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PLOS | NEGLECTED TROPICAL DISEASES

Administration of E2 and NS1 siRNAs Inhibit Chikungunya Virus Replication In Vitro and Protects Mice Infected with the Virus

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Abstract

Background: Chikungunya virus (CHIKV) has reemerged as a life threatening pathogen and caused large epidemics in several countries. So far, no licensed vaccine or effective antivirals are available and the treatment remains symptomatic. In this context, development of effective and safe prophylactics and therapeutics assumes priority.

Methods: We evaluated the efficacy of the siRNAs against ns1 and E2 genes of CHIKV both *in vitro* and *in vivo*. Four siRNAs each, targeting the E2 (Chik-1 to Chik-4) and ns1 (Chik-5 to Chik-8) genes were designed and evaluated for efficiency in inhibiting CHIKV growth *in vitro* and *in vivo*. Chik-1 and Chik-5 siRNAs were effective in controlling CHIKV replication *in vitro* as assessed by real time PCR, IFA and plaque assay.

Conclusions: CHIKV replication was completely inhibited in the virus-infected mice when administered 72 hours post infection. The combination of Chik-1 and Chik-5 siRNAs exhibited additive effect leading to early and complete inhibition of virus replication. These findings suggest that RNAi capable of inhibiting CHIKV growth might constitute a new therapeutic strategy for controlling CHIKV infection and transmission.

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Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to the family *Togaviridae*. CHIKV is responsible for an acute infection, characterized by high fever, arthralgia, myalgia, headache, and rash [1,2,3]. Although having immense medical importance, effective vaccine or specific therapy is not commercially available. Currently, strict attention is given to good infection control practices that emphasizes mosquito control program.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing (PTGS) in animals and plants, which is induced by 21- to 23-nucleotide (nt) small interfering RNA (siRNA) that is homologous in sequence to the silenced gene [4,5,6]. RNAi not only regulates gene expression in the mammalian cells but also acts as a cellular defense mechanism against the invaders, including the viruses. In recent years, inhibition of specific genes by siRNAs has proven to be a potential therapeutic strategy against viral infection. For instance, inhibition of virus replication and gene expression by directly introducing siRNAs into the cells have been reported for several RNA viruses, including several important human pathogens, such as poliovirus, HIV, Hepatitis, Chandipura and influenza virus [7–24]. It has been also shown that alphaviruses such as Semliki Forest virus [7], Venezuelan equine encephalitis [20], O'nyong-nyong virus [14] are susceptible to small interfering RNA action. Recently Dash et al., [22] have demonstrated that introduction of exogenous siRNAs can inhibit replication of CHIKV *in vitro*. The success of this study is limited as siRNAs used against ns3 and E1 genes of CHIKV were shown to reduce replication by 65% by 48 h p.i. and not evaluated in-vivo [22]. Cell clones expressing shRNAs against CHIKV E1 and nsP1 genes showed significant inhibition of CHIKV replication as compared to the scrambled shRNA cell clones and non-transfected cell controls [25].

Alphaviruses contain a linear, positive sense, single stranded RNA genome of approximately 11.8 kb. RNA genome consists of a capped 5′ non-coding region (NCR) and 3′ polyadenylated NCR. The non-structural proteins, nsP1, nsP2, nsP3 and nsP4 are required for the virus replication; the structural proteins, which consist of capsid and envelope proteins (E1, E2, E3 and 6K), are synthesized as polyproteins and are cleaved by capsid autoproteinase and signalases [26]. Given the similarity of the CHIKV genomic structure to those of other alphaviruses, CHIKV is expected to encode spikes on the virion surface that is each formed by three E1–E2 heterodimers where the E1 glycoproteins mediate the fusion and the E2 glycoproteins interact with the host receptor [26,27,28]. Nsp1 protein is involved in the RNA synthesis and capping. E2 and ns1 genes are highly conserved in CHIKV strains

Author Summary

Despite having immense medical importance, still vaccine, chemoprophylactic, or effective therapeutic measures are not commercially available for chikungunya. Only strict attention to good infection control practices may prevent CHIKV infection. The pathogenic properties of CHIKV necessitate the development of an efficient antiviral therapies. Four siRNAs each, targeting the E2 and ns1 genes of chikungunya were designed and evaluated for their efficiency in inhibiting CHIKV growth in *in vitro* and *in vivo* model systems. Efficiency of these siRNAs in controlling CHIKV replication *in vitro* and *in vivo* was assessed by the real time PCR, IFA and plaque assay. Chik-1 and Chik-5 siRNA ids efficiently inhibited CHIKV replication in the virus-infected Vero-E6 cells and mice. CHIKV replication was completely inhibited in the virus-infected mice when administered 72 hours post infection (p.i.). The combination of Chik-1 and Chik-5 siRNAs exhibited additive effect leading to early and potent inhibition of virus replication. Taken together, these findings suggest the promising efficacy of RNAi ids in silencing sequence-specific genes of CHIKV and might constitute a new therapeutic strategy for controlling the CHIKV infection and transmission.

and are important in the entry and the multiplication in the host cell and, therefore, represent the rational targets for antiviral therapy.

In the current study, based on consensus sequence of CHIKV strains, the siRNA were designed to target the conserved regions in the E2 and ns1 genes of CHIKV. The efficacy of siRNAs targeted against E2 and ns1 genes individually or in combination in inhibiting the replication of CHIKV were evaluated *in vitro* (Vero cells) and *in vivo* (mice).

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by Institutional Animal Ethics Committee (IAEC). The experiments were done in a biosafety level-2 animal facility at the National Institute of Virology. All animal work was approved by the IAEC. Animal experiments were carried out in strict compliance with Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) guidelines, India.

Animals and route of siRNA delivery

Swiss albino and C57BL/6 mice (3–4 wks old; 20–25 grams) were maintained in the BSL-2 facility with controlled temperature (22°C), humidity, and a 12 h light/dark cycle. Mice received the CHIKV via one of three delivery methods: 1) Intra nasal (i.n.) 100 µl, 2) standard intra venous tail vein injection (i.v.) 200 µl, 3) Intra muscular injection (i.m.) 200 µl. siRNA (~20–25 µg/mouse) mixed with Hiperfect transfection reagent (Qiagen, Germany) and PBS (final volume 200 µl) via i.v. delivery method.

Vero E6 cells and virus strains

African Green monkey kidney (Vero-E6) cells were maintained in minimum essential medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and Neomycine 50 µg/mL. Vero-E6 cells grown under similar conditions were used for the propagation of CHIKV (African genotype, Strain No. 061573; Andhra Pradesh 2006; Accession Number EF027134), Dengue-2 (DENV-2) (Trinidad; TR1751) virus and Chandipura virus (CHPV) (Strain No. 034627; Andhra Pradesh; 2003) stock. CHIKV, DENV-2 and CHPV strains were obtained from virus repository of National Institute of Virology, Pune, India. Virus strains were passaged twice in Vero-E06 cells. Cell supernatants were harvested when 75% of the cells showed cytopathic effect, aliquoted, and stored at −80°C and used throughout the study. The virus stock titers were determined using real time PCR (8.26×10^8 CHIKV RNA copies/ml) and standard plaque assay (7×10^7 plaque-forming units/mL).

siRNA

CHIKV whole genome sequences were retrieved from GenBank NCBI database (http://www.ncbi.nlm.nih.gov) and consensus sequence was used to design the siRNA. All siRNAs were designed using HP OnGuard siRNA design (Table 1 and Fig. 1). siRNAs were then checked for the homology to all other sequences of the genome using non-redundant sequence database and the homology analysis tool. Four siRNAs each, targeting E2 and ns1 genes were designed and synthesized (Qiagen, Germany) (Table 1, Fig. 1). Negative control siRNA [ncsiRNA; siRNA against Chandipura virus (24) with no significant homology to any known mammalian gene was used as a non-silencing control in all RNAi experiments and were purchased from Qiagen, Germany. Fluorescent labeling of siRNA was performed using the Cy3 Silencer labeling kit (Ambion, USA) and modified as described in the manufacturer's protocol.

CHIKV infection and transfection

Vero E6 cells were infected with CHIKV (Multiplicity of infection MOI 5. Two h post infection (p.i.), cells were transfected with E2 (Chik-1, Chik-2, Chik-3, Chik-4), ns1 (Chik-5, Chik-6, Chik-7, Chik-8) siRNA and control using the Amaxa Nucleofector device II (Amaxa biosystems). After electroporation, Vero-E6 cells were incubated at 37°C until analyzed for inhibition of CHIKV replication. Cells were harvested at 24, 36 and 48 h p.i. and inhibition of CHIKV replication was determined by quantitative RT-PCR (qRT-PCR), plaque assay and ImmunoFluorescnce Assay (IFA).

Optimization of siRNA concentration

At two hours p.i. with CHIKV, Vero E6 cells were transfected with Chik-1, Chik-5 and combination of both siRNAs at different concentration (10, 50, 100, 150 and 200 pmol). After 24 h post transfection, total RNA was isolated from the tissue culture supernatant and cells. One step qRT-PCR was carried out to evaluate the inhibitory effect of siRNA.

MTT assay. Cytotoxicity tests were performed with Vero E6 cells using an in vitro toxicology assay kit (TOX-1, Sigma) based on the reduction activity of methyl thiazolyl tetrazolium (MTT). Twenty-four hours before transfection, 5×10^3 cells were seeded in a 96-well plate. The transfection was performed using Hiperfect transfection reagent according to the manufacturer's instructions. The cells were divided into four groups: 1) control group (No Hiperfect and siRNA); 2) Hiperfect group; 3) Chik-1 siRNA group; 4) Chik-5 siRNA group; and 5) Combination of Chik-1+Chik-5 siRNA (Comb-siRNA) group. Transfection of siRNAs was carried out on the following day as described earlier in the CHIKV infection and transfection section. Experiments were conducted with non-transfected or siRNA transfected (100 pmol) cells at 24 and 48 hr post-transfection. 24 hours after the siRNA transfection, the cells of the appropriate groups were subjected to MTT assay (TOX-1, Sigma). Blue formazon, solubilized by adding MTT solubilization solution to the wells, produced by

Table 1. Nucleotide sequences of siRNA designed for CHIKV genes.

No.	Si RNA Name	Location on genome (nt number)	Location on gene (nt number)		5′ Sequence 3′
E2 gene					
1	Chik-1	8574–8591	30–47	Sense	r (GGA CAA CUU CAA UGU CUA U) dTdT
				Antisense	r (AUA GAC AUU GAA GUU GUC C) dTdT
2	Chik-2	8955–8973	411–429	Sense	r (CCA CGA CCC UCC UGU GAU A) dTdT
				Antisense	r (UAU CAC AGG AGG GUC GUG G) dTdG
3	Chik-3	8848–8866	304–322	Sense	r (GGA ACA AUG GGA CAC UUC A) dTdT
				Antisense	r (UGA AGU GUC CCA UUG UUC C) dAdG
4	Chik-4	9386–9404	842–860	Sense	r (CCA CCG UGA CGU ACG GGA A) dTdT
				Antisense	r (UUC CCG UAC GUC ACG GUG G) dGdG
ns1 gene					
5	Chik-5	1641–1659	1563–1581	Sense	r (GGU CGA AAU CGA CGU GGA A) dTdT
				Antisense	r (UUC CAC GUC GAU UUC GAC C) dTdG
6	Chik-6	695–713	617–635	Sense	r (GGC UAA GAA CAU AGG AUU A) dTdT
				Antisense	r (UAA UCC UAU GUU CUU AGC C) dTdT
7	Chik-7	1107–1125	1029–1047	Sense	r (CGG CAU CCU UGC UAC AGA A) dTdT
				Antisense	r (UUC UGU AGC AAG GAU GCC G) dGdT
8	Chik-8	290–308	212–230	Sense	r (GGA UGA UGU CGG ACA GGA A) dTdT
				Antisense	r (UUC CUG UCC GAC AUC AUC C) dTdC

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viable cells was quantified in ELISA reader (Biorad, USA) at 570 nm after subtracting the background reading at 650 nm. The data were presented as the percentage of viable cell numbers in the siRNA treated and untreated control wells.

In vivo inhibition of CHIKV using Chik-1 and Chik-5 siRNA

Swiss albino and C57BL/6 mice (3–4 weeks) were infected with approximately 1×10^6 pfu of CHIKV (100 µl of 10^7 pfu/ml; $\sim4.5\times10^8$ RNA copies/ml) by three routes viz.; i.v., i.n. and i.m. and RNA copies were checked in muscles on 2^{nd}, 4^{th}, 7^{th} and 14^{th} day p.i.. C57BL/6 mice (4–6 weeks) were infected by CHIKV (100 µl of 10^7 pfu/ml; $\sim4.5\times10^8$ RNA copies/ml) and CHIKV RNA copies were measured daily in the blood and muscles by one step qRT-PCR for fourteen days. siRNAs were complexed with HiPerfect^{TM} (QIAGEN, Valencia CA) according to the manufacturer's instructions and ~25 µg/mouse (1 mg/Kg body wt) was administered i.v. once after 48 or 72 h p.i.. Chik-1 siRNA, Chik-5 siRNA and combination of Chik-1 and Chik-5 siRNAs (Comb-siRNA) were used in different mice groups. Blood (~200 µl) was collected from siRNA, ncsiRNA, or saline injected mice groups at 0, 1, 2, 3 and 4 day post treatment. CHIKV E3 RNA was quantitated from the sera using qRT-PCR. In C57BL/6 mice, the 72 h time point was chosen for siRNA treatment. Blood and hind limb muscle tissues were harvested from C57BL/6 mice at 0, 1, 2, 3 and 4 day post siRNA injection. The tissues were dissected, weighed, crushed and macerated in liquid nitrogen using mortar pestle, and used for the RNA isolation.

Quantitative RT-PCR

RNA from the Vero-E6 cells, serum and mice tissues was extracted using QIAmp viral RNA minikit (QIAGEN, Valencia, CA) and trizol (Invitrogen USA) method respectively following the manufacturer's instructions. Viral load in serum and/or tissue samples were determined by absolute quantification using the

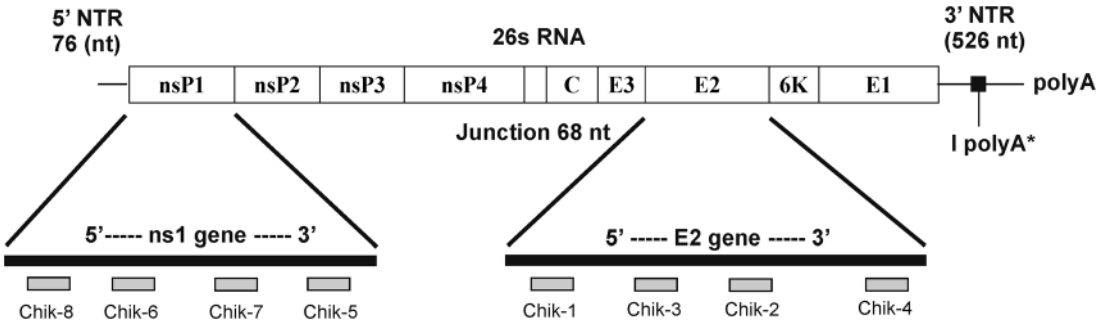

Figure 1. Schematic representation of the sites of the eight siRNA target sequence in CHIKV genome. (Exact location on CHIKV genome is depicted in Table 1.)
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standard curve method. One step RT-PCR was performed in 25 µl reaction mixture containing 5 µl RNA, 12.5 µl TaqMan One-Step RT-PCR 2× Master Mix, 1 µl 40×(RT+RNAasin) (Applied Biosystems) each 1 µl sense (µM), 1 µl anti-sense (µM) primer and 1 µl TaqMan probe. Primers were selected from the E3 structural protein region. Real-time one step RT-PCR was performed in a 96-well format using 7300 real time PCR system and SDS software V 1.0.2 (Applied Biosystems). The amplification program included: reverse transcription at 48°C for 30 min, initial denaturation at 95°C for 10 min, and 50 cycles of denaturation (95°C for 15 sec) and annealing and extension (60°C for 1 min). After the amplification, a melting curve was acquired to check the specificity of PCR products. A standard curve was generated by the amplification of serial dilutions of in vitro transcribed RNA of CHIKV (10^8 to 10^2 serial dilutions). After generation of standard curve, we compared unknowns to the standard curve and extrapolated the value. Viral titers were expressed as RNA copies per ml of serum or per ml of tissue culture suspension or per well or per mg tissue. Detection limit of real time PCR was 10 copies per reaction.

Plaque assay

Serial dilutions of tissue culture supernatants of infected and siRNA transfected cells were added to a monolayer of Vero E6 cells and the plates were incubated at 37°C for 1 h. After the incubation, the medium was replaced by overlay medium (2× MEM, 2% CMC and 10% FBS (Gibco, USA). The plates were incubated at 37°C for 72 h and the cells were stained with amido black and the plaques were counted.

Immune sera preparation against the CHIKV genotypes in mice

Groups of 3–4 weeks old swiss albino mice were inoculated intra-peritonealy with the CHIKV African strain (1:1 vol:vol mixture of CHIKV and Freund's complete adjuvant) and were maintained under standard laboratory conditions. Two booster doses with CHIKV along with Freund's incomplete adjuvant (1:1) were administered at weekly intervals. Blood samples were collected at pre and post immunization (7 days after the last dose). IgG antibodies were then purified using protein A column (Merck Biosciences, India) according to the instructions of the manufacturer.

