-21 cells. After 2 h of incubation at 37 °C, cells were covered by 0.5% agarose supplemented with DMEM and 3% FBS. Plaques were stained by crystal violet 3 days post electroporation. RNA infectivities were determined as PFU/µg of the *in vitro*-synthesized RNAs.

The parental wCHIKV (AF15561$^{E2K200R:\Delta UTR}$) and its derivatives were rescued in BSL3 containment by transfecting plasmid DNA into BHK-21 cells using TransIT-X2 Transfection Reagent according to the manufacturer's recommendations (Mirus). Viruses were harvested at 48 h post transfection, and titers were determined by plaque assay on BHK-21 cells.

Analysis of viral RNA and protein synthesis. NIH 3T3 cells in 6-well Costar plates were infected with CHIKV 181/25 variants at a multiplicity of infection (MOI) 20 PFU/cell. Virus-specific RNAs were metabolically labeled between 4 and 8 h post infection (PI) in 0.8 ml of complete medium supplemented with [^{3}H]uridine (20 µCi/ml) and Actinomycin D (1 µg/ml). RNAs were isolated from the cells by TRIzol reagent according to the manufacturer's recommendations (Invitrogen). RNAs were denatured by glyoxal and analyzed by agarose gel electrophoresis in sodium phosphate buffer (Fayzulin and Frolov, 2004). After impregnation with 2,5-diphenyoxazol (PPO), the gel was dried and used for autoradiography.

For protein labeling, cells in 6-well Costar plates were infected with CHIKV mutants at an MOI of 20 PFU/cell. At 7 h PI, they were washed with PBS, and proteins were metabolically labeled for 30 min at 37 °C in 0.8 ml of DMEM lacking methionine, and supplemented with [^{35}S] methionine (20 µCi/ml) and 0.1% FBS. Cells were harvested and lyzed in the standard protein loading buffer for gel electrophoresis. Equal amounts of lysates were analyzed by gel electrophoresis in 10% NuPAGE gels (Invitrogen) followed by radioautography.

RT-qPCR. Cells were infected with CHIKV variants indicated in the figure at an MOI of 20 PFU/cell. Cellular RNAs were isolated from

5×10^5 cells using the Direct-zol RNA MiniPrep kit according to the manufacturer's instructions (Zymo Research). These RNA samples were used for cDNA synthesis by QuantiTect reverse transcription (RT) kit according to the manufacturer's instructions (Qiagen). The cDNAs were used for qPCR analysis with primers for the following mouse genes: IFN-β (NM_010510), IFIT1 (NM_008331), IFIT3 (NM_010501), ISG15 (NM_015783), and human genes: IFN-β (NM_002176), IFIT1 (NM_005101), IFIT3 (NM_001548), ISG15 (NM_001549). The qPCRs were performed using SsoFast EvaGreen supermix (Bio-Rad) in a CFX96 real-time PCR detection system (Bio-Rad). The specificities of the qPCR products were confirmed by analyzing their melting temperatures. The data were normalized to the mean threshold cycle (CT) of 18 S ribosomal RNA in each sample. The fold difference was calculated using the ΔΔCT method.

Animal studies. To evaluate the ability of candidate viruses to cause viremia in mice, and to assess the resulting Ab response, 2-to-3-week old C57Bl/6 mice were inoculated into left footpad with 5×10^3 PFU of indicated viruses diluted in PBS containing 1% mouse serum. All of the animal studies were carried out under the approval of the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (UAB). The experiments with CHIKV 181/25- and wCHIKV-based mutants were carried out in the ABSL2 and ABSL3 facilities, respectively. Animals were monitored daily for weight change, signs of disease or any abnormalities during the course of the experiment. At the times PI indicated in the figure legends, blood samples were taken from the retro-orbital sinus and sera were analyzed for either the levels of viremia or neutralizing Abs. All the mice were sacrificed humanely after the completion of the study.

Neutralizing antibody titers ($PRNT_{50}$). Serum samples were incubated at 50 °C for 1 h and then serially (2-fold) diluted in PBS supplemented with 1% FBS and 250 PFU/ml of CHIKV 181/25. Samples were incubated at 37 °C for 2 h, and 0.2 ml aliquots were applied to monolayers of BHK-21 cells in 6-well Costar plates. After 1 h-long incubation at 37 °C, cells were overlaid with 0.5% agarose supplemented with DMEM and 3% FBS. After 3 days of incubation, plaques were stained with crystal violet. The percentage of reduction was plotted against the dilution to generate slope and intercept values using the best fit non-linear curves that were used to calculate the 50% reduction dilution (GraphPad Prism software).

Statistical analysis. Where possible, data were analyzed by using one-way ANOVA followed by either Dunnett's or Tukey's multiple comparisons tests (see figure legends for details) of GraphPad Prism software.

IFN-β induction. Cells were infected with CHIKV variants at MOIs indicated in the figure legends. Harvested samples were used to assess viral titers, and the levels of the mouse or human IFN-β were measured with a VeriKine Mouse or Human IFN-β ELISA kits, respectively, according to manufacturer's recommendations (PBL Interferon Source).

3. Results

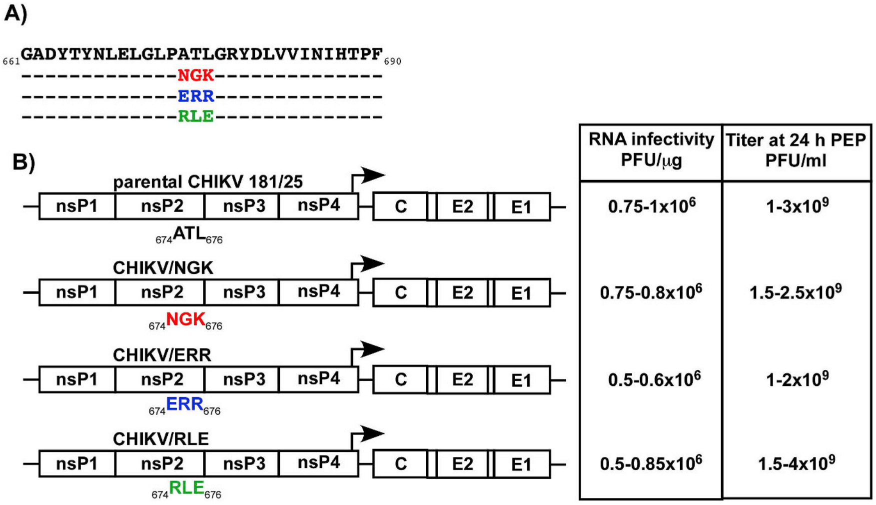
CHIKV 181/25 variants with mutated VLoop are viable and less cytopathic. In our previous study, we selected a variety of CHIKV/GFP variants that contained mutations in the C-terminal SAM MTase-like domain of nsP2 (Akhrymuk et al., 2019). These modifications altered the cytopathogenicity of the virus and made the designed mutants less cytopathic for rodent cells, but did not have negative effects on viral replication rates. These changes in viral phenotype resulted from replacements of VLoop ($_{674}$ATL$_{676}$ peptide) in CHIKV nsP2 by heterologous amino acid (aa) sequences (Akhrymuk et al., 2019). In this study, we further explored the effects of the mutations on viral replication rates *in vivo* and *in vitro,* and on CHIKV immunogenicity.