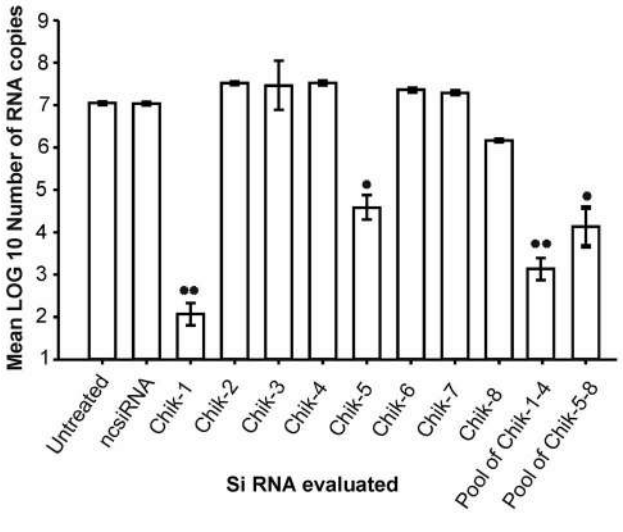

Figure 2. Effect of different siRNAs on CHIKV replication. Vero E6 cells seeded in 6 well plates were infected with CHIKV and after 2 h p.i., transfected with different siRNAs (100 pmol). Total RNA was isolated 24 h after virus infection. Amount CHIKV RNA was detected by measuring E3 RNA copies by real-time RT-PCR. siRNA Chik-1 and Chik-5 showed substantially stronger inhibition of CHIKV as compared to other siRNAs. Values are given as mean $\log_{10}$RNA copies/well ± SD. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pntd.0002405.g002

Immunofluorescence assay

Immunofluorescence assay (IFA) was carried out as described by Sudeep et al. (29). Vero E6 cells were fixed with acetone and blocked with 2% BSA in the phosphate buffered saline (pH 7.4) for 1 h. The cells were incubated with (1:100) mouse anti CHIKV antibody followed by incubation with FITC-conjugated rabbit anti-mouse (1:500) antibodies (Invitrogen, USA). Cells were counter stained with Evan's blue for one min. The slides were visualized using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software). Negative controls were similarly processed using pre-immune sera.

Histopathology

Hind limb tissues, excluding the femur bone, were fixed in 4% formaldehyde and were embedded in the paraffin. Thin section of 8 µm size were prepared. Tissues were stained with haematoxylin and eosin. Histopathological evaluation was performed on the muscle tissues of the hind legs from the control (saline injected, ncsiRNA), CHIKV infected (4, 5, 6 and 7 day p.i.), treatment groups (Chik-1 siRNA, Chik-5 siRNA and Comb-siRNA). siRNA treatment was given on third day p.i. and the tissues were harvested at 4, 5, 6 and 7 days p.i. and evaluated for necrosis, inflammation, regeneration, mineralization, fibrosis and the edema. Concurrently IFA was carried out to check the presence of CHIKV. Immunofluorescence assay (IFA) was carried out as described by Sudeep et al. [29]. The slides were incubated with (1:100) mouse anti CHIKV antibody followed by incubation with Alexa flor 546-conjugated rabbit anti-mouse (1:200) antibodies (Invitrogen USA). Cells were counter stained with DAPI for 10 seconds. The slides were visualized using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software). Negative controls were processed similarly.

Interferon gene expression analysis employing qPCR

For real-time reverse transcription RT-PCR analysis, hind limb muscle tissues were crushed in liquid nitrogen. RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One step RT-PCR was performed using Quantitect SYBR Green RT PCR kit (Qiagen, Germany). Real-time PCR analysis used the following nucleotide primers: 5′-GGCCGAGGACTTTGATTGCACATT-3′ and 5′- AGGATGGCAAGGGACTTCCTGTAA-3′ for actin beta, 5′- AGGAGGAGTTTGATGGCAACCAGT -3′ and 5′- TCCTCATCCCAAGCAGCAGATGAA-3′ for Interferon alpha (INF-α) (NM_010502), 5′- TGTGGCAATTGAATGGGAGGCTTG-3′ and 5′- TCTCATAGATGGTCAATGCGGCGT -3′ for interferon beta (IFN-β), and 5′-AGCGGCTGACTGAACTCAGATTGT-3′ and 5′- ACTGCTTTCTTTCAGGGACAGCCT-3′ for interferon gamma (IFN-γ) (NM_008337). The 25-µl amplification reaction mixture contained 500 ng total RNA, 0.5 µM each primer pair, 0.25 of reverse transcriptase enzyme and 12.5 µl of 2× SYBR green qPCR Supermix (Qiagen, Germany). Cycling conditions were as follows: one cycle of 50°C for 30 min and one cycle of 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 57°C for 30 s, 72°C for 30 s and 68°C for 15 s. The real-time PCR was performed by using a Rotor-Gene 3000 PCR

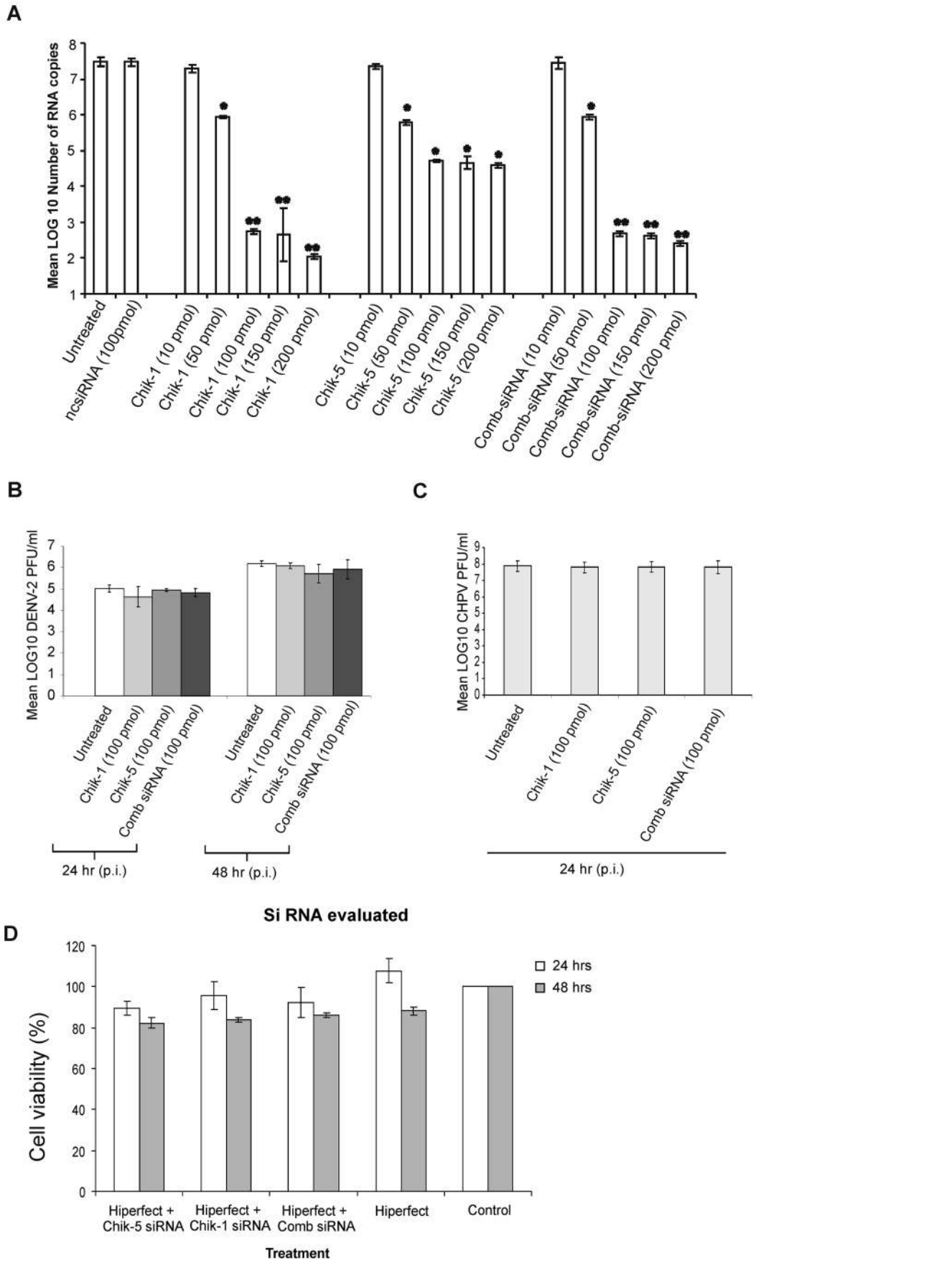
A
Mean LOG 10 Number of RNA copies
Untreated
ncsiRNA (100pmol)
Chik-1 (10 pmol)
Chik-1 (50 pmol)
Chik-1 (100 pmol)
Chik-1 (150 pmol)
Chik-1 (200 pmol)
Chik-5 (10 pmol)
Chik-5 (50 pmol)
Chik-5 (100 pmol)
Chik-5 (150 pmol)
Chik-5 (200 pmol)
Comb-siRNA (10 pmol)
Comb-siRNA (50 pmol)
Comb-siRNA (100 pmol)
Comb-siRNA (150 pmol)
Comb-siRNA (200 pmol)
B
Mean LOG10 DENV-2 PFU/ml
Untreated
Chik-1 (100 pmol)
Chik-5 (100 pmol)
Comb siRNA (100 pmol)
24 hr (p.i.)
48 hr (p.i.)
C
Mean LOG10 CHPV PFU/ml
24 hr (p.i.)
D
Si RNA evaluated
Cell viability (%)
24 hrs
48 hrs
Hiperfect + Chik-5 siRNA
Hiperfect + Chik-1 siRNA
Hiperfect + Comb siRNA
Hiperfect
Control
Treatment

Figure 3. Optimization of siRNA concentration. A) Vero E6 cells were transfected with Chik-1 and Chik-5 siRNAs individually as well as in combination at different concentration (10, 50, 100, 150 and 200 pmol). Total RNA was isolated 24 h after virus infection. Amount of CHIKV RNA was detected by measuring E3 RNA copies by real-time RT-PCR. siRNA Chik-1 and Chik-5 treatment at a concentration of 10 pM failed to reduce CHIKV RNA copies. Whereas all other concentrations of Chik-1 and Chik-5 significantly reduced the viral RNA copies. Values are given as mean $\log_{10}$ RNA copies/well ± SD. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$. **B**) Effect of Chik-1 and Chik-5 siRNAs on DENV-2 growth: Vero-E6 cells were infected with DENV-2 and Chik-1 (100 pmol) CHIK-5 (100 pmol) and Comb-siRNA (100 pmol) transfected after 2 h p.i. and DENV-2 titers were measured by plaque assay. **C**) Effect of Chik-1 and Chik-5 siRNAs on Chandipura virus (CHPV) growth: Vero-E6 cells were infected with CHPV and Chik-1 (100 pmol) CHIK-5 (100 pmol) and Comb-siRNA (100 pmol) transfected after 2 h p.i. and CHPV titers were measured by plaque assay **D**) Effect of siRNA–Hiperfect complex treatment on survival of Vero-E6 cells was accessed by MTT assay. siRNAs were used in 100 pmol concentration.
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machine. The data were analyzed with Rotor-Gene real-time analysis software. Each sample was analyzed in duplicate and normalized to actin beta mRNA. Expression changes in interferon alpha, beta and gamma genes in CHIKV infected group, CHIKV infected mice treated with Chik-1, Chik-5 and Comb-siRNA group, and the control mice with Chik-1, Chik-5 and Comb-siRNA treatment were investigated using real time PCR analysis. Mice were mock-infected with CHIKV and treated with siRNA on third day p.i. and then gene expression determined at days 4, 5, 6, and 7 p.i.. Three mice were used for each treatment and each time point.

Statistical analysis

All data were expressed as mean ± standard deviation. The viral loads were log-transformed for improvement of normality. Statistical significance was determined by Dunnet's test using ANOVA. A value of $p<0.05$ was considered statistically significant. Fold change was compared using one way ANOVA and the groups were also compared by nonparametric Kruskal-Wallis test for confirmation of results.

Results

Based on the consensus sequences of CHIKV genomes, four siRNAs each were designed to have an antisense strand complementary to the E2 and ns1 RNA. The sequences and the corresponding genomic positions are shown in Table 1 and Fig. 1 respectively.

Efficiency of different siRNAs in reducing CHIKV replication in vitro

For the initial comparison of antiviral activity of different siRNAs, Vero-E6 cells were infected with CHIKV and transfected with different siRNAs (Chik-1 to Chik-8) 2 h p.i.. Chik-1 and Chik-5 were the most effective siRNAs, suppressing CHIKV copies by 5 $\log_{10}$ ($p<0.001$) and ~2.5$\log_{10}$ ($p<0.05$) RNA copies respectively (Fig. 2). The pool of siRNAs Chik 1–4 (4 $\log_{10}$; $p<0.001$) and Chik5–8 (3 $\log_{10}$; $p<0.001$) did not increased the CHIKV suppression in Vero E6 cells (Fig. 2). Results obtained with the individual siRNAs and pool of siRNAs indicated that only siRNAs Chik-1 and Chik-5 possessed the antiviral activity against CHIKV. Therefore only Chik-1 and Chik-5 and Comb-siRNA were used for further studies. The reduction in the CHIKV copies by Chik-1 and Chik-5 was initiated at the siRNA concentrations of 50 pmol, and reached a plateau at 100 pmol (Fig. 3 A). Chik-1 and Chik-5 showed sequence dependent inhibition and showed no reduction in the dengue-2 (Fig. 3B) and the Chandipura virus (Fig. 3C) replication in Vero-E6 cells.

Effect of siRNA treatment on proliferation of VeroE6 cells (MTT assay)

Effect of Chik-1, Chik-5 and Comb-siRNAs (100 pmol) transfection on survival of Vero-E6 cells was assessed by the MTT assay. At 24 h, transfection of Chik-1 siRNA (95.1±6.51), Chik-5 siRNA (89.46±3.19), Comb-siRNA (92.11±7.11) and Hiperfect reagent (95.88±11.47) do not exhibited any significant change in proliferation of Vero E6 cells (Fig. 3D). At 48 h,

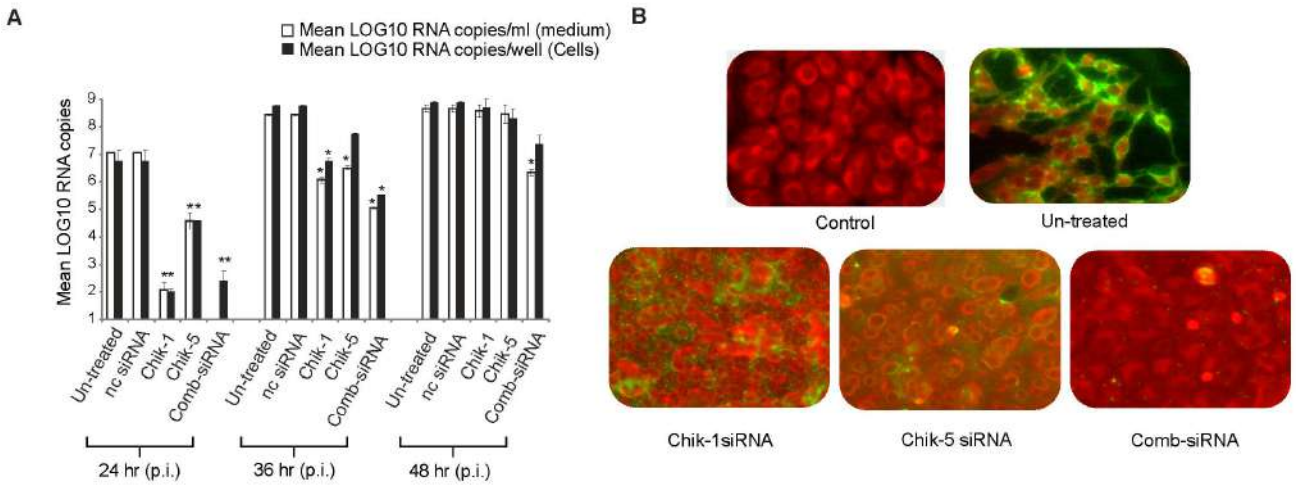

Figure 4. Evaluation of siRNAs directed against CHIKV E2 and ns1. A) Quantitative analysis of intra cellular and extra cellular CHIKV RNA copies using real time PCR: Total RNA was isolated at 24, 36 and 48 h after virus infection and E3 RNA copies determined by real-time RT-PCR. Values are given as mean RNA copies/well ± SD for cells and mean RNA copies/ml ± SD for culture medium. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$; ***$p<0.001$. **B**) Detection of CHIKV in Vero E6 cells using immuno-fluorescence microscopy: Vero E6 cells seeded in 6 well plates were infected with CHIKV and after 1 h transfected with 100 pmol of the indicated siRNAs. Cells were fixed 48 h later and CHIKV (green) were detected with fluorescein labeled antibodies. Cells were co-stained with Evan's blue (red) to visualize the cell morphology.
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Table 2. Plaque assay showing inhibitory effect of siRNAs on CHIK virus replication in Vero E6 cell line at 48 h.

Material	Virus titer pfu/ml (SD)	Virus inhibition compared to untreated
Untreated	4×10^7 (1.9×10^7)	Nil
CHIKV+Chik-1 siRNA	2×10^2 (1.1×10^2)	>5 $\log_{10}$**
CHIKV+Chik-5 siRNA	4×10^4 (1.8×10^4)	3 $\log_{10}$*
CHIKV+Comb-siRNA	7.5×10^4 (6.3×10^4)	~3 $\log_{10}$*
CHIKV+ncsiRNA	4×10^7 (1.8×10^7)	Nil

Values are given as mean pfu/ml (SD).
Significance ANOVA, Dunnett's test:
*$p<0.05$;
**$p<0.01$.
doi:10.1371/journal.pntd.0002405.t002

transfection of Chik-1 siRNA (83.71±9.24), Chik-5 siRNA (82.13±2.71), Comb-siRNA (86.1±1.65) and Hiperfect reagent (88.02±2.58) displayed small reduction in viable cell number (Fig. 3D).

siRNA stability

Transfected Cyanine 3 dye labeled siRNAs showed signal at 4 h and 6 h whereas at 24 h signal was minimal, but still present compared to control treatment (Supplementary Information Fig. S1). Chik-1 and Chik-5 siRNAs were stable till 24 h.

Effect of Chik-1 and Chik-5 siRNAs on CHIKV replication

Figure 4A depicts the effect of Chik-1, Chik-5 and Comb-siRNAs on the CHIKV replication at different time points. At 24 h p.i., treatment of Chik-1 and Chik-5 siRNAs resulted in the reduction of 5 $\log_{10}$ and 3 $\log_{10}$ CHIKV RNA copies respectively in cells and the supernatant (Fig. 4A). At 36 h p.i., 3 $\log_{10}$ (Chik-1), and 2 $\log_{10}$ (Chik-5) reduction in CHIKV RNA copies was observed in tissue culture supernatant whereas 2 $\log_{10}$ reduction was recorded in cells with Comb-siRNAs (Fig. 4A). At 48 h p.i., no significant reduction in CHIKV RNA copies was noted in the cells and the supernatant. Overall, the siRNAs directed against E2 gene (Chik-1) were more efficient in inhibiting CHIKV replication than the siRNA directed against ns1 region (Chik-5). We further evaluated the additive advantage of treatment with Comb-siRNAs. In supernatant, 5 $\log_{10}$ ($p<0.001$), 2.5 $\log_{10}$ ($p<0.05$) and 2.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) reduction in CHIKV copies was observed at 24, 36 and 48 h respectively when compared to virus

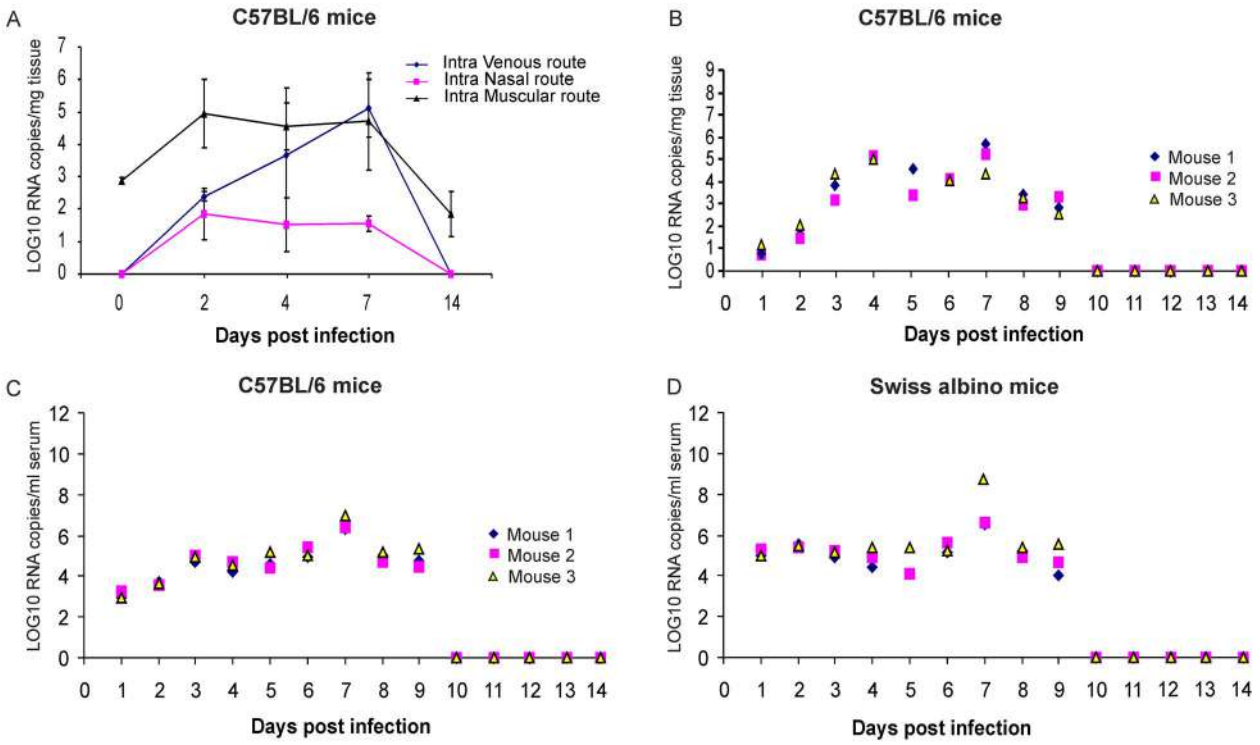

Figure 5. CHIKV replication in C57BL/6 and Swiss albino mice. C57BL/6 mice were infected with CHIKV (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) by three different routes viz. intra venous, intra nasal and intra muscular. **A**) CHIKV copies were measured in hind limb muscle tissues on 0, 2, 4, 7, and 14 days p.i.. **B**) CHIKV RNA copies in hind limb muscle tissues of C57BL/6 mice on 1–14 days p.i. **C**) CHIKV RNA copies in serum of C57BL/6 mice on 1–14 days **D**) CHIKV RNA copies in serum of swiss albino mice.
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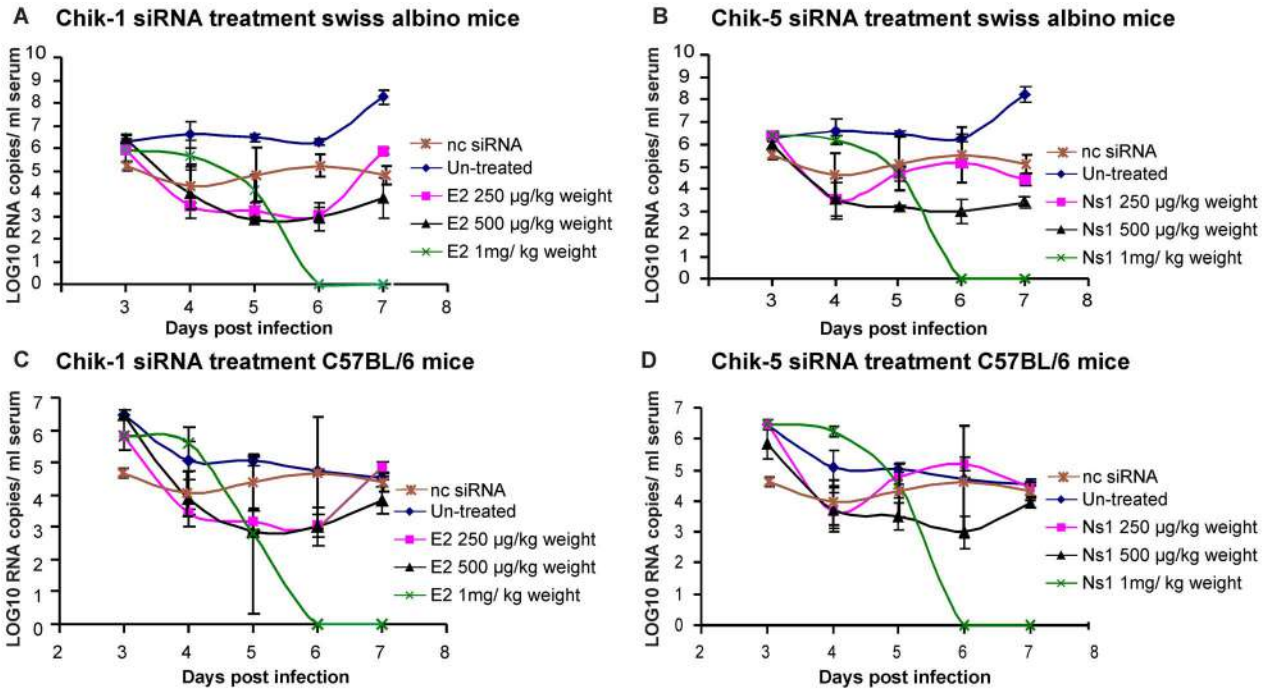

Figure 6. Dose dependent reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in swiss albino and C57BL/6 mice infected with CHIKV. Swiss albino and C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). After 72 h p.i. swiss albino mice were inoculated i.v. with **A**) Scrambled siRNA (siRNA against chandipura virus), 250 µg, 500 µg and 1 mg/kg body weight Chik-1 siRNA (n = 3 for each treatment and time point), **B**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-5 siRNA (n = 3 for each treatment and time point). After 72 h of p.i. C57BL/6 mice were inoculated i.v. with **C**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-1 siRNA (n = 3 for each treatment and time point), **D**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-5 siRNA (n = 3 for each treatment and time point). At indicated time after injection of siRNA blood was collected from eye and RNA was isolated from serum. CHIKV E3 RNA copies were quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum.
doi:10.1371/journal.pntd.0002405.g006

infected control. In cells, 4.5 $\log_{10}$ ($p<0.001$), 3 $\log_{10}$ ($p<0.05$) and 2 $\log_{10}$ ($p<0.05$) reduction was obtained at 24, 36 and 48 h respectively. Importantly, the Comb-siRNA could prolong the inhibitory effect as compared to individual siRNAs (Fig. 4A).

When plaque assay was used as the measure of CHIKV replication, Chik-1 siRNA yielded a reduction of 5 $\log_{10}$ at 48 h p.i. (Table 2). Chik-5 reduced 3 $\log_{10}$ and Comb-siRNA showed reduction of 3 $\log_{10}$ in the virus titer. At 24 and 36 h p.i., cytopathic effects were not observed in the treated cultures where as commencement of cytopathic effects was observed in the untreated control from 24 h p.i. demonstrating the inhibitory effect of the siRNAs. These results were consistent with our real time RT-PCR results and the plaque assay results, IFA also showed the reduction of viral antigen in Chik-1 and Chik-5 siRNAs treated cells (Fig. 4 B).

Swiss albino and C57 BL/6 mice are permissive to CHIKV infection

As human muscle cells are the target of CHIKV infection, we evaluated the i.m. route along with i.v. and i.n. route for CHIKV infection. Infection by all the three route resulted in CHIKV replication in the thigh muscles (Fig. 5A). CHIKV RNA copies were not detected in uninfected mice at 0, 2, 7 and 14 days p.i.. Mice inoculated with in i.v., i.m. and i.n. routes; CHIKV was not detected in the muscle tissues at 0 day p.i. (Fig. 5A). CHIKV appeared in the muscle tissues by 2 days p.i., persisted till 7 days p.i. and disappeared on 14 days p.i. (Fig. 5A). CHIKV inoculated via i.m. route could be detected in thigh muscle tissues at 2 h p.i. (753±101 CHIKV RNA copies). Therefore i.m. route was not preferred as it was difficult to distinguish newly replicated virus from the virus innoculum. Intra nasally inoculated mice exhibited the lower viral RNA copies in the thigh muscles (Fig. 5A). Since i.m. and i.n. routes were not yielded satisfactory results, therefore we used i.v. route for CHIKV infection.

Swiss albino and C57 BL/6 mice were infected by i.v. route and CHIKV RNA copies were measured in serum and muscle tissues from 1 day p.i. to 14 day p.i.. Infection of adult swiss albino and C57BL/6 mice with 1×10^6 PFU CHKV (100 µl of 10^7pfu/ml) CHIKV by i.v. route did not cause mortality. Clinical symptoms such as sluggishness and foot swelling were observed. A definite evidence of the replication of the virus was observed in muscle tissues (Fig. 5B). CHIKV RNA copies were detected in mice serum from 1 day p.i. till 9 days p.i. (Fig. 5 C & D). Viremia in i.v inoculated mice reached a peak by 3 days p.i., with viral loads ranging from 7×10^5 to 5×10^7 viral RNA copies/ml (Fig. 5 C & D).

siRNA inhibits the CHIKV replication in swiss albino mice

To assess whether siRNAs could protect mice from CHIKV infection, groups of CHIKV infected mice (1×10^6 PFU CHIKV; 100 µl of 10^7pfu/ml) were administered Chik-1 and Chik-5 siRNAs at 72 h p.i.. Swiss albino mice treated with E2 or ns1 siRNA with 250 µg per kg body weight (~6 µg/mice) showed ~3$\log_{10}$ inhibition, 500 µg per kg body weight (~12 µg/mice) showed 3$\log_{10}$ inhibition of CHIKV whereas at 1 mg per kg body weight (~25 µg/mice) siRNA led to 7$\log_{10}$ reduction in CHIKV copies (Fig. 6 A & B). Similar results were obtained in C57BL/6 mice (Fig. 6 C & D). We therefore administered 1 mg/kg body

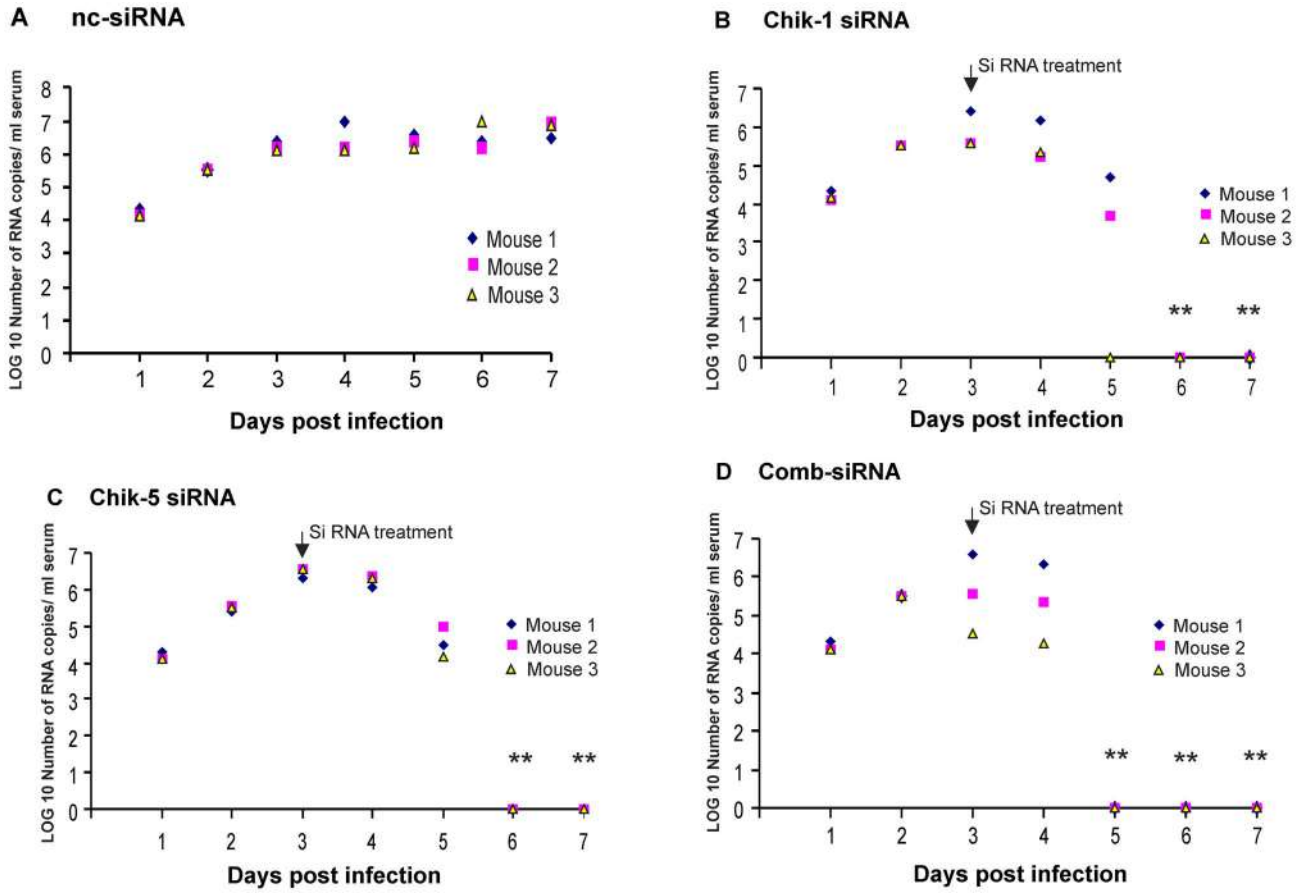

Figure 7. The reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in Swiss albino mice infected with CHIKV. Swiss albino mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). After 72 h of p.i. mice were inoculated i.v. with **A**) ncsiRNA 1 mg/kg body weight **B**) 1 mg/kg body weight Chik-1 (n = 3), **C**) 1 mg/kg body weight Chik-5 (n = 3) and **D**) Comb-siRNA (n = 3) 1 mg/kg body weight each. At indicated time after injection of siRNA blood was collected from eye and RNA was isolated from serum. CHIKV E3 RNA was quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pntd.0002405.g007

weight (~25 µg/mice) siRNA in subsequent experiments. For all *in-vivo* experiments, Hiperfect reagent was used for delivery of siRNA. Chik-1, Chik-5 and Comb-siRNA administered at 72 h p. i. provided significant reduction in serum viral load as assessed by real time PCR (Fig. 7). At 48 h post siRNA injection, reduction with Chik-1 and Chik-5 was around 2.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) as compared to 0 h and ncsiRNA whereas 100% inhibition ($7\log_{10}$) was observed with Comb-siRNA (ANOVA Dunnet's test $p<0.01$). At 72 h p.i., administration of Chik-1, Chik-5 and Comb-siRNAs showed complete inhibition ($7\log_{10}$, ANOVA Dunnet's test $p<0.01$).