The genome of original CHIKV 181/25 was modified to contain the natural $_{674}$ATL$_{676}$ peptide substituted by NGK, ERR, and RLE aa sequences (Fig. 1A). The newly designed mutants, termed CHIKV/NGK, CHIKV/ERR, and CHIKV/RLE, encoded no heterogenous genes, such as GFP, which could attenuate viral replication. Infectivities of the *in vitro*-synthesized RNAs were evaluated in the infectious center assay (ICA) (see Materials and Methods for details), and viral stocks were harvested at 24 h post RNA transfection. In repeat experiments, the newly designed and parental CHIKV 181/25 constructs reproducibly demonstrated identical RNA infectivities (Fig. 1B). This precluded the possibility that the acquisition of additional adaptive mutations was necessary for their viability. Similar infectious titers in the harvested stocks of CHIKV 181/25 and the mutants also suggested their efficient replication in BHK-21 cells (Fig. 1B). Notably, we observed a complete cytopathic effect (CPE) within 24 h post electroporation of RNA of parental CHIKV 181/25. However, despite efficient replication, the designed mutants did not induce CPE, and electroporated BHK-21 cells continued to grow. To our convenience, the mutants were still able to develop plaques in this cell line under agarose cover, but only in the presence of FBS at low concentration. This strongly simplified the assessments of viral titers.

The designed CHIKV nsP2 mutants replicate in a variety of cell lines. Next experiments were aimed at comparing replication rates of the designed mutants with those of parental CHIKV 181/25 in the cells of different origins (Fig. 2). Some of the used cell lines, such as human fibroblasts MRC-5, BJ-5ta and mouse NIH 3T3 fibroblasts, are fully competent in type I IFN induction and signaling (see the followng sections). HEK 293 cells were used as a less efficient type I IFN inducer (data not shown), and others, Vero and BHK-21 cells, were applied as cell lines having defects in either type I IFN induction and signaling, respectively. All of the above cell lines were infected at an MOI of 0.01 PFU/cell, and virus release was assessed at different times post infection (PI). CHIKV nsP2 mutants demonstrated efficient replication in all cell types with the highest titers in BHK-21 and HEK 293 cells. However, in the cells competent in type I IFN induction and signaling (BJ-5ta, MRC-5 and NIH 3T3), at the late times PI, final titers of the mutants were reproducibly lower than those of the parental CHIKV 181/25. The detected 50–100-fold decrease in viral titers at 24 h PI was not a result of alterations in either G and SG RNA synthesis or translation of structural proteins. NsP2 mutants and parental CHIKV 181/25 exhibited similar efficiencies of RNA synthesis and translation of structural proteins (Fig. 3). Thus, the plausible explanation for the detected lower titers of the mutants in the experiments performed at low MOI was that in contrast to CHIKV 181/25, they became efficient type I IFN inducers.

NsP2-specific mutations make CHIKV a potent IFN-β inducer. Next, we evaluated the ability of CHIKV nsP2 mutants to induce type I IFN in mouse NIH 3T3 and human MRC-5 and HFF-1fibroblasts (Fig. 4). Cells were infected with parental CHIKV 181/25 and the designed mutants at high MOI (20 PFU/cell). Media were harvested at 18 h PI, and viral titers and concentrations of the released IFN-β were determined in the same samples. In all of the used cell lines, CHIKV 181/25 induced IFN-β very inefficiently, if at all (Fig. 4), and its concentrations remained at the limit of detection. In repeat experiments, the nsP2 mutants reproducibly replicated to essentially the same titers as the parental CHIKV 181/25 (see the left panels in Fig. 4), but, in contrast, were very potent IFN-β inducers, particularly in MRC-5 cells. Taken together, these results demonstrated that mutations in VLoop altered the interferon-inhibiting effect of nsP2 protein in both mouse and, importantly, in human cells.

Consequently, the inability of the viruses to interfere with induction of IFN-β made them incapable of forming plaques in the cell lines having no defects in type I IFN induction and signaling (Fig. 5). On BHK-21 cells, the mutants and parental virus produced readily detectable plaques of similar sizes. In NIH 3T3, MRC-5 and BJ-5ta cells, mutants failed to develop plaques. However, we observed cytopathogenicity in MRC-5 and BJ-5ta cell lines when they were infected at higher MOI by less diluted samples. The most likely explanation for the inability to form plaques was that IFN-β released by primarily infected cells activated the antiviral state in the surrounding cells and protected



	RNA infectivity PFU/μg	Titer at 24 h PEP PFU/ml
parental CHIKV 181/25	0.75-1x10^{6}	1-3x10^{9}
CHIKV/NGK	0.75-0.8x10^{6}	1.5-2.5x10^{9}
CHIKV/ERR	0.5-0.6x10^{6}	1-2x10^{9}
CHIKV/RLE	0.5-0.85x10^{6}	1.5-4x10^{9}

Fig. 1. CHIKV 181/25 derivatives with mutated VLoop are viable. (A) The alignment of CHIKV nsP2 fragment containing indicated mutations. Dashes indicate identical aa. The numbers correspond to aa in nsP2 of parental CHIKV 181/25. (B) RNA infectivity and viral titers at 24 h post electroporation. BHK-21 cells were electroporated with 3 μg of *in vitro*-synthesized RNAs of parental CHIKV 181/25 and the designed mutants. Electroporated cells were used for the infectious center assay (see Materials and Methods for details) and for generating viral stocks. Viral titers were assessed by plaque assay on BHK-21 cells. Transfection experiments were reproducibly repeated three times and the presented values are ranges from three individual experiments.