Inhibition of the CHIKV replication in C57BL/6 mice after treatment of siRNA

Chik-1, Chik-5, Comb-siRNA and ncsiRNA administered 72 h p.i. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) provided significant reduction in the serum viral load as assessed daily by real time PCR (Fig. 8). At 24 h and 48 h post siRNA treatment, 2.5 $\log_{10}$ and 3.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) reduction was recorded for all siRNAs, when compared to ncsiRNA. At 72 h post treatment, reduction with siRNA Chik-1, and Chik-5 was around 3.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) while Comb-siRNA showed 100% inhibition ($7\log_{10}$, ANOVA Dunnet's test $p<0.01$). Importantly, Comb-siRNA produced prolonged inhibitory effect when compared to individual siRNAs. In muscle tissues, CHIKV RNA reached peak by third day p.i., with viral loads ranging from 1×10^4 to 7×10^5 viral RNA copies/mg tissue (Fig. 8). At 24 h post-siRNA treatment ~2.5 $\log_{10}$ reduction in CHIKV RNA was noted with all the three siRNAs as compared to ncsiRNA control. At 72 h, all the siRNAs produced $4\log_{10}$ reduction in CHIKV RNA (100% inhibition, ANOVA Dunnet's test $p<0.01$). Similar results were seen when IFA was used to evaluate the effect of siRNA on CHIKV replication in muscle tissues that corroborated with real time PCR-based data (Fig. 9).

Histopathological evaluation of mice muscle tissues after CHIKV infection and siRNA treatment

Having demonstrated that Chik-1 and Chik-5 siRNA treatment significantly reduced the CHIKV titer in serum and muscle tissues, histopathology analysis was performed to determine the inflammation and infiltration in chikungunya infected and siRNAs treated tissues. Histopathological examination of CHIKV infected mice (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) showed pathological changes such as extensive necrosis, inflammation, pronounced monocyte/macrophage infiltrates and edema (Fig. 10). Such histopathological changes were prevented by systemic treatment either with Chik-1, Chik-5 individually or in Comb-siRNAs. At 3 days p.i., the muscle

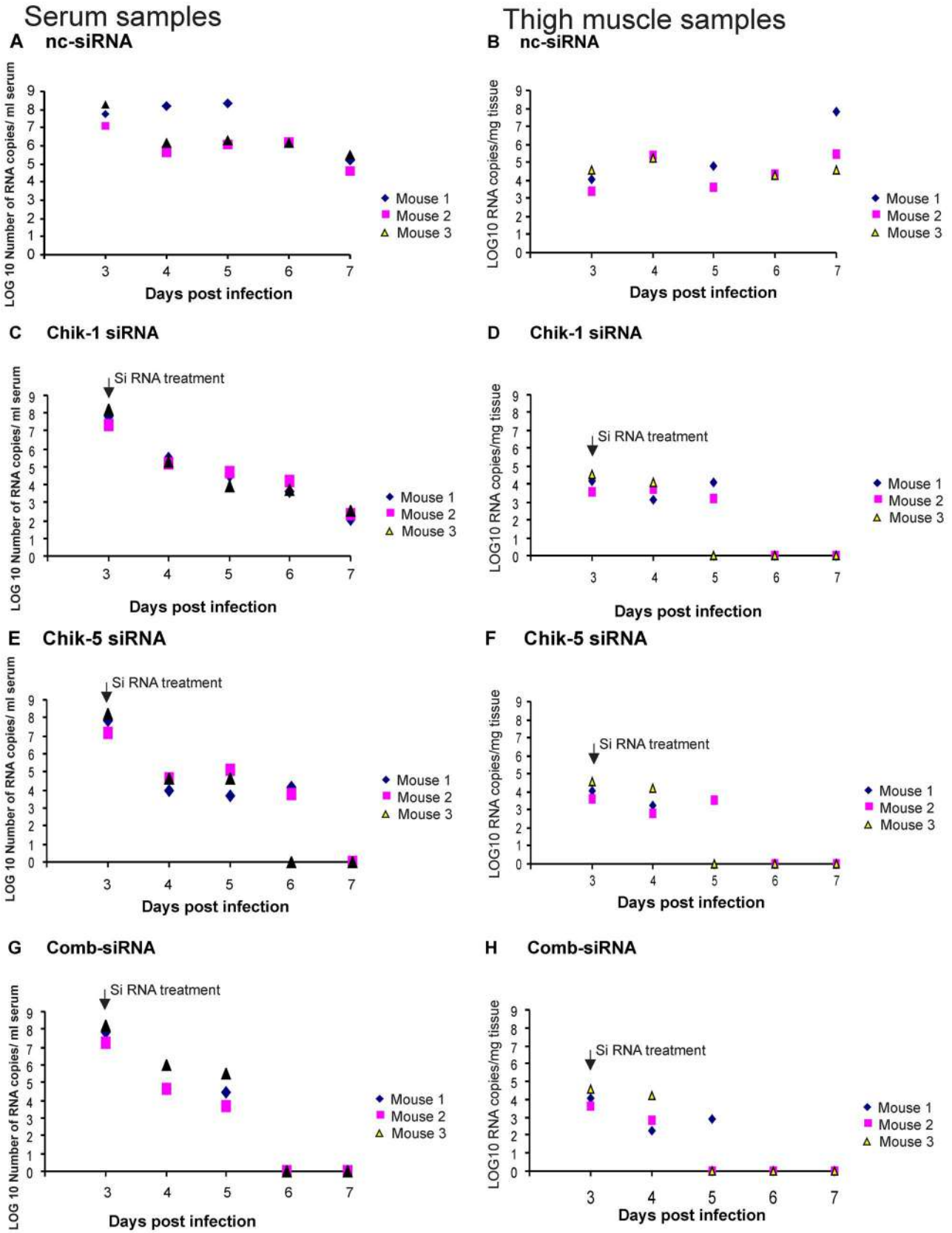

Figure 8. The reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in C57BL/6 mice infected with CHIKV. C57BL/6 mice (n = 15) were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) and viral RNA copies were checked in serum and muscle tissues. After 72 h p.i. mice were inoculated i.v. with 1 mg/kg body weight nc siRNA (**A** and **B**), 1 mg/kg body weight Chik-1 (n = 15) (**C** and **D**), 1 mg/kg body weight Chik-5 (n = 15) (E and F) and Comb-siRNA (n = 15) 1 mg/kg body weight each (**G** and **H**) and viral RNA copies were checked

in serum and muscle tissues at indicated time after injection. CHIKV E3 RNA was quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum and LOG10 RNA copies/mg of tissue. Significance Dunnett's test: *$p<0.05$; **$p<0.01$.
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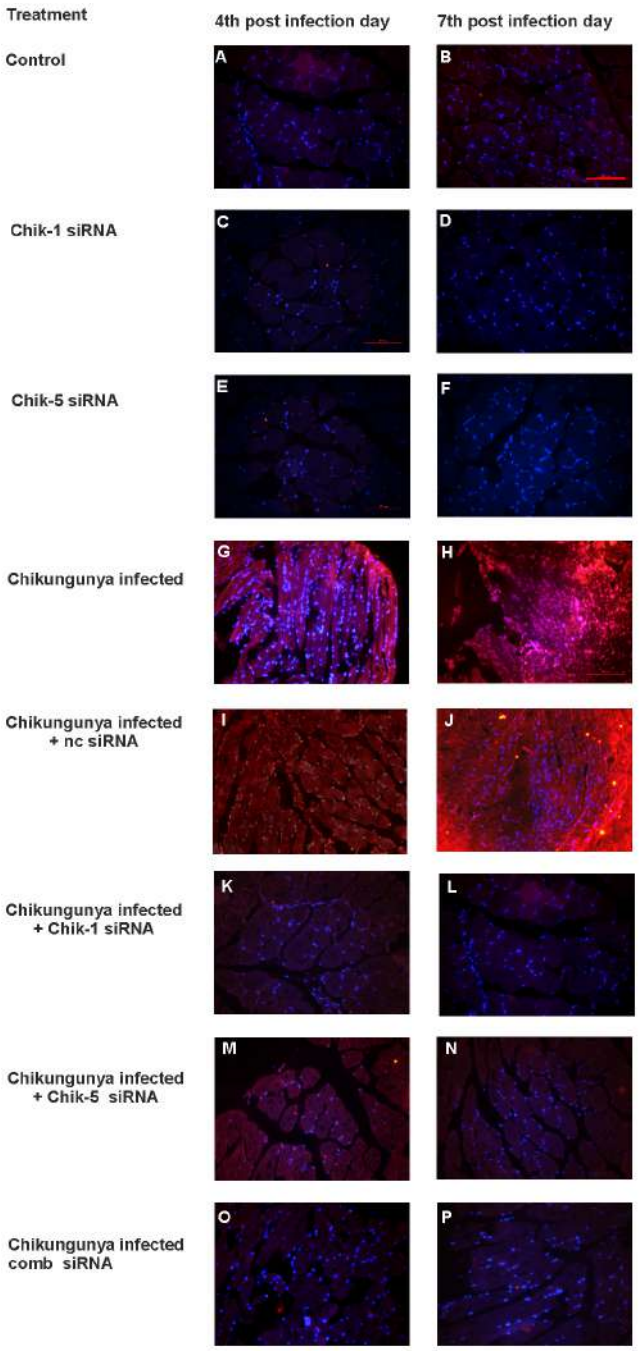

Figure 9. Detection of chikungunya in mouse muscle tissues using Immunofluorescence assay after chikungunya infection and siRNA treatment. C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). PBS, ncsiRNA, E2 siRNA and ns1 siRNA injected mice showed absence of chikungunya antigen at 4th and 7th days p.i. (**A**, **B**, **C**, **D**, **E** and **F**). CHIKV infected muscles showed presence of chikungunya antigen (**G** and **H**), ncsiRNA treated CHIKV infected muscles showed presence of chikungunya antigen (**I** and **J**) whereas siRNA treated CHIKV infected mice muscle tissues showed the faint staining of chikungunya antigen (**K**, **L**, **M**, **N**, **O** and **P**) (Magnification ×200).
doi:10.1371/journal.pntd.0002405.g009

tissues chikungunya infected mice showed mild inflammation of lymphocytes and monocytes. At 4 days p.i., chikungunya infected mice muscle tissues showed moderate inflammation of lymphocytes and monocytes, focal edema and focal necrosis whereas siRNA treated mice muscle tissues showed only mild inflammation. At 7 days p.i., extensive muscular necrosis with dense inflammation of lymphocytes and monocytes was observed in CHIKV infected and ncsiRNA treatment mice. On other hand, siRNA treatment preserved the morphological integrity of the muscle tissues with regeneration (Fig. 10). The muscle tissues from control mice infected with saline showed no pathological changes such as necrosis, edema, inflammation and infiltration of polymorphs (Fig. 10).

Expression levels of interferon genes after siRNA treatment

We tested if inhibition of CHIKV replication in mice was indeed sequence dependent and not because of non-specific antiviral interferon response. In the absence of CHIKV, Chik-1, Chik-5 and Comb-siRNA treatment did not significantly induced the α, β and γ interferon mRNA expression in hind limb muscle tissues (Table 3; Kruksal Wallis $p>0.05$). Similarly, siRNA treatment of CHIKV-infected mice did not show significant elevations in α, β and γ interferon gene expression in the hind limb muscle tissues (Table 3; Kruksal Wallis $p>0.05$). These results suggest that siRNA mediated reduction in CHIKV replication is sequence specific without any deleterious effect on the host.

Taken together, this first in vivo experiment clearly revealed that siRNA therapy is effective *in vivo* by reducing the clinical symptoms in the challenge-infected animals and was capable of significantly reducing virus replication in the serum and muscles.

Discussion

This study for the first time clearly shows the efficacy of ns1 and E2 siRNAs in combination, in inhibiting CHIKV replication in mice infected with the CHIKV (1×10^6 PFU CHIKV; 100 µl of 10^7pfu/ml, ANOVA Dunnet's test $p<0.01$). Importantly, a single i.v. inoculation of the siRNAs, 72 h after CHIKV infection could completely inhibit viral replication as evidenced by the absence of viral RNA in the muscles and serum. Though attractive, the therapeutic potential of siRNAs in treating viral diseases has been limited primarily because of the failure when evaluated in animal models and the absence of appropriate delivery systems. However, the success of *in-vivo* use of siRNAs in viral infection is noteworthy [17,23,24].

E2 and ns1 genes were chosen as the target genes because of the critical roles in viral replication. E2 and ns1 genes are highly conserved in CHIKV strains. CHIKV is expected to encode spikes on the virion surface, each formed by three E1–E2 heterodimers where the E1 glycoproteins mediate fusion and the E2 glycoproteins interact with the host receptor [26,27,28,30]. Together with nsP4, nsP1 is expected to catalyze the initiation of negative strand RNA synthesis. Nsp1 protein is also involved in methylation and capping of positive RNA [31]. Indeed, CHIKV nsp1, a 535 amino acid long protein contains consensus sequence at the N terminal region which is characteristic of Alphaviruses. Elimination of nsp1 abolishes the ability of CHIKV to replicate. We evaluated four different siRNAs each against ns1 and E2 genes when administered two h p.i. *in vitro*. Of these, Chik-1 (siRNA for E2 gene) and

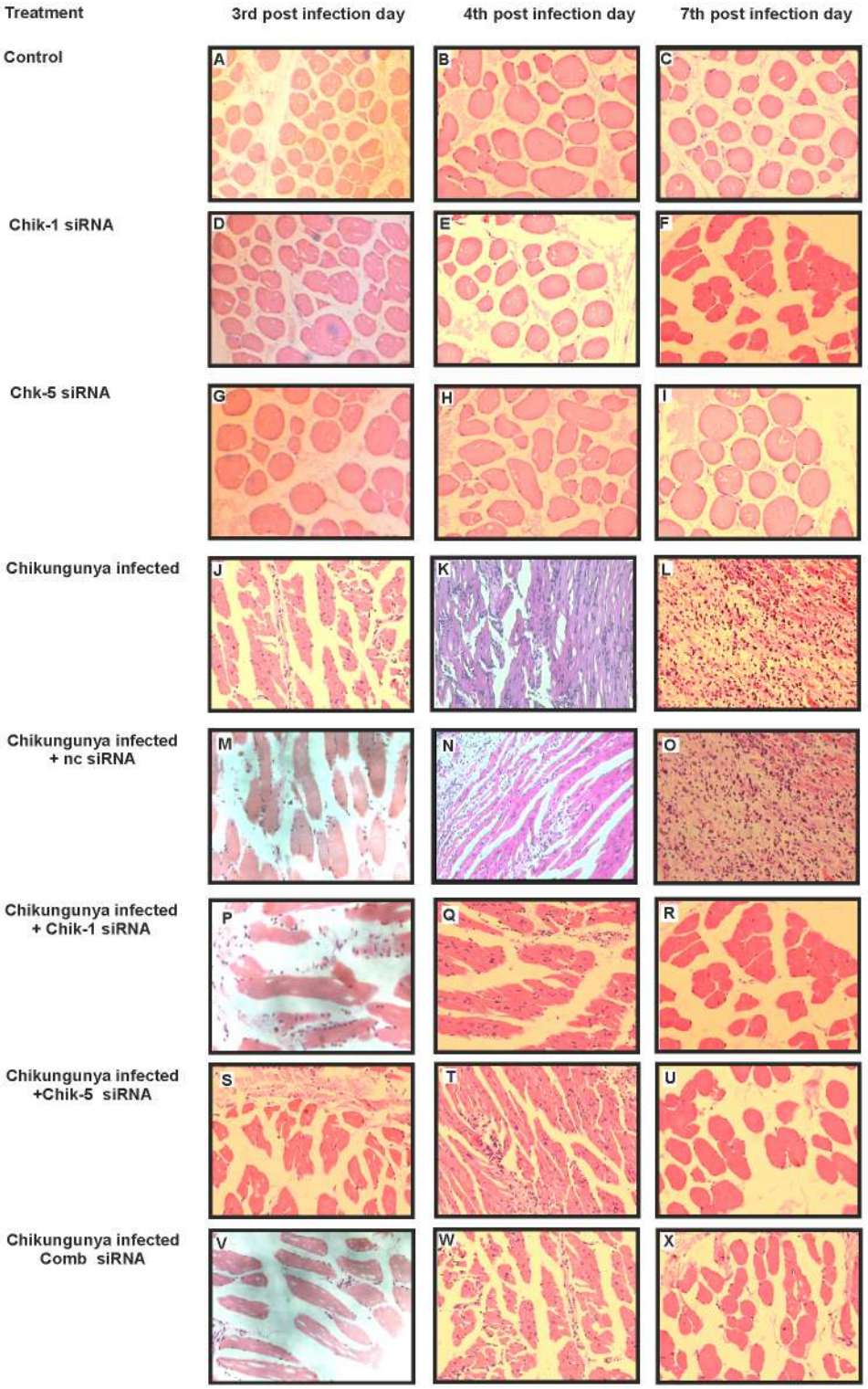

Figure 10. Histopathological changes in mouse muscle tissues after chikungunya infection and siRNA treatment. C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). Hematoxylin/eosin-stained tissue sections were screened to investigate the pathological effects of siRNA treatment. PBS injected mice showed normal cellular organization (**A**, **B** and **C**). No significant cellular changes were observed in E2 siRNA treated mice (**D**, **E** and **F**) and ns1 siRNA treated mice (**G**, **H** and **I**). At 3 days p.i. mild inflammation and mild monocyte/macrophage infiltrates were observed (**J**, **M**, **P**, **S** and **V**). At 4 days p.i. and 7 days p.i. CHIKV infected and ncsiRNA treated muscles showed pronounced monocyte/macrophage infiltrates, necrosis and edema (**K**, **L**, N and **O**), whereas siRNA treated CHIKV infected mice muscle tissues showed the regeneration after treatment (**Q**, **R**, **T**, **U**, **W** and **X**) (Magnification ×400).
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Table 3. Interferon response in hind limb muscle tissue of C57BL/6 mice after treatment with siRNAs.