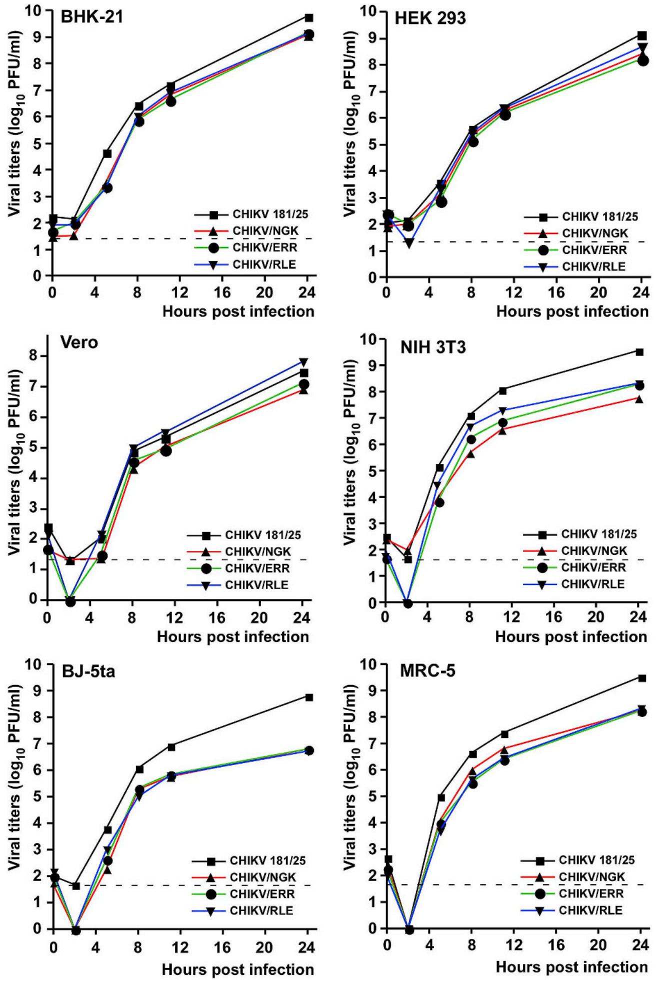


Fig. 2. CHIKV nsP2 mutants efficiently replicate in rodent and human cells. 5×10^{5} BHK-21, Vero, NIH 3T3, HEK 293, MRC-5 and BJ-5ta cells in 6-well Costar plates were infected with CHIKV 181/25 or designed mutants at an MOI of 0.01 PFU/cell. At the indicated time points, media were replaced, and viral titers were determined by plaque assay on BHK-21 cells. The experiments were reproducibly repeated 3 times, and the results of one representative experiment are presented.

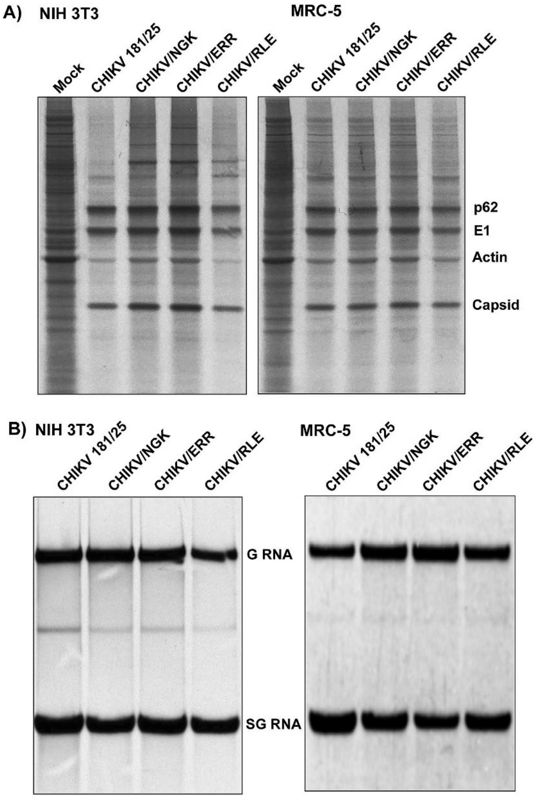


Fig. 3. The designed CHIKV nsP2 mutants demonstrate an efficient synthesis of virus-specific RNAs and viral structural proteins. (A) 5×10^{5} cells in the 6-well Costar plate were infected with parental CHIKV 181/25 and designed mutants at an MOI of 20 PFU/cell. At 7 h PI, they were washed with PBS, and proteins were metabolically labeled for 30 min with [^{35}S]methionine (see Materials and Methods for details). Equal amounts of lysates were analyzed by gel electrophoresis in 10% NuPAGE gels (Invitrogen), followed by autoradiography. (B) 5×10^{5} cells in the 6-well Costar plate were infected with the indicated viruses at an MOI of 20 PFU/cell. Virus-specific RNAs were metabolically labeled between 4 and 8 h PI in complete media, supplemented with [^{3}H]uridine (20 μCi/ml) and Actinomycin D (1 μg/ml), then RNAs were isolated and analyzed by agarose gel electrophoresis as described in Materials and Methods.

them against subsequent rounds of infection.

Replication of CHIKV nsP2 mutants results in activation of interferon-stimulated genes (ISGs). In additional experiments, we tested whether cells infected with the mutants were not only releasing IFN-β, but also remained competent in IFN signaling and ultimate activation of ISGs. NIH 3T3 and MRC-5 cells were infected at an MOI of 20 PFU/cell and, at 16 h PI, the induction of selected ISGs and IFN-β

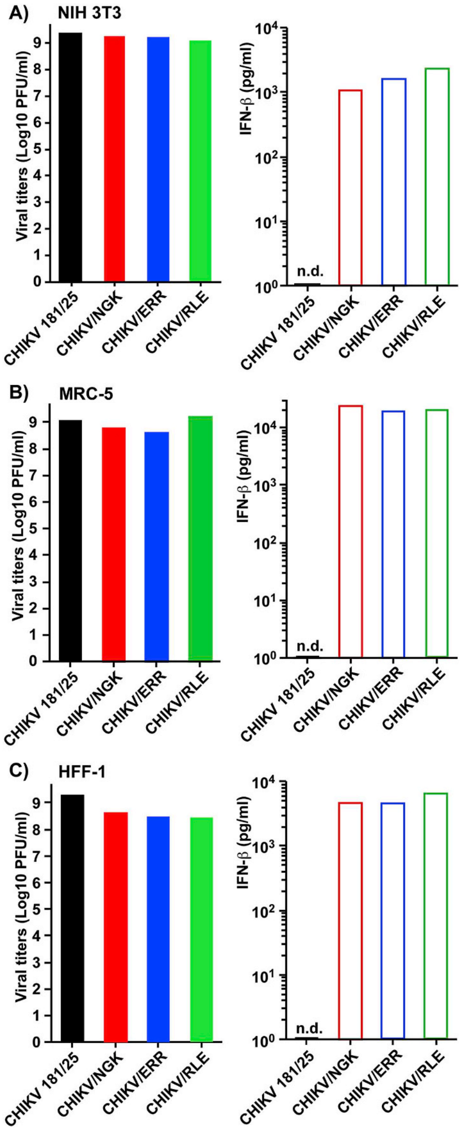


Fig. 4. CHIKV nsP2 mutants, but not the parental CHIKV 181/25, are potent inducers of IFN-β in both murine and human cell lines. NIH 3T3 (A), MRC-5 (B), and HFF-1 (C) cells were infected at an MOI of 20 PFU/cell with nsP2 mutants and parental CHIKV 181/25. At 18 h PI, the supernatants were harvested to determine viral titers and the levels of IFN-β. n. d. indicates that the concentration of IFN-β was below the limit of detection. Similar experiments were made multiple times with consistent results. The results of one of the representative experiments are presented.

was evaluated by RT-qPCR. Compared to parental CHIKV 181/25, cells infected with the mutants demonstrated a few orders of magnitude more efficient activation of ISGs (Fig. 6). Thus, CHIKV nsP2 mutants were incapable of inhibiting ISG-dependent antiviral response in the cells of both human and mouse origins.

CHIKV nsP2 mutants remain immunogenic in mice. Taken together, the accumulated data demonstrated that nsP2 VLoop-specific mutations affected CHIKV 181/25 infection spread *in vitro*, in the cells having no defects in type I IFN induction and signaling. Infected cells remained capable of efficient type I IFN induction and responded by ISG activation. These characteristics suggested that the introduced mutations could improve the currently available, attenuated strain CHIKV 181/25 in terms of its safety. However, the enhanced attenuation could potentially make it less capable of inducing an adaptive immune response.

Thus, firstly, to understand the effect of the mutations on CHIKV

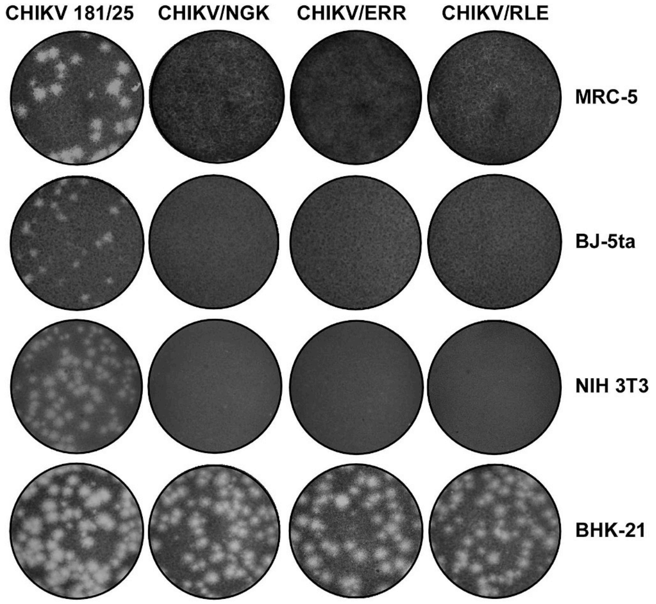


Fig. 5. CHIKV nsP2 mutants do not form plaques on the cells competent in type I IFN induction and signaling. CHIKV nsP2 mutants and parental CHIKV 181/25 were used in plaque assay performed on the indicated cell lines. This figure displays wells with monolayers of different cells, which were infected with the same dilutions of the indicated viruses. All of the plates were stained with crystal violet after 3 days of incubation at 37 °C.

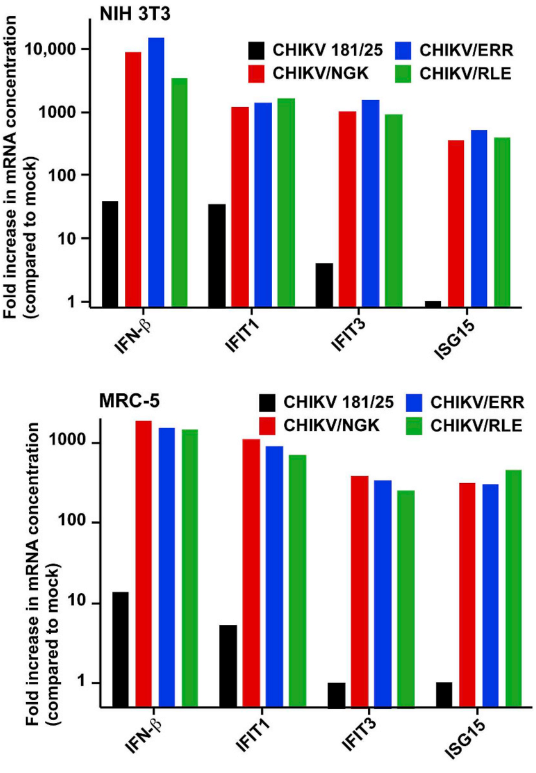


Fig. 6. Infections of murine NIH 3T3 and human MRC-5 fibroblasts with CHIKV nsP2 mutants result in ISG activation. NIH 3T3 and MRC-5 cells were infected with the indicated viruses at an MOI of 20 PFU/cell, and, at 16 h PI, the induction of indicated ISGs and IFN-β was evaluated by RT-qPCR as described in Materials and Methods. The fold increase in the ISGs transcript level in virus-infected cells relative to the mock-infected cells were calculated using ΔΔCT method. The experiment was reproducibly repeated three times with similar results, and the data from one representative experiment are shown. Average values from triplicate samples are presented, but the error bars are too small to be visible at this scale.

replication *in vivo*, 2-to-3-week-old C57BL/6 mice were infected with the same doses of parental CHIKV 181/25 and nsP2 mutants in the left footpad. The blood samples were collected on days 1, 2 and 3 PI and tested for levels of viremia. Only two samples taken on days 1 and 2 from CHIKV 181/25-infected mice showed the presence of the virus at the levels of 100 and 50 PFU/ml, respectively (data not shown). In all other mice, the levels of viremia caused by either parental virus or the mutants were below the limit of detection. We monitored swelling of the joints or weight loss that are indications of morbidity, but in all groups, no significant changes were detected (data not shown). Thus, these experiments did not generate direct, conclusive data about changes in replication levels of the CHIKV 181/25-derived viruses with mutated nsP2 *in vivo*.

To confirm replication of the mutants and their immunogenicity if any, we collected blood samples on day 21 PI and tested them for the levels of neutralizing antibodies (see Materials and Methods for details). The results demonstrated that mutants remained competent in replication *in vivo*. They induced readily detectable levels of CHIKV-specific, neutralizing antibodies, albeit less efficiently than the parental CHIKV181/25 (Fig. 7A). This was an indirect indication that viral replication *in vivo* was affected. At day 25 PI, the immunized mice were also challenged with a more pathogenic variant of CHIKV, wCHIKV (see Materials and Methods for details), to show the protective effect of vaccination. Blood samples were collected on days 1, 2, and 3 post challenge to assess the levels of viremia. In the unimmunized mice (PBS group), wCHIKV induced high levels of viremia that remained detectable on days 2 and 3 (Fig. 7B). Only two mice immunized with CHIKV/NGK demonstrated low viremia on day 1 post challenge, as well as did one mouse immunized with CHIKV 181/25 on day 2. No infectious virus was detected in mice immunized with CHIKV/ERR and CHIKV/RLE at any time post challenge. Thus, the designed CHIKV 181/25-based mutants remained immunogenic and offered protection from wCHIKV infection.