Post infection period	Treatment			Relative fold change compared to control		
	Chikungunya infection	Chik-5 siRNA	Chik-1 siRNA	Interferon α	Interferon β	Interferon γ
				Mean (SD)	Mean (SD)	Mean (SD)
4 days p.i.						
(n = 3)	+			0.94 (0.88)	2.03 (1.31)	25.22 (21.12)
(n = 3)	+	+		2.83 (2.19)	1.81 (1.61)	8.25 (5.06)
(n = 3)	+		+	2.06 (1.77)	2.69 (2.21)	18.00 (16.80)
(n = 3)	+	+	+	1.21 (0.80)	3.99 (3.82)	4.55 (1.23)*
(n = 3)		+		1.54 (1.21)	5.18 (3.17)	1.09 (0.60)
(n = 3)			+	0.95 (0.66)	4.76 (3.23)	1.08 (0.64)
(n = 3)		+	+	2.19 (0.46)	6.00 (4.55)	7.09 (5.18)
5 days p.i.						
(n = 3)	+			0.11 (0.05)	0.41 (0.33)	371.73 (236.04)
(n = 3)	+	+		1.88 (1.48)	1.71 (1.54)	19.44 (11.93)
(n = 3)	+		+	4.65 (2.08)	4.74 (4.14)	44.38 (41.70)
(n = 3)	+	+	+	0.81 (0.30)	3.59 (3.49)	163.09 (65.36)*
(n = 3)		+		1.22 (0.53)	4.69 (1.06)	3.31 (1.91)
(n = 3)			+	0.60 (0.44)	6.54 (0.74)	0.97 (0.06)
(n = 3)		+	+	3.38 (2.63)	5.55 (4.75)	1.49 (2.00)
6 days p.i.						
(n = 3)	+			2.15 (2.05)	0.42 (0.27)	9.37 (5.87)
(n = 3)	+	+		2.50 (1.27)	3.61 (3.06)	6.06 (4.64)
(n = 3)	+		+	14.27 (9.47)	1.54 (1.35)	50.74 (42.74)
(n = 3)	+	+	+	5.15 (4.80)	4.85 (2.20)	344.23 (294.86)
(n = 3)		+		0.45 (0.10)	4.89 (3.59)	1.10 (0.74)
(n = 3)			+	0.62 (0.42)	5.71 (1.40)	0.68 (0.65)
(n = 3)		+	+	3.67 (3.47)	6.16 (5.59)	0.78 (0.64)
7 days p.i.						
(n = 3)	+			0.67 (0.56)	2.23 (1.90)	4071.74 (705.74)
(n = 3)	+	+		2.36 (1.98)	0.54 (0.05)	7.51 (1.50)
(n = 3)	+		+	18.10 (13.29)	6.44 (5.12)	2.15 (1.87)
(n = 3)	+	+	+	1.32 (1.31)	4.83 (0.33)	119.31 (91.42)
(n = 3)		+		0.58 (0.07)	2.67 (0.18)	1.13 (0.17)
(n = 3)			+	0.52 (0.02)	6.35 (3.68)	2.39 (0.28)
(n = 3)		+	+	2.17 (1.53)	5.74 (5.13)	27.38 (20.14)

Mice were treated with siRNA and gene expression changes in interferon alpha, beta and gamma was monitored in hind limb muscle tissues (n = 3 for each treatment) at 24 h, 48 h, 72 h and 96 h p.i.. Results expressed as $2^{-\Delta\Delta CT}$ were reported as mean ± standard deviation and were analyzed using Kruksal Wallis test.
*$p<0.05$ significantly different gene expression change as compared to chikungunya infected mice of respective time point.
doi:10.1371/journal.pntd.0002405.t003

Chik-5 (siRNA for ns1 gene) were most efficient in inhibiting CHIKV replication as assessed by multiple parameters such as real time PCR, plaque assay and IFA (Fig. 2, Fig. 3, Fig. 4, Table 2). The work described here utilizes targets against viral RNA sequences that are conserved, and invariant between different strains. The target sites of Chik-1 and Chik-5 siRNA are 100% conserved in all sequenced CHIKV genome. As compared to individual Chik-1 and Chik-5, the pool of Chik-1–4 and Chik5–8 siRNAs does not have any additive effect on the CHIKV inhibition. The combination therapy of Chik-1 and Chik-5 siRNAs provided additive effect and was found to be most effective as compared to individual siRNA in inhibiting CHIKV replication even at later time points. Thus, simultaneous silencing of multiple genes of CHIKV appeared to be better strategy for preventing viral replication. Recently Dash et al., [22] have used siRNA against ns3 and E1 genes of CHIKV and showed reduction in CHIKV replication by 65% by 48 h p.i. and not evaluated *in-vivo* [22]. *In vitro* studies employing Chik-1, Chik-5 and Comb-siRNAs showed more than 90% reduction in CHIKV replication with all three formulations, whereas complete inhibition of CHIKV replication was observed in *in vivo* studies. The observed efficient inhibition of CHIKV might be due to targeting of conserved sites, route of delivery or combination of both.

The next step was to evaluate these siRNAs in a suitable animal model. Recently C57BL/6 mice were established as susceptible murine models for CHIKV infection [32,33]. In the absence of an

immunocompetent mouse model replicating clinical symptoms in humans, we used Swiss albino and C57BL/6 adult mice for the evaluation of siRNAs. Though mice strains showed mild clinical symptoms (sluggishness and swelling of foot), replication of CHIKV was evident and the effect of siRNAs on CHIKV replication could be assessed employing several parameters (Fig. 5, Fig. 6, Fig. 7 and Fig. 8). SiRNAs were stable in Vero-E6 cells till 24 h post transfection (Fig. S1). We could not study the stability of siRNAs in mice because of low signal of cyanine 3 dye labeled siRNAs. However, it may be noted that previous studies have demonstrated uptake of siRNAs in the liver, kidney, lung endothelium and jejunum using standard i.v. injection of siRNA [34,35,36]. Despite the weaker siRNA uptake with standard intra venous administration, earlier reports suggest that this technique is still effective and may offer a potential route for systemic therapeutic use. Standard i.v. administration of siRNA efficiently delivered the siRNAs to various organs and resulted in efficient reduction of CHIKV titer.

Although chikungunya viremia has not been extensively studied in humans, studies on non human primates and mice suggest viremia of short duration, with viral loads ranging from 1×10^3 to 1.2×10^{10} viral RNA copies/ml [33,37,38,39,40]. It has been demonstrated that CHIKV can persist for longer time in animal models [37,40]. The findings of Morrison et al., [40] and Labadie et al., [37] indicate that though CHIKV is readily cleared from most tissues after the acute stage of infection, CHIKV RNA persists in joint tissues for at least 3–4 weeks after inoculation. In monkey model a clear relationship has been demonstrated between the inoculation dose and the period and magnitude of the viremia [37]. In current study we used the high dose of CHIKV (1×10^6 PFU) which might resulted in longer persistence of CHIKV RNA (7–8 days post inoculation). The results obtained in this study are consistent with findings of Morrison et al., [40] and Labadie et al., [37]. It has been demonstrated that CHIKV actively suppresses STAT activation by both type I and type II interferon [37,41]. In current study moderate suppression in Interferon α and Interferon β was observed in CHIKV infected mice. It is possible that CHIKV persistence observed in current study might be the combined effect of high dose of CHIKV, route of inoculation, active evasion of host innate or adaptive immune responses by the CHIKV. It will be interesting to evaluate the mechanisms involved in CHIKV persistence in C57BL/6 mice.

Similar to *in-vitro* studies, Comb-siRNA was more efficient than the individual components *in-vivo* studies also. Important point is that the siRNA was administered 48 or 72 h p.i. suggesting utility in CHIKV-infected hosts. CHIKV was detected in muscle tissues of infected mice inducing pathological changes such as severe necrosis and dense infiltration of monocytes and lymphocytes (Fig. 9 and 10). On the contrary, ~1 day after siRNA treatment, mild inflammation and infiltration of monocytes was observed while after ~4 days, regeneration and intact muscle morphology with no evidence of inflammation was recorded (Fig. 10). These results clearly demonstrate the therapeutic effect of siRNAs, especially Comb-siRNA, in virus-infected mice. Even a single dose administered 3 days p.i. could protect mice suggesting ability of the siRNAs in treating an established virus infection.

Under certain circumstances, siRNAs can induce the interferon (IFN) pathway and trigger inflammation [42,43,44]. It has been suggested that canonical small interfering RNA (siRNA) duplexes are potent activators of the mammalian innate immune system [42,43,44]. Synthetic siRNA in delivery vehicles that facilitate cellular uptake can induce high levels of inflammatory cytokines and interferons after systemic administration in mammals and in primary human blood cell cultures [42,43]. To differentiate the modes of protection offered by siRNAs, we determined expression levels of interferon α, β and γ interferon genes in the muscle tissues of different mice groups (Table 3). siRNAs alone did not induce significant induction of interferon genes; CHIKV-infected, siRNA treated mice did not show siRNA-induced interferon gene expression when compared to the virus infected mice. These results revealed that the observed inhibition of CHIKV replication was mainly because of characteristic activity of siRNAs.

In conclusion, Comb-siRNAs (E2 and ns1 genes) described by us provide an excellent therapeutic agent for chikungunya and should be further assessed in non-human primates. Need for a proper delivery system for use in humans remains an important issue.

Supporting Information

Figure S1 Stability of siRNAs. Cyanine 3 dye labelled Chik-1 and Chik-5 were transfected in Vero-E6 cells using Hiperfect reagent (Qiagen, Germany). After 4 h and 24 h cyanine 3 dye fluorescence signal was detected using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software).
(DOC)

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Author Contributions

Conceived and designed the experiments: DP MSP VAA. Performed the experiments: DP MSP SK MDG ABS SBS. Analyzed the data: MSP. Contributed reagents/materials/analysis tools: DP MSP SK MDG ABS SBS VAA. Wrote the paper: DP MSP VAA.

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Effect of Cationic Lipid Nanoparticle Loaded siRNA with Stearylamine against Chikungunya Virus

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Abstract: Chikungunya is an infectious disease caused by mosquito-transmitted chikungunya virus (CHIKV). It was reported that NS1 and E2 siRNAs administration demonstrated CHIKV inhibition in in vitro as well as in vivo systems. Cationic lipids are promising for designing safe non-viral vectors and are beneficial in treating chikungunya. In this study, nanodelivery systems (hybrid polymeric/solid lipid nanoparticles) using cationic lipids (stearylamine, C9 lipid, and dioctadecylamine) and polymers (branched PEI-g-PEG -PEG) were prepared, characterized, and complexed with siRNA. The four developed delivery systems (F1, F2, F3, and F4) were assessed for stability and potential toxicities against CHIKV. In comparison to the other nanodelivery systems, F4 containing stearylamine (Octadecylamine; ODA), with an induced optimum cationic charge of 45.7 mV in the range of 152.1 nm, allowed maximum siRNA complexation, better stability, and higher transfection, with strong inhibition against the E2 and NS1 genes of CHIKV. The study concludes that cationic lipid-like ODA with ease of synthesis and characterization showed maximum complexation by structural condensation of siRNA owing to high transfection alone. Synergistic inhibition of CHIKV along with siRNA was demonstrated in both in vitro and in vivo models. Therefore, ODA-based cationic lipid nanoparticles can be explored as safe, potent, and efficient nonviral vectors overcoming siRNA in vivo complexities against chikungunya.

Keywords: chikungunya; non-viral vectors; cationic lipids; siRNA; nanodelivery systems; stearylamine

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1. Introduction

Chikungunya (CHIK) is a mosquito-borne disease caused by chikungunya virus (CHIKV). The resurgence of CHIK in the Indian Ocean Islands and India drew worldwide attention in 2005–2006 due to its explosive nature [1]. CHIKV is a positive-sense single-stranded RNA virus that is 11.8 kb long, belonging to the alphavirus genus and the Togaviridae family. Chikungunya fever is usually associated with headache, joint pain, rash, and arthralgia. Humans have become a reservoir of CHIKV for mosquitoes [2]. As per the National Vector-Borne Disease Control Program (NVBDCP), the epidemiology profile of chikungunya fever in India since its reemergence in 2005–2006 is alarming [3]. The majority of the initiatives to combat this disease are preventive rather than therapeutic. This is largely due to the lack of specific prophylactic or effective therapeutics available in the market. There is a strong urge to addresses this unmet need [4].

Gene therapy has gained enormous attention as a novel therapeutic approach for diversified pathologies including neoplastic, genetic, and infectious diseases. Therapy includes a functional procedure to treat or alleviate disease by genetically amending the cell of the patient [5]. RNA interference (RNAi) regulates both gene expression and cellular-defense mechanisms against viruses, especially in mammalian cells. Small interfering RNAs (siRNA) are pivotal in RNAi, a process of sequence-specific gene silencing without activating an interferon response in animal cells. This discovery has opened up newer technologies for molecular targeting of various cancer and viral infections [6]. Earlier, we have designed, screened, and evaluated the therapeutic potential of two promising siRNAs (NS1 and E2) against CHIKV in a mouse model [7]. However, the siRNA degradation by endogenous nucleases minimizes their half-life, and the smaller size aids rapid clearance by the kidney, limiting their in vivo efficacy [8]. Thus, there is a need for a stable, inert system that can efficiently encapsulate and transfect into the cell, allowing an effective siRNA-induced knockdown with minimal degradation or rapid in vivo clearance. Designing a stealth deliverables system to bypass the reticuloendothelial system for siRNA is quite promising for better targeting, sustained-release minimizing the dosage regimen, and unwanted cytotoxicity [9].

An efficient delivery system is essential for effective gene therapy. Both viral- and non-viral-based vectors have been evaluated to date [10]. Viral vectors are commonly used because of high transfection efficiency, but due to their low carrying capacity, immunogenicity, limited specificity, and unwanted cytotoxicity, their use has been limited [11]. The development of nucleic-acid-based therapy for chikungunya has been hampered due to methodological delivery limitations and undesirable side effects. These restrictions can be addressed with advances in the nanotechnology fields with the development of a nanoparticle-based siRNA complex as a non-viral gene delivery system. High biocompatibility, low cytotoxicity, and cost favor their use in comparison to viral vectors [12,13].

One of the best designated and optimized delivery systems among non-viral vectors for gene therapy are cationic solid lipid nanoparticles (SLN) [14]. SLN are easily fabricated using biocompatible materials, owing to their safety with better nucleic-acid encapsulation, stability, and cellular uptake [15]. The ease of surface trimming of SLN for site-specific targeting and extended blood circulation time makes them an ideal choice for gene delivery [16]. Presently, there is one FDA-approved, siRNA-based therapy for treatment of hATTR amyloidosis [17].

Cationic lipids (amphiphilic molecules) have gained significance in recent years due to their ease of synthesis, are extensively characterized, and can enable the elucidation of structure–activity relationships by functional domains' modifications [18]. The polar head group of the cationic lipid enables the condensation of nucleic acid by electrostatic interactions with the phosphate groups of genes having a negative charge and further regulates the transfection efficiency [19]. Cationic lipids are categorized based on the nature and charged density of hydrophilic head groups. Slight modifications in the head groups can help to bypass varied in vitro and in vivo transfection barriers [20]. Stearylamine/octadecylamine (ODA) is a cationic lipid that has not yet been fully explored for its intrinsic properties and for its role in formulating drug or gene deliverables [21]. It was reported for the uniform distribution of cationic charge in and around the nanocarrier formulations like liposome even at the transmembrane pH gradient [22]. The nitrogen hydrophilic head group of ODA has a strong potential to become cytotoxic by interacting with critical enzymes like PKC along with good transfection [23]. It has even been reported for its anti-helminthic property [24], and its activities can be altered by other neutral or helper lipids that can be beneficial [25]. Therefore, we utilized ODA to induce a cationic charge in lipid nanoparticles for better complexation and transfection of siRNA.

In the present study, cationic charged systems for the effective complexation and delivery of siRNA against CHIKV using various lipids were developed, which was not reported earlier. The four formulated delivery systems were characterized and evaluated in the in vitro system for their potential in catering two different siRNAs, i.e., CHIK1 and

CHIK 5 (designed against the ns1 and the E2 gene region) alone or in mixed form to inhibit the CHIKV replication. The optimized siRNA-based delivery system was further assessed for its safety, stability, and efficacy. Among the four different delivery systems developed, we inform about utilizing and evaluating the synergistic or antagonistic or additive effect of ODA in formulating delivery system against CHIKV for the good transfection of siRNA, with retained functionality.