NsP2-specific mutations attenuate wCHIKV replication *in vivo*. In order to generate better data about the effects of VLoop replacements on viral replication *in vivo*, we cloned the above-described mutant VLoop-encoding sequences into the genome of pathogenic wCHIKV (see Materials and Methods for details). The latter virus was used in challenge experiments described in the previous section. The designed variants were termed wCHIKV/NGK, wCHIKV/ERR, and wCHIKV/RLE. These viruses and parental wCHIKV were rescued by transfecting the plasmids containing cDNAs of viral genomes under control of the CMV promoter (see Materials and Methods for details). Two-to-three-week-old C57BL/6 mice were infected with the same dose, 5 × 10^3 PFU, of the rescued viruses. Since the differences in viremia levels correlate most efficiently with the differences in CHIKV pathogenicities (Ashbrook et al., 2014), we assessed the induced viremia on days 1, 2 and 3 PI (Fig. 8A). On day 1, mice infected with parental wCHIKV exhibited viremia at the level of 10^6 PFU/ml, and it continued on day 2 PI. At day 3, viremia above the level of detection was found in one mouse. Mice infected with wCHIKV also demonstrated the delay in gaining weight (Fig. 8B). Whereas, the designed nsP2 mutants developed almost 3 orders of magnitude lower viremia even on day 1 (Fig. 8A), and only 2 mice infected with wCHIKV/NGK remained positive for viremia on day 2. In other samples collected on days 2 and 3, the presence of the viruses was below the limit of detection. Mice infected with the mutants gained weight more efficiently than those in wCHIKV-infected group (Fig. 8B). Thus, the modifications in VLoop had strong negative effects on the ability of wCHIKV to develop viremia in a mouse model.

Additional CHIKV attenuation by mutations in the macro domain of nsP3. The nsP2 mutants, which were developed on the bases of either CHIKV 181/25 or wCHIKV, demonstrated dramatically lower cytopathogenicity in mouse cells, if any, and induced lower viremia in mice. However, we were concerned that the designed recombinant viruses retained cytopathogenicity in the tested cell lines of human origin. In contrast to NIH 3T3 cells, at the MOIs higher than 1 PFU/cell, human cells were slowly developing complete CPE. This was an indication that in contrast to mouse fibroblasts, in human cells, ISG activation (Fig. 6) by virus-induced type I IFN was insufficient for preventing cell death and virus clearance. Therefore, we explored additional means of CHIKV attenuation by introducing mutations into nsP3 macro domain. As in our previous studies of the mechanism responsible for the SINV-induced cytopathic effect (Akhrymuk et al., 2018), N24T and N24A/D32G mutations were aimed to inactivate the nsP3 macro domain-associated mono-ADP-ribosylhydrolase activity without compromising viral replication rates (Eckei et al., 2017). In other studies, alterations of this nsP3 macro domain-specific function affected CHIKV pathogenesis (McPherson et al., 2017). N24T and N24A/D32G substitutions were introduced into genomes of both CHIKV/NGK/GFP (Fig. 9A) and wCHIKV/NGK, which already had the nsP2-specific VLoop replaced. In CHIKV 181/25-based constructs, GFP was left under control of the subgenomic promoter to better monitor

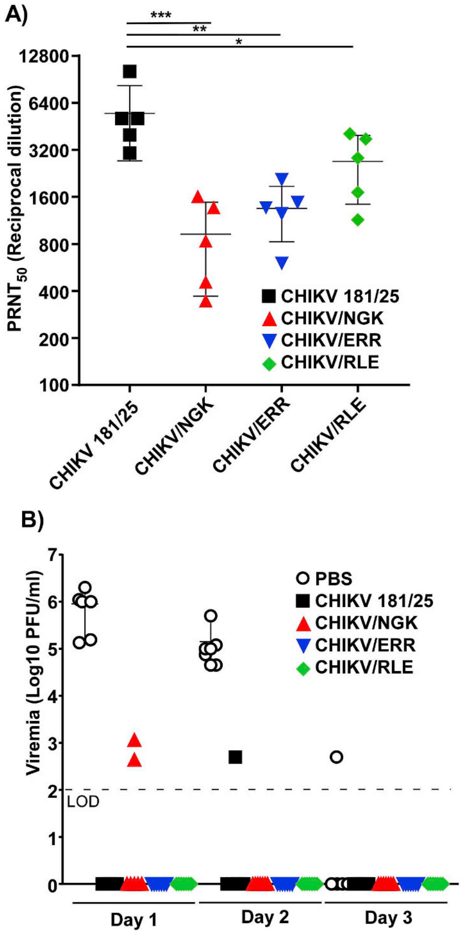


Fig. 7. The designed CHIKV 181/25-based nsP2 mutants remain immunogenic and protect mice against following challenge with wCHIKV. Two-to-three-week old C57BL/6 mice (n = 6/group) were infected in the left footpad with 5 × 10^3 PFU of CHIKV 181/25 and its indicated nsP2 mutants. (A) The levels of neutralizing Abs were assessed at day 21 PI as described in Materials and Methods. Results are presented as means with standard deviations. Significances of differences among the values were determined by one-way ANOVA, followed by Dunnett's multiple comparisons test. They are indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. CHIKV181/25 is compared to other groups. (B) Mice were challenged on day 25 PI with 10^5 PFU of wCHIKV (see Materials and Methods for details) using the same route of infection. Blood samples were collected on days 1, 2, and 3, and levels of viremia were assessed by plaque assay on BHK-21 cells. Each data point represents a value from an individual mouse. The dashed line indicates the limit of detection (LOD) in the plaque assay.

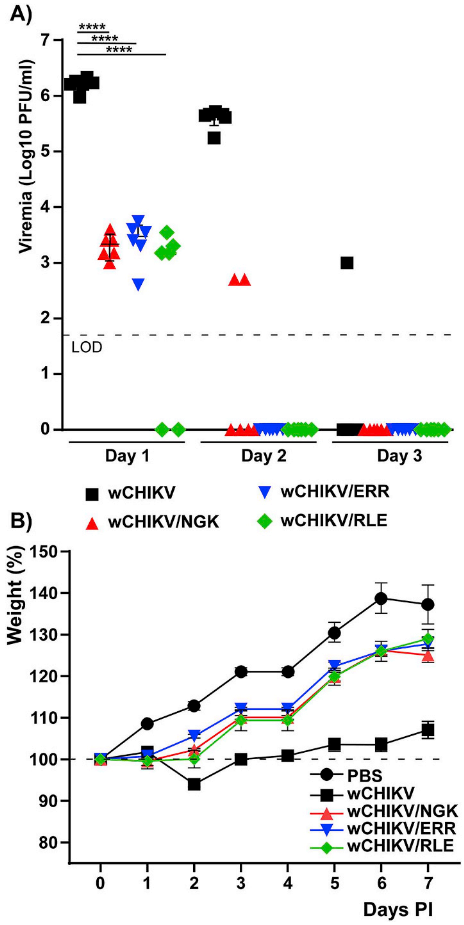


Fig. 8. Mutations in nsP2 of wCHIKV strongly affect the development of viremia in mice. (A) Two-to-three-week old C57BL/6 mice (n = 6/group) were infected in the left footpad with 5×10^3 PFU of wCHIKV nsP2 mutants and parental wCHIKV. Blood samples were taken on days 1, 2 and 3 PI, and the levels of viremia were assessed by plaque assay on BHK-21 cells. The dashed line indicates the limit of detection (LOD). Results are presented as means with standard deviations and significant differences, determined by one-way ANOVA, followed by Tukey's multiple comparisons test. They are indicated by asterisks as follows: ****, $P < 0.0001$. wCHIKV is compared to other groups. (B) Weight change post infection with indicated viruses. Results are presented as means with standard deviations.

viral replication in the absence of obvious CPE development.

The newly designed CHIKV 181/25-based nsP2/nsP3 mutants, CHIKV/NGK/N24A/D31G/GFP and CHIKV/NGK/N24T/GFP (Fig. 9A), exhibited remarkably reduced cytopathogenicity. Unlike the parental CHIKV/NGK/GFP, they could not even form clear plaques in BHK-21 cells under agarose cover (data not shown). These mutants were also able to persistently replicate in Vero and Huh7 cells, which are deficient in type I IFN response (Fig. 9B), while the parental CHIKV/GFP and CHIKV/NGK/GFP variants were causing complete CPE within 2–3 (data not shown) and 5–6 (Fig. 9B) days PI, respectively. Interestingly, the persistence of CHIKV/NGK/N24A/D31G/GFP and CHIKV/NGK/N24T/GFP in Huh7 cells developed in an unusual manner. In replicate experiments, a few days PI, these cells accelerated growth, and the mutants also demonstrated higher levels of replication. This was suggestive of viral adaptation/selection; however, further investigation of this phenomenon was beyond the scope of this study. In contrast to Vero and Huh7 cells, the IFN competent human MRC-5 fibroblasts were able to stop and clear the established replication of nsP2/nsP3 mutants (Fig. 9B), while essentially all cell infected with parental CHIKV/NGK/GFP were non-viable by day 5 PI.

The described above nsP3 mutations were also introduced into the genome of wCHIKV/NGK (Fig. 10A). The designed wCHIKV/NGK/N24A/D31G and wCHIKV/NGK/N24T variants were rescued and used for *in vivo* study (Fig. 10B). Two-to-three-week-old C57BL/6 mice were infected with equal doses (5×10^3 PFU) of nsP2/nsP3 mutants or parental wCHIKV and wCHIKV/NGK in the left footpad. In this experiment (Fig. 10B), wCHIKV showed the highest level of viremia, which was $\sim 10^6$ PFU/ml on day 1, then decreased to $\sim 10^5$ PFU/ml on day 2, and dropped to the lowest level of detection on day 3. For all of the mutants, viremia on day 1 was more than 3 orders of magnitude lower and below the detection threshold on the subsequent days. Similar levels of viremia induced by nsP2 and nsP2/nsP3 mutants also suggested that mutations in nsP3 had no negative effects on viral replication in mice.

Despite 1000-fold lower and shorter viremia, the nsP2 and nsP2/nsP3 mutants induced neutralizing Abs with the efficiencies similar to that of the parental wCHIKV (Fig. 10C). wCHIKV/NGK/N24A/D31G and wCHIKV/NGK/N24T variants, which demonstrated an additional reduction in cytopathogenicity in human cells, remained as immunogenic as the parental wCHIKV/NGK.

4. Discussion

To date, live attenuated viral vaccines remain desirable due to their efficient induction of protective immunity against viral infections. The most common approach in the development of live attenuated vaccines is the serial passage of the wt viruses either in cultured cells or in chicken embryos (Alevizatos et al., 1967; Barrett, 2001; Berge et al., 1961; Levitt et al., 1986a). Such passaging of alphaviruses usually leads to an accumulation of mutations in viral structural proteins and, in some cases, in the promoter of G RNA located in the 5′UTR (Gorchakov et al., 2012; Kinney et al., 1989, 1993). Mutations in E2 glycoprotein may make viral spikes capable of more efficient interaction with heparan sulfate at the plasma membrane (Ashbrook et al., 2014; Gorchakov et al., 2012; Klimstra et al., 1998). They increase alphavirus infectivity during propagation in cultured cells (Ashbrook et al., 2014). Furthermore, the 5′UTR-specific mutations destabilize the RNA secondary structure (Kulasegaran-Shylini et al., 2009a, 2009b) and release the very 5′-terminal nucleotides from the stems, which are predicted for both 5′UTR of G RNA and the 3'end of the negative strand RNA intermediate. These structural changes improve the rates of G RNA replication and likely translation of nsPs (Kulasegaran-Shylini et al., 2009b). However, they also make evolved alphaviruses more sensitive to the antiviral effect of one of the ISG products, IFIT1 (Hyde et al., 2014; Reynaud et al., 2015), and thus, contribute to their attenuation. The passaging-based approach has previously been applied for the development of attenuated VEEV and CHIKV variants, TC-83 and 181/25 strains, respectively (Alevizatos et al., 1967; Levitt et al., 1986b). The attenuated phenotypes of the selected CHIKV and VEEV mutants rely on only two point mutations (Gorchakov et al., 2012; Kinney et al., 1993), and both viruses remain capable of causing adverse effects in some vaccinees (Edelman et al., 2000; Powers, 2018). Thus, in the case of CHIKV and VEEV, application of passaging-based approach had likely reached its limit; however, the developed strains, VEEV TC-83 and CHIKV 181/25, remained insufficiently attenuated. Nevertheless, they did become stable upon propagation in tissue culture, demonstrated significant attenuation and may be used for further improvement of their safety.

Interference with the development of the innate immune response is a common characteristic of many viral taxonomic groups (Zinzula and Tramontano, 2013). Alphaviruses are not an exception and have developed the abilities to downregulate cellular response to their replication and to inhibit cell signaling that is aimed at the establishment of the antiviral state in yet uninfected cells (Garmashova et al., 2007). Some of the alphaviruses, including CHIKV, induce type I IFN very inefficiently if at all (Burke et al., 2009) (Fig. 4). As do most of the positive sense RNA viruses, alphaviruses isolate their dsRNA