2. Results

2.1. Formulation Development and Optimization of Delivery Systems

Different formulations were prepared using various cationic lipids to evaluate and rationalize an optimum delivery system. F1, F2, and F3 delivery systems prepared using cationic polymers indicated low complexation with siRNA in comparison to F4. Octadecylamine used in the preparation of F4 delivery systems indicated the maximum entrapment of siRNA. Additionally, in our previous studies, pure ODA (8 mg) alone has indicated strong inhibition of CHIKV in vitro and in vivo. Therefore, we anticipated that the delivery system prepared with ODA would exhibit a strong inhibition of CHIKV, and it was further optimized.

2.2. Particle Size, Polydispersity, and Zeta-Potential Determination

The average nanoparticle size, the polydispersity index (PDI), and the surface charges of the F1, F2, F3, and F4 formulations were determined and recorded (Figure 1). Among the cationic lipids used, the F4 formulation indicated a maximum cationic charge density (45.7 ± 4.2) in comparison to F1, F2, and F3, which had weak cationic charges. F4 indicated an average size (152.4 ± 0.36) and polydispersity of nanoparticles (<0.5) in comparison to other delivery systems. All formulations were filtered through a 0.45 μM filter before performing further studies, i.e., an MTT assay and conjugation with siRNA.

2.3. Evaluation of Optimal Lipid to siRNA Complexation Ratio for F4

As a part of the F4-formulation optimization and in order to assess the critical lipid concentration for maximum complexation of siRNA, the role of change in both particle size and zeta potential depending on the N/P ratio was determined (Figure 2). The experiments revealed that the size of the lipid nanoparticles increased with siRNA complexation, and the surface charge varied depending upon the lipid concentration used. However, our studies revealed that increasing the cationic lipid concentration did not exhibit a logarithmic increase in cationic charge density over nanoparticles. For example, for an increase from 2 to 8 mg of ODA, we observed a maximum of 45.7 ± 4.2 mV for the surface charge, and a further increase in the ODA concentration to 10 mg did not increase the cationic charge proportionally. With the direct-method complexation of cationic lipid with siRNA, the particle size was seen at a maximum at an N/P ratio of 6, and the surface charge increased from 20 to 45 mV as the N/P ratio increased from 1 to 8.

2.4. In Vitro Release Studies

The siRNA release was performed in two different pH conditions (pH 7.4 and 6.4). The results indicated that at the neutral condition the percent release of siRNA was higher in comparison to the acidic condition. There was a burst effect observed in the acidic condition that was minimized indicating a prolonged release in neutral conditions (Figure 3).

2.5. In Vitro Cytotoxicity Studies

The effect of different delivery systems F1, F2, F3, and F4 on the Vero cells in the culture was studied using an MTT assay. Based on these findings, the developed delivery systems F3 and F4, having a CC-50 value >100 μg/mL, were considered non-toxic to Vero cells, and both were further selected for preliminary screening for an optimal and a suitable delivery vehicle for siRNA in treating CHIK infection (Figure 4).

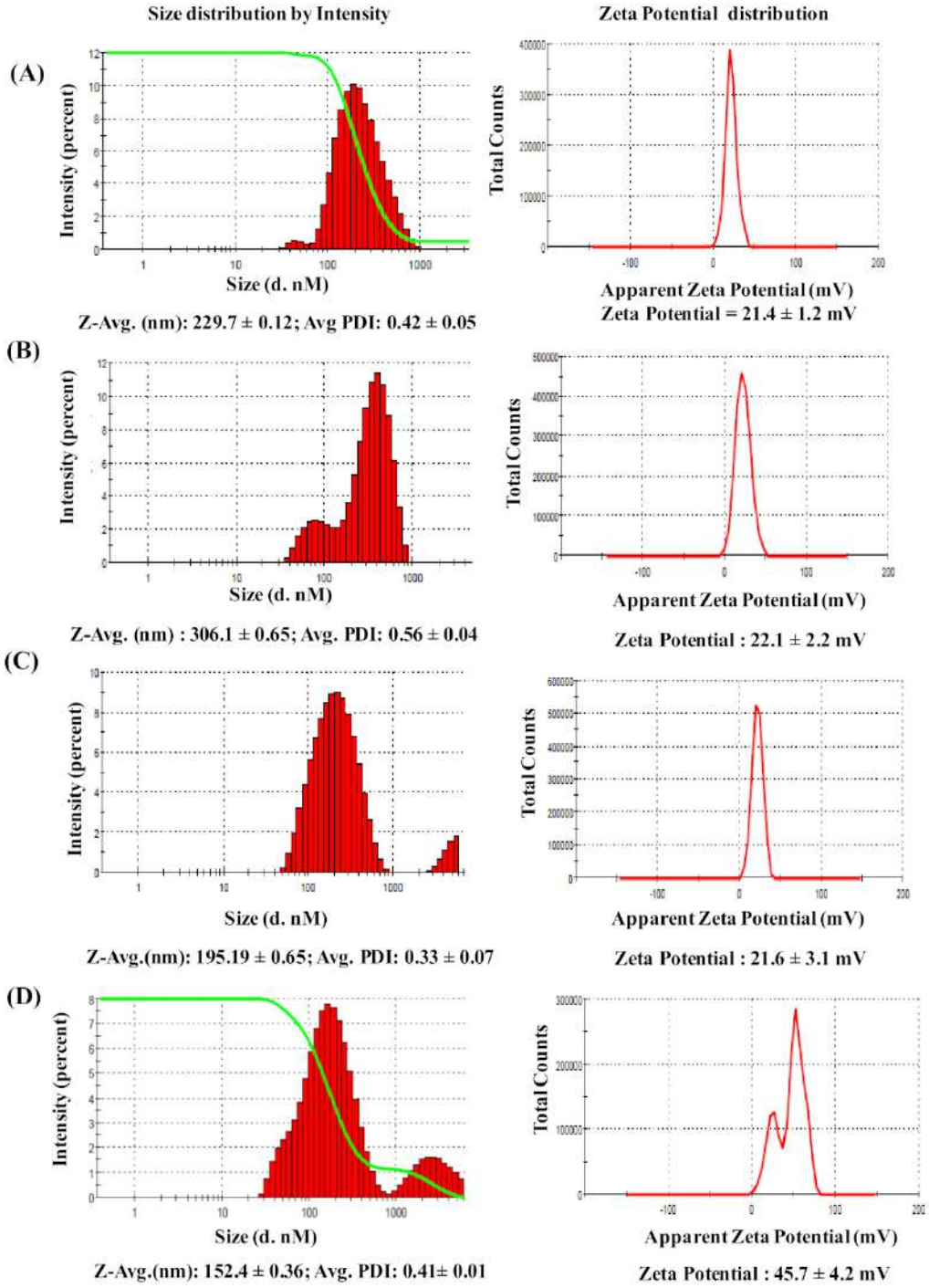

Figure 1. Dynamic light scattering (DLS) particle-size distributionsby intensity of experiments and Zeta potential of finally optimized (**A**) F1, (**B**) F2, (**C**) F3, and (**D**) F4 formulations measured by a Malvern Zetasizer Nano ZS. The average particle size in nm and polydispersity index (PDI) and Zeta potential in mV were shown for each formulation. All experiments were performed in triplicates; values are expressed as mean ± SEM. Besides, F1, F3 and F4 nanoparticles showed well polydispersity with PDI < 0.5. Zeta-potential analysis showed that F4 exhibited an indicated maximum cationic charge density (45.7 ± 4.2) in comparison to other formulations with average size (152.4 ± 0.36), which was better in comparison to all other formulations. It is also reported that nanoparticles with a Zeta potential above (+/−) 30 mV are stable in suspension.

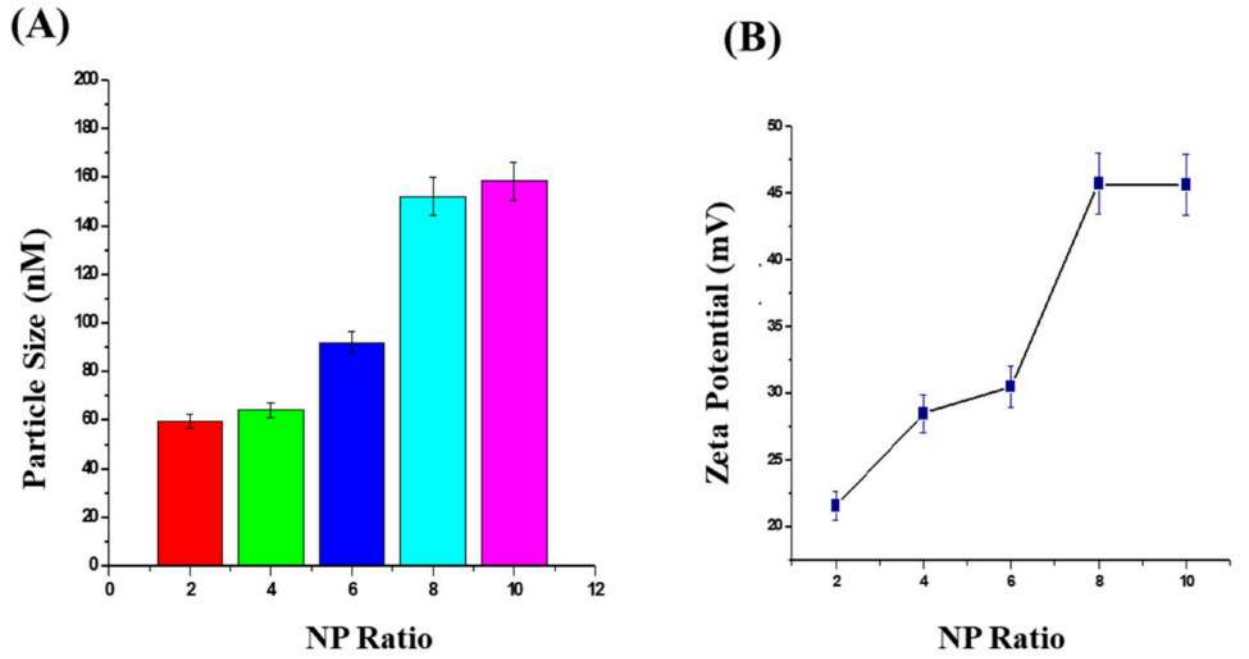

Figure 2. Representation of the trend of solid lipid nanoparticles particle size (nm) (**A**) and Zeta potential with N/P ratio at fixed siRNA concentration (**B**). All the values are expressed as mean ± SEM (n = 3).

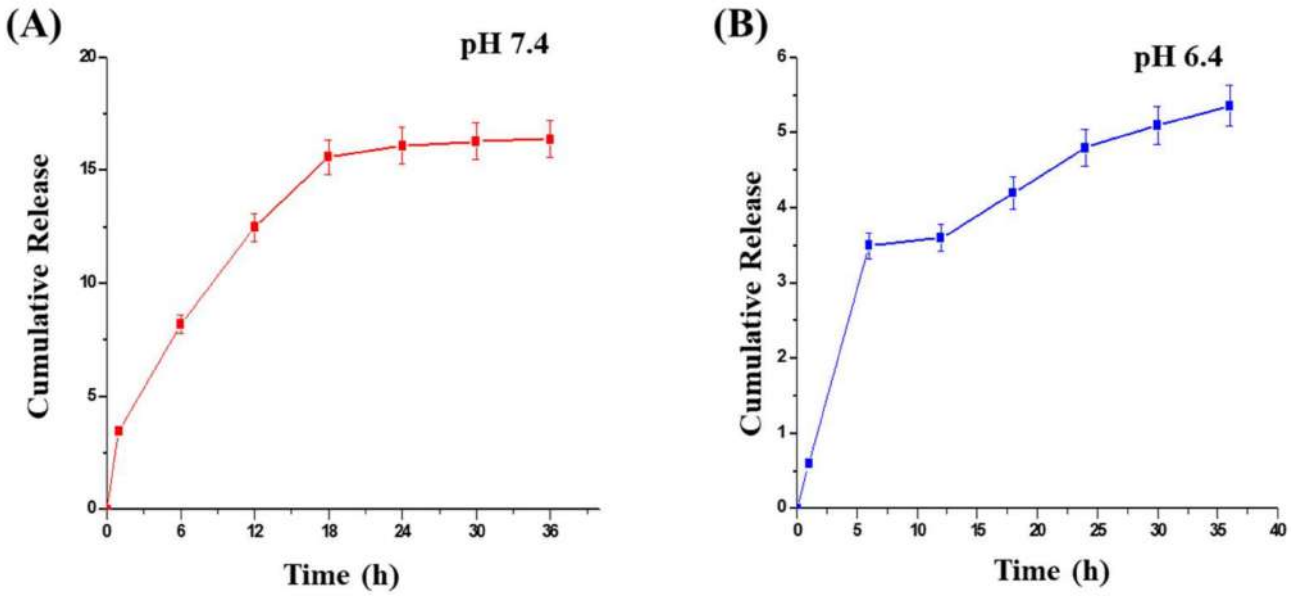

Figure 3. In vitro release profile of F4 formulation at different pH: (**A**) pH 7.4 and (**B**) pH 6.4; all the values are expressed as mean ± SEM (n = 3).

2.6. Optimization of siRNA Effective Concentration

To determine the optimal antiviral concentration of siRNA, the Vero cells were infected with the CHIKV and after that transfected with different concentrations of siRNA Chik-1 ranging from 200 pmol to 2000 pmol using lipofectamine reagent to the culture medium 1 h after infection and incubation were done for 24 h. The culture supernatant was evaluated for viral genomic RNA levels using real-time RT-PCR. The results show that rgw concentration equal to or greater than 400 pmol concentration showed a significant reduction in viral load (Figure 5A), so we selected the lowest conc. i.e., 400 pmol.

2.7. Transfection Efficiency Comparison between F3 and F4

siRNA (Chik1 concentration 400 pmol) loaded with F3 and F4 delivery systems was transfected to Vero cells after CHIKV infection. No significant decrease in viral load was observed in Chik-1 loaded with the F3-delivery-system-treated cells compared to viral control cells (Figure 5B). The Chik-1-loaded F4 delivery system showed a significant decrease in viral load compared to VC- ($p < 0.001$) and F3 + Chik1- ($p < 0.01$) treated cells. F4 + chik1-treated cells showed a significant decrease in viral load; hence, the F4 delivery system was selected over F3, for further studies (Figure 5B).

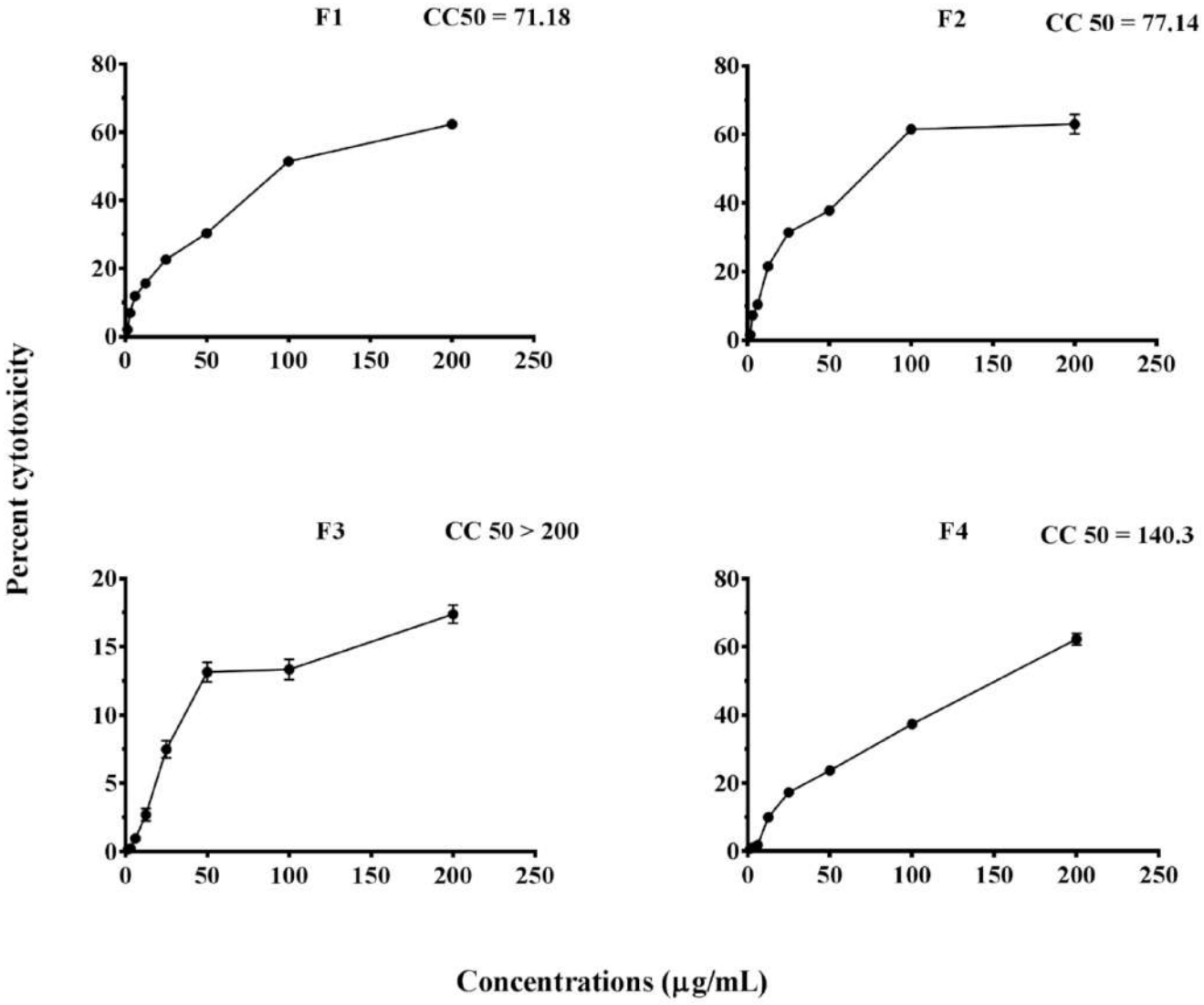

Figure 4. Cell cytotoxicity of different formulations against Vero E6 cells at various concentrations using an MTT assay. All the values are expressed as mean ± SEM ($n = 3$).