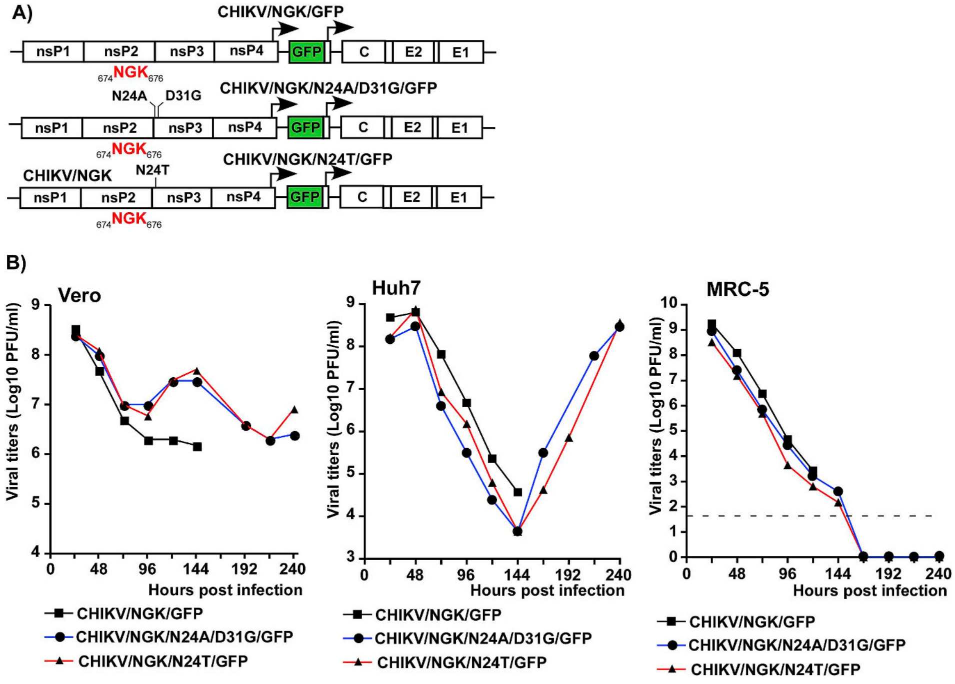


Fig. 9. Additional mutations in the macro domain of nsP3 in CHIKV/NGK/GFP reduce viral cytopathogenicity in human and Vero cells. (A) The schematic presentation of the genomes of recombinant viruses. (B) 5×10^5 cells of the indicated cell lines in 6-well Costar plates were infected with the mutants at an MOI of 20 PFU/cell. Media were replaced at the indicated time points, and cells were split upon reaching confluency. Viral titers were determined by plaque assay on BHK-21 cells. The dashed line indicates the limit of detection (LOD).

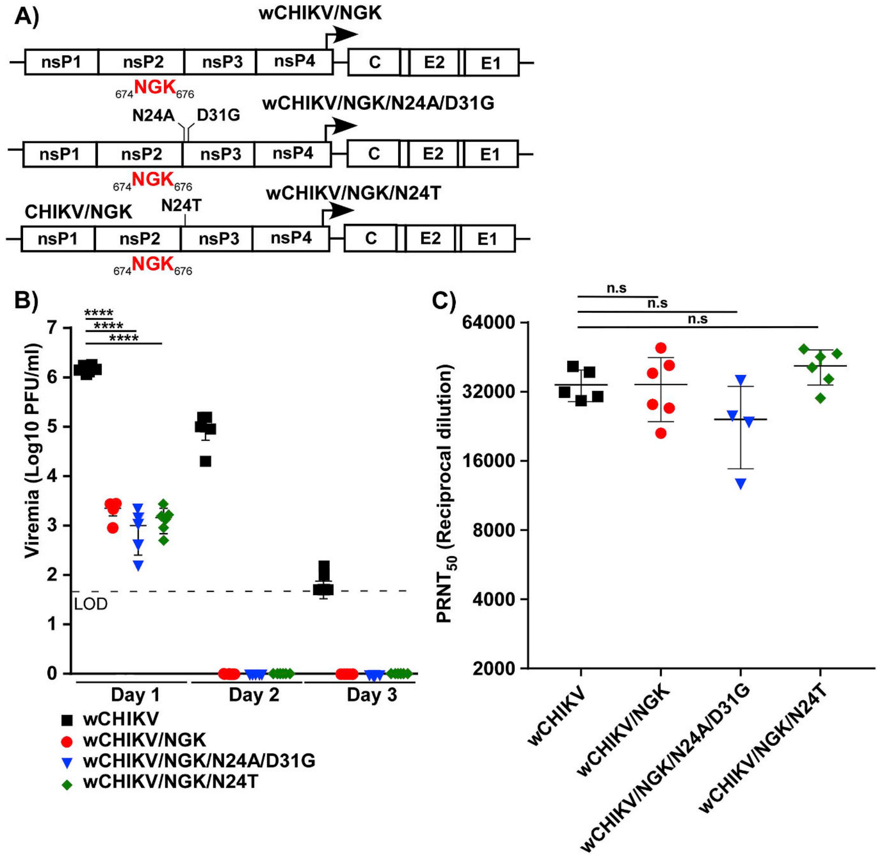


Fig. 10. Mutations in the macro domain of nsP3 in wCHIKV/NGK variant have no negative effect on viral replication in mice. (A) The schematic presentation of the recombinant genomes. (B) Two-to-three-week old C57BL/6 mice ($n = 6$/group) were infected in the left footpad with 5×10^3 PFU of the indicated mutants and parental wCHIKV. Blood samples were collected on days 1, 2, and 3 PI to analyze the levels of viremia. The dashed line indicates the limit of detection (LOD). Results are presented as means with standard deviations and significant differences, determined by one-way ANOVA, followed by Tukey's multiple comparisons test. wCHIKV is compared to other groups. **** indicates $P < 0.0001$ (C) Titers of neutralizing antibodies were evaluated on day 21 PI. Results are presented as means with standard deviations. Values are not significantly different, as determined by one-way ANOVA, followed by Dunnett's multiple comparisons test. $P > 0.05$.

intermediates into membrane spherules (Frolova et al., 2010). This likely complicates sensing of these pathogen-associated molecular patterns (PAMPs) by cytoplasmic receptors (pattern recognition receptors, PRRs), such as RIG-I, MDA5 and PKR (Akhrymuk et al., 2016). However, this isolation appears to be incomplete, and some of the viral mutants that demonstrate no alterations in spherule formation become potent type I IFN inducers (Frolova et al., 2002; Gorchakov et al., 2008). Moreover, alphavirus RCs can utilize cellular mRNA as templates for dsRNA synthesis (Nikonov et al., 2013). These dsRNA molecules may be included into spherules less efficiently and be also detected by cellular PRRs.