2.8. Determination of siRNA Complex with F4 SLN Using Agarose Gel Electrophoresis

As depicted in Supplementary Figure S1, the siRNA movement was retarded in the ODA-loaded F4 siRNA delivery system in comparison to the naked siRNA at 4 °C and 37 °C temperatures. Therefore, the studies strongly indicate that ODA formed a stable complex and resulted in strong complexation with siRNA.

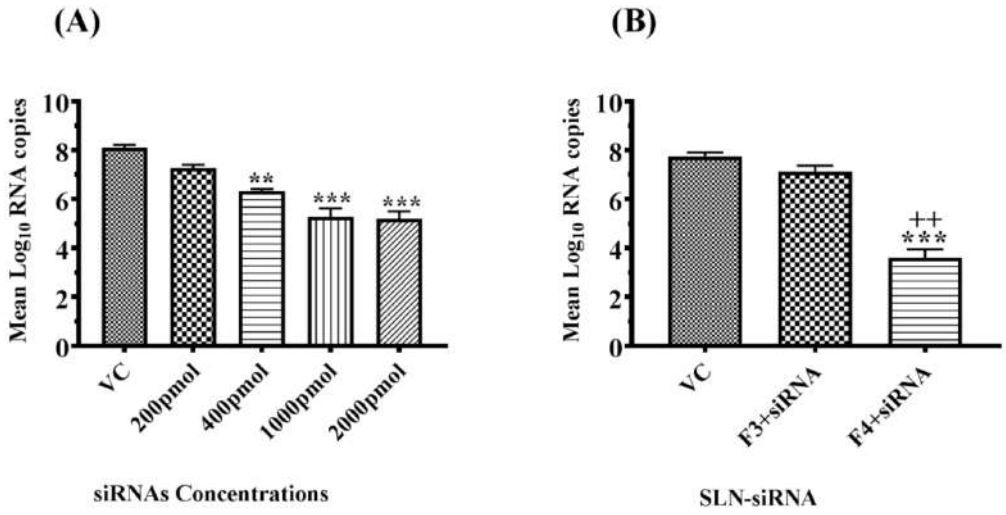

Figure 5. (**A**) Effect of transfection of different siRNA concentrations using lipofectamine reagent against CHIKV at 24 h treatment using qRT-PCR analysis, (**B**) Transfection efficiency comparison between F-3 and F4 RT-PCR results. All the values are expressed as mean ± SEM (n = 3); ** $p < 0.01$, *** $p < 0.001$ vs. VC, virus control group; ++ $p < 0.01$ vs. F3 + SiRNA treated group.

2.9. In Vitro Anti-CHIKV Activity of the siRNA-loaded F4 SLN Delivery System

siRNA Chik 1 and Chik-5, at individual and combination levels (400 pmol), were transfected using lipofectamine reagent and incubated for 24 h and 48 h post-treatment. In a similar way, siRNAs were loaded with the F4 SLN delivery system and incubated with CHIKV-infected Vero cells for 24 h and 48 h. The RT-PCR results showed that the siRNA complexed with the F4 delivery system significantly decreased the viral load at both time points; in the case of the Chik 1 and Chik 5 combination delivered with F4, the viral-load inhibition was greater when compared with that transfected with lipofectamine (Figure 6A,B). A decreased viral load at 24 h by the treatment of Chik 1 + F4 SLN was significantly ($p < 0.01$) greater compared to Chik 1-treated cells; similarly, Chik1 + Chik 5 + F4 was significantly ($p < 0.001$) more effective when compared to Chik 1 + Chik 5-treated cells (Figure 6A). Figure 6B showsthat all siRNA + F4-treated cells showed more significant ($p < 0.001$) inhibition of viral load compared to their respective naked siRNA-treated cells. The FFU assay also showed a 100% reduction using siRNA Chik1 and Chik5 loaded with the F4 SLN delivery system at 24 h and 48 h (Figure 6C,D) These results were also confirmed with immunofluorescence assay, and even the percentage of infected cells also showed the same effect (Figure 6E,F).

2.10. In Vivo Anti-CHIKV Activity of siRNA-loaded F4 SLN Delivery System

To investigate the anti-CHIKV activities of the siRNA-loaded F4 delivery system, in vivo female C57BL/6 mice (4–5 weeks) were used. The mice were intramuscularly (I.M.) inoculated with 100 µL CHIKV, i.e., 10^7 pfu on day 0. siRNA Chik 1, Chik 5, and Chik1 + Chik 5 of both were incubated with an F4 delivery system and administered intravenously on the 3rd day. Blood collection was done on days 3, 5, and 7 for viremia analysis. Muscle tissue was collected from the hind feet of mice, and histopathology and RT-PCR analysis was done at different time points. Treatment with siRNA complexed with F4 SLN exhibited a significant reduction in the serum viral load as evaluated at days 5 and 7 using real-time RT-PCR. The Chik-1 and Chik-5 siRNA combination delivered with F4 showed a ~99% reduction in viremia compared to the control group at days 5 and 7 in both serum (Figure 7A) and muscle tissue (Figure 7B). Histopathology results (Figure 8) also indicated that treatment of the siRNA F4 complex reduced the inflammatory cell infiltration, atrophy, and muscular necrosis from day 5 onwards.

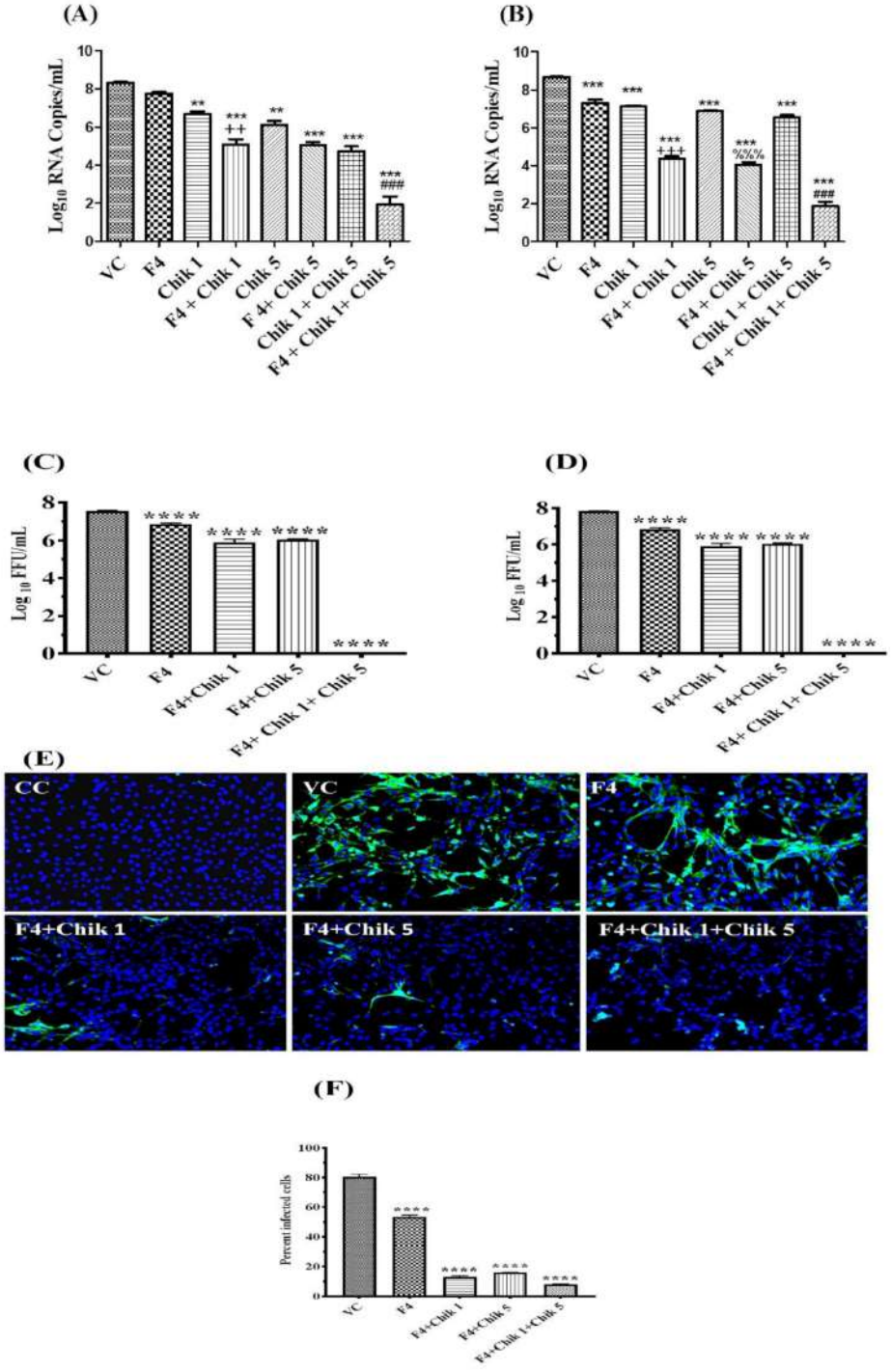

Figure 6. Effect of siRNA treatment against CHIKV replication. Naked siRNA (transfected with lipofectamine) and siRNA conjugated with F4 SLN effect at 24 h (**A**) and 48 h (**B**) using qRT-PCR analysis; siRNA conjugated with F4 SLN formulation using focus forming units (FFU) assay at 24 h (**C**) and 48 h (**D**); immunofluorescence assay (24 h treatment); (**E**) and percentage of infected cells (**F**).All values are given as mean log10 RNA copies/well ± SEM; **** $p < 0.0001$,*** $p < 0.001$, ** $p < 0.01$ vs. VC, virus control. ++ $p < 0.01$, +++ $p < 0.001$ vs. Chik 1, $^{\%\%\%}$ $p < 0.001$ vs. Chik 5, $^{\#\#\#}$ $p < 0.001$ vs. Chik 1 + Chik 5. All the siRNA combination conjugated with F4 SLN showed significant ($p < 0.001$) reduction to their respective treatment of naked siRNA with lipofectamine at 48 h; it indicated the superiority of the F4 SLN delivery system over transfection with lipofectamine.

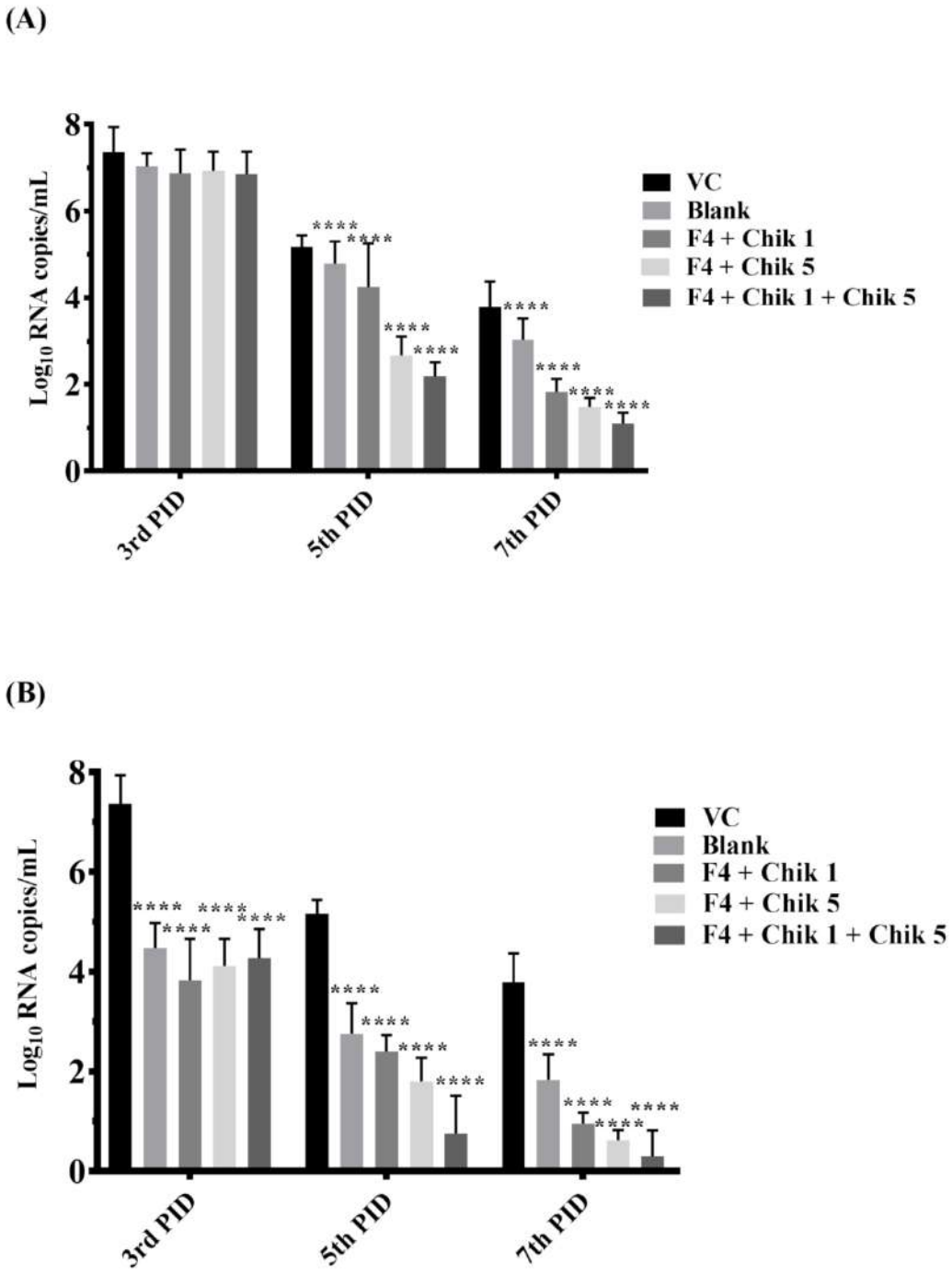

Figure 7. In vivo anti-CHIKV activity of siRNA-complexed SLN delivery system. The reduction in CHIKV copies/mL in (**A**) serum and (**B**) muscle tissue after treatment with the siRNA-loaded F4 delivery system. C57BL/6 mice (n = 9) were infected with CHIKV intramuscular route (100 μL of 10^7 pfu/mL). After 72 h p.i. (3rd day), mice were treated with the siRNA-loaded F4 delivery system by intravenous route with different treatment combinations. Viral RNA copies were checked in serum and muscle tissue at different time intervals. CHIKV E3 RNA was quantitated using real-time RT-PCR. Values are given as LOG10 RNA copies/mL serum and LOG10 RNA copies/mg of tissue. **** $p < 0.0001$ VC, virus control.

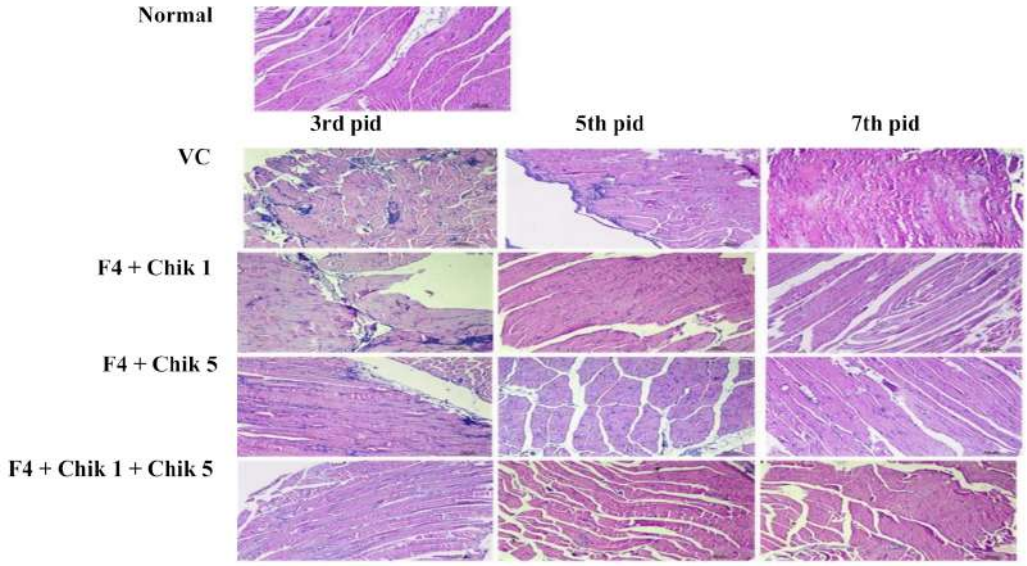

Figure 8. Histopathological changes in mouse muscle tissues after chikungunya infection and siRNA-loaded F4-delivery-system treatment.