Consequently, besides the membrane spherule formation, alphaviruses employ another powerful mechanism of interfering with the induction of antiviral response. Geographically isolated viral species induce robust transcriptional shutoff in vertebrate, but not in mosquito cells, despite using very different means of achieving this goal (Garmashova et al., 2007). In case of OW alphaviruses, including CHIKV, inhibition of transcription is mediated by the nonstructural protein nsP2. A large fraction of nsP2 accumulates in the nucleus (Akhrymuk et al., 2019; Fros et al., 2010), and within 4 h PI, presence of RPB1, the catalytic subunit of cellular DNA-dependent RNA polymerase II, drops to undetectable levels (Akhrymuk et al., 2012, 2019). Expression of CHIKV or SINV nsP2 proteins alone has deleterious effect on the overall cellular transcription and ultimately causes cell death (Garmashova et al., 2006). Thus, alterations of the nuclear functions of CHIKV nsP2 could make virus i) less cytopathic, ii) transform it into a potent type I IFN inducer, iii) attenuate viral infection *in vivo* and iv) improve the safety of already available, attenuated strain CHIKV 181/25. These possibilities were supported by the results of our previous studies, in which we designed recombinant VEE/CHIKV variants encoding VEEV-specific nsPs and CHIKV structural proteins (Kim et al., 2011; Wang et al., 2008, 2011). The distinguishing characteristic of the latter viruses was that they expressed no viral proteins with transcription inhibitory functions and demonstrated very attenuated phenotypes in mice, but remained immunogenic. Similarly, in other studies, VEEV TC-83 and EEEV FL93 viruses were designed to have mutations in the nuclear localization signals of their capsid proteins (Aguilar et al., 2008; Atasheva et al., 2015). Those modifications made capsids incapable of forming tetrameric complexes with CRM1 and importin-α/β and blocking nuclear pores (Atasheva et al., 2008, 2010). Consequently, viral mutants were also no longer able to inhibit cellular transcription and became attenuated *in vivo*. Taken together, these data suggested that alphavirus-specific nuclear functions play critical roles in viral pathogenesis, and thus, CHIKV nsP2 can be exploited as a target for mutations aimed at viral attenuation. However, modifications of nsP2 to make it incapable of interfering with nuclear functions is a more delicate task than introducing mutations into the RNA-binding domain of VEEV or EEEV capsid proteins. Such point mutations are well tolerated by the highly variable, disordered, positively charged, RNA-binding domain of capsid protein (Atasheva et al., 2015). NsP2, in contrast, exhibits a variety of enzymatic functions in viral RNA synthesis. Their alteration by mutagenesis may either have deleterious effects on viral RNA replication (Utt et al., 2015) or be lethal for the virus.

In the recent study, we have identified a small peptide on the surface of the C-terminal domain of the OW nsP2 (VLoop) that can be modified without affecting protease, helicase or other protein's functions (Akhrymuk et al., 2018, 2019). Here, the replacements of VLoop in CHIKV 181/25 by selected heterologous peptides affected only the nuclear function(s) of nsP2 without having any negative effects on viral replication in the cells with defects in type I IFN induction or signaling (Fig. 2). In IFN-competent mouse and, importantly, human cells, the designed CHIKV 181/25 variants, but not the parental CHIKV 181/25, were capable of inducing very high levels of IFN-β (Fig. 4). The infected cells also remained able to respond to IFN-β release by activating ISGs (Fig. 6). Most importantly, CHIKV 181/25 mutants remained immunogenic, and the levels of neutralizing Abs were comparable to those induced by parental CHIKV 181/25 (Fig. 7A). This was an indication that new mutations did not make virus overattenuated.

The same replacements of nsP2-specific VLoop in the more pathogenic wCHIKV variant decreased viral replication *in vivo* (Figs. 8A and 10B). On day 1 PI, the levels of viremia developed by wCHIKV/NKG, wCHICKV/ERR, and wCHIKV/RLE were lower than those of parental wCHIKV by 1000-fold. Interestingly, despite 3 orders of magnitude lower and very short viremia, wCHIKV nsP2 mutants induced very high levels of neutralizing Abs. Most likely, as it was described for VEEV replicons (Carroll et al., 2011), the lack of transcription inhibitory functions made these mutants potent inducers of the cytokines, most importantly IFN-β. Their release may have stimulatory effect on the antibody response. This was likely the case also for CHIKV 181/25-based mutants because they also induced readily detectable levels of neutralizing Abs after a single vaccination of mice.

A distinguishing characteristic of alpha- and other RNA + viruses is the high rate of their evolution (Kim et al., 2011). Viruses containing point mutations that affect replication are highly unstable. Their passaging in cultured cells leads to rapid generation and selection of more efficiently replicating variants, which usually accumulate either true reverting or second site mutations. The essentially wt levels of replication of the newly designed CHIKV nsP2 mutants *in vitro* and the replacements of the entire VLoop peptide instead of making point mutations suggested that reversion of the mutants to parental phenotype is a less probable event. Importantly, the original strain CHIKV 181/25 is already highly attenuated and so far, there is no indication that it can cause persistent arthritis. Therefore, in the unlikely case that the nsP2 mutations revert to the CHIKV 181/25-specific sequence, this will not result in the generation of pathogenic wild type CHIKV. The reversion of all of the nsP2- and E2-specific mutations at the same time to produce a natural strain of CHIKV is an even less likely event.

Since mice are not an adequate animal model for CHIK fever, the possibility that designed CHIKV 181/25 nsP2 mutants will be capable of inducing adverse effects in humans cannot be completely ruled out. Moreover, the nsP2 mutants were noncytopathic in mouse cells but remained cytopathic in human cell lines. However, previously, we found that to become noncytopathic, SINV nsP2 mutants required additional modifications in the macro domain of nsP3, which altered its mono-ADP-ribosylhydrolase activity and virus-induced translational shutoff (Akhrymuk et al., 2018). In this study, such nsP3 macrodomain-specific mutations also affected the cytopathogenicity of CHIKV in human cells without deleterious effects on viral replication rates (Fig. 9). As expected, these mutations also did not alter replication of wCHIKV nsP2 mutants, because they had been designed to affect virus-induced cell death, but not the efficiency of its replication. Such mutations in nsP3 can be likely applied for additional attenuation of CHIKV. However, their effects CHIKV-induced pathologies need better experimental support. Moreover, based on our previous studies of the NW and chimeric alphaviruses that exhibit no nuclear functions, the described modifications in nsP2 are likely sufficient for the development of safer alternatives of CHIKV 181/25.

In conclusion, the results of this study demonstrate that i) CHIKV-specific nsP2 can be modified to make the virus incapable of inhibiting transcriptional shutoff. ii) They make CHIKV a potent type I IFN inducer in mouse and human cells without affecting its *in vitro* replication rates. iii) The introduced mutations have a negative effect on the levels of viremia caused by pathogenic variant wCHIKV. iv) Both CHIKV 181/25 and wCHIKV nsP2 mutants remain capable of inducing neutralizing Abs. v) If necessary, CHIKV can be additionally modified through the introduction of the selected mutations into the macro domain of nsP3 to alter its mono-ADP-ribosylhydrolase function. Thus, CHIKV attenuation can be achieved through a rational design of mutations in its nonstructural genes. This rationale can improve attenuation, safety, and stability of CHIKV variants attenuated by other approaches. Importantly, similar VLoop mutants can be rapidly developed for other pathogenic OW alphaviruses.

Acknowledgments

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