3. Discussion

There is no specific antiviral drug treatment for chikungunya, and it is still a challenge on the clinical front. siRNA are ideal chemically synthesized drug candidates and can directly work on a target gene in a sequence-dependent manner. Since siRNAs are polyvalent anionic and highly hydrophilic mid-sized molecules, delivery of these molecules into cells is very difficult. They are easily degraded by nucleases in the blood, resulting in poor accumulation of siRNA in a target tissue. Hence, it is crucial to find a proper drug-delivery system for the development of siRNA-based drugs. Lipid-based nanoparticles are a suitable carrier for drug and nucleic-acid delivery. They have low toxicity and immunogenicity and excellent biocompatibility, biodegradability, structural flexibility, and ease of large-scale preparation. Several lipid-based formulations have been permitted and are being used around the world for several disease treatments. In this study, we prepared four different formulations using various cationic lipids to evaluate and rationalize an optimum delivery system that can show maximum siRNA complexation with a sufficient cationic surface charge. Previously, we reported that ODA itself has the potential to inhibit the CHIKV [26]. In the case of four formulations, earlier reports have indicated nanoparticles of PLGA-polyethylene glycol (PLGAPEG), with a strong potential to cross the blood–brain barrier and used to cater drug payloads [27,28]. It is highly uncertain that siRNA can cross the cell membrane due to its negative charge. However, a few reports indicate that nanoparticles facilitate siRNA internalization via endocytosis and as reasonable vectors for siRNA payloads owing great protection to the siRNA before the delivery of siRNA to the target [29]. Cationic lipids used can lockdown the negatively charged siRNA molecules inside the nanoparticles through electrostatic interactions.

Four delivery systems were evaluated for their toxicity, and F3 and F4, having a CC-50 value >100 μg/mL, were selected for further comparison of their in vitro CHIKV inhibition potential. Among F3 and F4 delivery systems, F3 indicated weak cationic charges and did not demonstrate high complexation with siRNA; therefore, it did not effectively downregulate the target genes in vitro. Further agarose-gel-electrophoresis results showed retardation of siRNA movement by siRNA-F4 SLN compared to naked SiRNA, which indicated the strength of complexes by exhibiting stable complexation. Hence, the F4 delivery system was selected for siRNA and further evaluation of their in vitro and in vivo anti-CHIKV potential.

Formulation F4 prepared using ODA acted as a cationic charge inducer in the protonated form and strongly interacted with the negatively charged siRNA, thereby facilitating

the self-assembly of the delivery-system components. Earlier reports of octadecylamine-based liposomes indicated strong inhibition (80%) against baculovirus (BV) in a dose-dependent manner. The binding of ODA liposomes to the cell membranes was high and was not cytotoxic to normal cells. Aditionally, ODA liposomes indicated good antiviral effects on herpes simplex virus type 1 in A549 cells in comparison to acyclovir [30]. In another study, Chitosan oligosaccharide-SS-Octadecylamine (CSSO), a redox-responsive nano-sized polymeric carrier was developed that can entrap DrzBC and DrzBS (10–23 DNAzyme), and it could block the expression of HBV e- and s- genes, respectively. In comparison to Lipo2000, the polymeric carrier can be as efficient as anti-hepatitis B virus gene therapy [31]. Therefore, the combinatorial effects of ODA with siRNA cationic nanoparticles showed enhanced growth inhibition of CHIKV suggesting their potential advantages in clinical settings.

From ODA, we observed a maximum of 45.7 $\pm$ 4.2 mV for the surface charge, and a further increase in ODA concentration did not increase the charge proportionally. With the direct-method complexation of cationic lipids with siRNA, the particle size was observed at a maximum at an N/P ratio of 6, and the surface charge increased from 20 to 45 mV as the N/P ratio increased from 1 to 8. The results were in good correlation with previously reported data [32–34]. The in vitro results were in a similar trend to those earlier reported for anti-miR-191 delivery using ODA-based liposomes [35]. Earlier reports indicated that ODA-based nanoparticles aided better stability, minimizing the leakage of entrapped drugs with a controlled release [36]. Studies even found that ODA-based lipid systems have greatly enhanced the delivery and bioavailability of several anticancer drugs [37–39]. Thus, we can affirm that ODA-based nanolipid formulation exhibits a combinatorial nature by CHIKV inhibition at a higher concentration aiding for effective transfection of siRNA at a lower concentration.

In vitro study results indicated more significant inhibition of viral load by siRNA loaded with the F4 SLN delivery system in Vero cells compared to the inhibition shown by naked siRNA transfected by the use of lipofectamine. In vivo anti-CHIKV activity of the siRNA-loaded F4 SLN delivery system was further evaluated in the in vivo system using C57BL/6 mice. We evaluated serum and skeleton muscle of infected feet for detection of the presence of CHIKV, which corresponds to the CHIKV infection in humans. Treatment with siRNA complexed with F4 SLN exhibited a significant reduction in the serum viral load as evaluated at days 5 and 7 by real-time PCR. The combination of Chik-1 and Chik 5 delivered with F4 showed a ~99% reduction in viremia compared to the virus control group at days 5 and 7 in both serum and muscle tissue. The results were further validated by histopathology, which found that treatment of the siRNA F4 complex reduced the inflammatory cell infiltration, atrophy, and muscular necrosis from day 5 onwards.

Our results indicate that siRNA complexed with the F4 delivery system inhibited the viral replication in infected Vero cells as well as decreased the viral burden and helped ameliorate acute disease symptoms in CHIKV-infected mice.

The study concludes that cationic lipids, such as octadecylamine with ease of synthesis and characterization, indicated maximum complexation by structural condensation of siRNA owing to high transfection alone and synergistic inhibition of CHIKV along with siRNA in both in vitroand in vivo models. Therefore, octadecylamine-based cationic lipid nanoparticles can be embraced and explored as safe, potent, and efficient nonviral vectors overcoming siRNA in vivo complexities against chikungunya. In the future, nanoparticles containing the siRNA approach can be used in developing a delivery system for the treatment of other viral disease treatments.

4. Materials and Methods

4.1. Materials

Octadecylamine (stearylamine),C9 lipid (composition from Dr. Surendar Reddy, IICT), dioctadecylamine, branched PEI-g-PEG -PEG Mn 5000; 18:1 (Δ9-Cis) PC–1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine

(DPPC), Resomer® R 202 S, poly (D, L-lactide) (PLGA 50:50), dimethyldioctadecylammonium bromide (DDAB), polyvinyl alcohol (85–90% hydrolysed) (PVA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt), powder (18:00 PEG 2000 PE), chloroform (DSEP PEG 2000), glyceryl-monostearate (GMS), Tween 80, cholesterol, lecithin, dicholoromethane (DCM), and chloroform from Sigma Aldrich (St. Louis, MO, USA) were purchased.

4.2. Vero Cells and Virus Strains

A vero cell line was maintained using MEM (Himedia, Mumbai, India), supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO_2. Chikungunya (CHIKV, strain no. 061573, P-2, African genotype) were used for this study. CHIKV stock was propagated in Vero cells at ICMR-NIV, Pune, and stored at −80 °C.

4.3. Animals, Housing, and Diets

C57BL/6 mice (3–4 weeks), which were bred in-house, were used for the in vivo experiments. Animals were maintained in the BSL-2 facility with a controlled temperature of 23 ± 2 °C and 40–70% relative humidity with a 12 h light–dark cycle. Animals had free access to a standard 9-pellet diet and fresh water. Animals were allowed to acclimatize for one week before the experimentation. All procedures described were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), National Institute of Virology (NIV), Pune, India (IAEC number CHK501 approved on 20 December 2017). The animal experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

4.4. siRNA

Two effective siRNAs, i.e., CHIK1 and CHIK 5 (designed earlier against the ns1 and E2 gene regions), were used in this study [7]. These siRNAs showed significant inhibition against CHIKV in the in vitro system and even in mice showing complete inhibition of CHIKV [7].

4.5. Formulation of Different Delivery Systems

For effective siRNA delivery, four different nano delivery systems were prepared using different cationic lipids by distinct techniques. The prepared individual delivery systems were designated as F1, F2, F3, and F4, respectively.

4.5.1. Preparation Method for F1 and F3 Delivery Systems

Both F1 and F3 delivery systems were hybrid polymeric lipid nanoparticles prepared by the double-emulsion solvent-evaporation method [40]. The F1 and F3 delivery systems had PEI-PEG (5 mg) and dioctadecylamine (3 mg) as cationic lipids, respectively, along with other components (Table 1). Briefly, all the required PEI-PEG, PLGA 50:50 (F1), dioctadecylamine, PLGA 50:50 (F3), DDAB, DOPC, DPPC, 18:00 PEG 2000 PE, cholesterol, and lecithin were accurately weighed and dissolved in 1 mL of DCM. The organic phase was slowly injected into 3 mL cold polyvinyl alcohol PVA (0.5% w/v) under probe sonication (Sonics Vibra cell, Newtown, CT, USA) at 50% amplitude for 15–20 min, forming a primary emulsion. The primary emulsion was added drop-wise to 10 mL cold PVA (0.5% w/v) under constant stirring at 900 rpm, leaving it for 30 min to form a double emulsion. Further, the emulsion was probe-sonicated at 50% amplitude for 25 min in an ice container. The resultant emulsion was left under stirring at 1200 rpm for 3 h at room temperature to evaporate the chloroform. The prepared formulations were stored in a sealed container at 4 °C.

Table 1. Compositions F1 and F3 delivery systems.

F1	F3
PLGA 50:50–3 mg PEI-PEG 5000–5 mg DDAB–5 mg Lecithin–2 mg PVA–0.3% (3 + 10 mL)	PLGA 50:50–3 mg Dioctadecylamine–3 mg DOPC–5 mg DPPC–5 mg Cholesterol–2 mg 18:00 PEG 2000 PE–2 mg PVA–0.3% (3 + 10 mL)
Total volume: 14 mL Total lipids: 15 mg	Total volume: 14 mL Total lipids: 20 mg

4.5.2. Preparation Method for F2 and F4 Delivery Systems

We determined that 8 mg of ODA was capable of inducing a firm cationic surface charge in comparison to 2, 4, 6, and 8 mg of ODA. Therefore, 8 mg of ODA was used as an optimal lipid concentration. Both the F2 and F4 delivery systems are based on SLN prepared by the emulsification-solvent evaporation method [41–43]. The F2 and F4 delivery systems had new C9 lipids (9 mg) and ODA (8 mg) as cationic lipids, respectively, along with glyceryl monostearate (GMS), cholesterol, DOPC, and DPPC (helper lipids). Briefly, for preparing both F2 and F4, a premixed solution of lipids in 1 mL of chloroform (organic phase) (Table 2) was added to 0.5% of Tween 80 (aqueous phase) and homogenized (Ultra Turrax, Reichenbach, Germany) for 5 min and sonicated at a 50% amplitude for 20 min and kept in stirring condition for 2–3 h until the solvent evaporated. The prepared formulations were stored in a sealed container at 4 °C.

Table 2. Compositions of the F2 and F4 delivery systems.

F2	F4
C9 lipid–9 mg DOPC–5 mg DDAB–5 mg Cholesterol–5 mg DSPE-PEG 2000–3 mg Tween 80 (0.5%)–10 mL	Stearylamine–8 mg GMS–60 mg DOPC–5 mg DPPC–5 mg DSEP PEG 2000–2 mg Cholesterol–2 mg Tween 80 (0.5%)–10 mL
Total volume: 11 mL Total lipids: 27 mg	Total volume: 11 mL Total lipids: 82 mg

4.6. Particle Size, Polydispersity, and Determination of Zeta Potential

The average particle size, surface charge, and size distribution of developed delivery systems were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). ODA-SLNs surface charge was elucidated by investigating the ζ-potential using the earlier reported method [44]. All measurements were performed using deionized water at 25 °C in triplicate (n = 3) in a 1:50 dilution.

4.7. Determination of Optimal Lipid-to-siRNA Complexation Ratio for F4

The particle size and the Zeta potential were used to determine the siRNA/optimal lipid nitrogen/phosphate (N/P) complexation ratio. The ODA-based lipoplex (F4) was prepared at different (N/P) atomic ratios that were tested: 1, 2, 4, 6, 8 and 10, keeping the siRNA quantity constant at 1000 pmol. The lipid nanoparticle size and charge were observed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [45].

4.8. Cytotoxicity Assay (MTT Assay)

The in vitro cytotoxicity of four delivery systems (F1, F2, F3, and F4) on Vero cells was evaluated using a 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

reduction assay [46]. Briefly, monolayers of Vero cells in 96 well plates were incubated with different concentrations of test formulations from 0 to 200 µg/mL for 48 h at 37 °C, incubated with a MTT solution (5 mg/mL) for an additional 3 h at 37 °C. The solubilized formazan crystals were measured using a microplate reader (BioTek Synergy HT, Winooski, VT, USA) at 570 nm with a reference filter of 690 nm. The percentage cell viability was calculated in comparison with control cells treated with the same volume of sterile PBS using GraphPad Prism 5.0 software [47].

4.9. Formation of siRNA-SLN Complexes

The siRNA-SLN complexes were prepared by incubating1000 pmol of the siRNA with 50 µg/mL of nanoparticulate formulation at 4 °C for 4 h. The total-lipid concentration needed to complex with siRNA was maintained with no toxic effect on the Vero cells. The siRNAdelivery complex was further evaluated for stability.

4.10. Determination of siRNA F4 SLN Complexes' Stability by Gel Retardation Assay

The complexation stability of the F4 delivery systems with the siRNA was analyzed using agarose-gel electrophoresis. The complexation of delivery systems with siRNA was carried out using different temperatures, lipid concentrations, and siRNA. Proper complexation was allowed by the incubating delivery system with siRNA for about 4 h at 4 °C. Gel electrophoresis was performed using 1% agarose gel at 65 V for 45 min in a TAE buffer solution [43].

4.11. In Vitro Release Studies

The siRNA release from the optimized ODA-SLN formulation (F4) was studied in phosphate buffer saline PBS (pH 7.4) and acidic conditions (pH 6.4). Formulations (4 mL) were centrifuged at 13,000× *g* for about 28 min at 37 °C in RNase-free eppendorfs. The obtained pellets were re-suspended in PBS and acidic solutions (3 mL). Further, the pellets were stirred at 100 rpm for 7 days at 37 °C. At different time points, the samples were aliquoted and were centrifuged at 13,000× *g* for 28 min at 25 °C [45]. The collected supernatant with an equal volume of fresh PBS or acidic solution was added to the sample. The siRNA concentration obtained in the supernatant was analyzed using a Thermo Scientific NanoDrop 1000 Spectrophotometer (Waltham, MA, USA) [48].

4.12. Determination of In Vitro Anti-CHIKV Activity of the siRNA-Loaded SLN Delivery System

Vero cells were infected with CHIKV and after one hour treated with naked siRNAs (Chik 1 and Chik 5) and siRNAs loaded with delivery systems individually and in combination at 37 °C. CHIKV replication inhibition was determined by quantitative RT-PCR (qRT-PCR) and focus forming units (FFU) assay at 24 and 48 h p.i. [49]. For the FFU assay, different dilutions of tissue-culture supernatants of infected and siRNA transfected cells were added to a monolayer of Vero cells and incubated at 37 °C for 1 h. After the incubation, the medium was replaced by an overlay medium and was further incubated for 24 h. The cells were fixed, and primary and secondary antibodies were added, followed by addition of true-blue peroxidase substrate. Cells were washed with PBST and were incubated at 37 °C between each step of additions. Foci were obtained and calculated as FFU per mL. For the qRT-PCR assay, RNA extraction from the cells was done using QIAmp viral RNA minikit (QIAGEN, Valencia, CA, USA) method. Total viral RNA was determined by real time-PCR using the standard-curve method. The primers and the probe targeted the E3 gene, and the sequences have been reported earlier [49]. In addition, the IFA assay for the quantitative estimation of virus infectivity as described earlier was also performed [49]. In brief, for IFA treated cells, cover slips were fixed, followed by addition of a primary antibody (immune mouse serum against a CHIKV clone) and a secondary (anti-mouse IgG FITC conjugate produced in goat) conjugate antibody. Cells were then mounted with a mounting solution containing DAPI (nuclear stain) and were observed under an EVOS

Floid Cells imaging station microscope with 20× fixed magnification. The percent cells infection was calculated by Image J software.

4.13. Determination of In Vivo Anti-CHIKV Activity of the siRNA-Loaded SLN Delivery System

To evaluate the in vivo antiviral activity of the siRNA-loaded F4 SLN formulation, C57BL/6 female mice were infected with CHIKV using the intramuscular route (i.m.) (100 µL of 10^7 pfu/mL), and mice were treated with different siRNA- (dose 1 mg/kg of body weight) loaded F4 formulations via the intravenous (i.v.) route on day 3 pid. Each mousereceived almost 50 µL of siRNA- (25 µg) loaded F4 SLN (3.75 mg/mL). Submandibular blood was collected at 3, 5, and 7 pid for viremia analysis. At each time point, mice were euthanized by cervical dislocation. Muscle tissue from hind feet from mice were collected; histopathology and RT-PCR analysis was performed at different time points [49].

Supplementary Materials: Supplementary materials are available online, Figure S1: Agarose Gel electrophoresis.

Author Contributions: Conceptualization, D.P. and R.S.; methodology, M.K.J., M.K., P.P., A.M., R.S. and D.P., formal analysis, M.K.J., M.K., P.P., A.M., D.P. and R.S.; resources, D.P. and R.S.; writing—original draft preparation, M.K. and D.P.; writing—review and editing, D.P., M.K.J. and R.S.; supervision, D.P. and R.S.; project administration, D.P. and R.S.; funding acquisition, D.P. and R.S. All authors have read and agreed to the published version of the manuscript.

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