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Lentivirus-mediated RNA interference against Japanese encephalitis virus infection *in vitro* and *in vivo*

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ABSTRACT

Japanese encephalitis virus, a serious mosquito-borne flavivirus, causes acute encephalitis in humans and many animals, with a high fatality rate. RNA interference is a reasonable antiviral mechanism for target gene silencing. In this study, four lentiviral shRNAs (LV-E1, LV-E2, LV-NS3 and LV-NS4b) were constructed. The results showed that four recombinant lentiviruses suppressed JEV replication *in vitro*. Through treatment with LV-E1 or LV-E2, the $TCID_{50}$ values were reduced by 10^3-fold during 120 h post-challenge; the relative expression of viral mRNA was <7% or 13% in mouse and human neuroblastoma cells. Lentiviral shRNAs displayed robust inhibitory activity in various cells and against different genotypes of JEV. *In vivo*, pre-treatments of LV-E1 or LV-E2 resulted in no viral particles being observed in suckling mice brain sections. For 21 days of observation, 100% of mice were protected against lethal JEV injection by two pre-treatments with LV-E1 or LV-E2; the survival of the mice pre-challenged with lethal JEV was 88.3%/66.7% by treatment with LV-E1 or LV-E2. LV-E1 and LV-E2 suppressed the induction of inflammatory mediators effectively in neuroblastoma cells and mice. Lentiviral shRNAs significantly inhibit JEV infection for long-term *in vitro* and *in vivo* and effectively reduce the inflammatory response and relieve encephalitis symptoms, highlighting the feasibility of using lentivirus-mediated RNAi for potential therapy in JEV infection.

1. Introduction

Japanese encephalitis virus (JEV), which belongs to the *Flavivirus* genus in family Flaviviridae, is the largest worldwide cause of epidemic viral encephalitis (van den Hurk et al., 2009). Clinically, JEV infection induces microglial activation and increases the levels of inflammatory mediators such as TNF-α, IL-6, IL-8, IL-12 and RANTES, causing massive neuronal death (Swarup et al., 2007; Das et al., 2011). Currently, approximately 68,000 JE cases are estimated to occur annually causing at least 10,000–15,000 deaths, mostly in infants and children, and leaving permanent sequelae in approximately 50% of cases (Campbell et al., 2011; Solomon, 2006). Despite large effective immunization campaigns, JE remains a disease of global health concern (Le Flohic et al., 2013).

JEV contains a positive single-stranded RNA genome of approximately 11 kb. The genome codes for three structural proteins (C, prM, E) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Chambers et al., 1990; Lindenbach et al., 2007). Mainly based on the E gene, JEV is divided into five genotypes (I–V). Genotypes I and III are epidemic in East and Southeast Asia (Morita, 2009; Gao et al., 2013).

RNA interference (RNAi), a post-transcriptional mechanism widely present in eukaryotes, is an evolutionarily conserved process of specific gene silencing (Bosher and Labouesse, 2000; Hannon, 2002). In this process, dsRNA is cleaved into siRNAs of 21–25 nt by the RNAase III-like enzyme dicer. These siRNAs are associated with a multiprotein complex known as the RNA-induced silencing complex (RISC) and ultimately degrade homologous mRNA and prevent protein translation (Dykxhoorn et al., 2003). There is a new field of drug discovery with RNAi therapeutics that target a wide variety of human diseases, ranging from cancer to metabolic diseases and viral infections (de Fougerolles et al., 2007). Recent studies have demonstrated the efficacy of RNAi in inhibiting several viruses, *in vitro* and *in vivo*, including hepatitis C virus (Pan et al., 2012; Suhy et al., 2012), hepatitis B virus (Pichard et al., 2012; Wang et al., 2013), West Nile virus (Anthony et al., 2009; Schnettler et al., 2012), and influenza virus (Langlois et al., 2013). It is known that the lentiviral vector (LV) possesses multiple advantages, such as a high efficiency of infec-

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tion in different cell types, a relatively large packaging capacity and stable transduction that is suitable for applications in disease therapies compared with synthetic and vector-borne siRNA and other virus carriers. LV is a good gene delivery system and has been explored for preclinical or clinical applications, showing promising results (Humeau et al., 2004; Scherr and Eder, 2002).

We previously performed shRNA screening of JEV entire genomes and selected potential anti-JEV shRNAs. In this study, four lentiviral shRNAs (LV-E1, LV-E2, LV-NS3 and LV-NS4b) were constructed. All of the lentiviral shRNAs suppressed JEV replication effectively, and LV-E1 and LV-E2 performed significant long-term inhibition of JEV infection *in vitro* and *in vivo*. Our results provide novel insights into the inhibition of JEV-induced encephalitis.

2. Materials and methods

2.1. Cells and virus

Mouse and human neuroblastoma cells (N2a and SK-N-SH), BHK-21, Vero, PK-15, A549 and 293T cells were used for the experiments. The cells were maintained at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Virulent JEV strains of genotype III (NJ2008, GQ918133; SA14, U14163) and genotype I (HEN0701, FJ495189) were used for the viral challenge.

2.2. Animals

Suckling mice and female BALB/c mice aged 4 weeks (purchased from Model Animal Research Center of Nanjing University) were housed in a dedicated SPF facility (Key Laboratory of Animal Diseases Diagnosis and Immunology, Ministry of Agriculture of China). All of the mouse experiments were performed in accordance with the institutional ethics committee guidelines.

2.3. Construction of lentiviral shRNAs

We previously performed the screening of shRNAs targeting JEV entire genomes using a siRNA target-finder (http://www.ambion.com/). Accordingly, we selected four target sequences to construct the lentiviral shRNAs (LV-E1, LV-E2, LV-NS3 and LV-NS4b) including the negative control (LV-NC) (Table. 1). The target sequences were used to design two complementary oligonucleotides, which were synthesized and cloned into pGLV1/U6/GFP (Genepharma, China). The positive purified lentiviral shRNA-expressing plasmids were transfected with the packaging plasmids into 293T cells for lentivirus generation (Genepharma, China). The viral supernatants were harvested and titered on 293T cells. The titers of the lentiviruses were generally up to 10^8 TU/ml.

2.4. Viral challenge in vitro

N2a and SK-N-SH cells were inoculated with each lentiviral shRNA at a multiplicity of infection (MOI) of 10 with 5 μg/ml polybrene in DMEM with 10% FBS. After 24 h, the cells were challenged with JEV at a MOI of 0.1. At 48–120 h post-challenge (hpc), the cells were examined microscopically for lentiviral GFP expression, harvested and analyzed by $TCID_{50}$, real-time PCR, flow cytometry and western blot assays. The other cells were treated with the same procedure.

To normalize the transduction efficiency of the lentiviral shRNAs in various cells (N2a, SK-N-SH, Vero, PK-15 and A549), the GFP-positive cells were evaluated by flow cytometry using the FACS Calibur Flow Cytometry System (BD Biosciences). The results are present in the Supplementary Data (Fig. S1).

Table 1
Target genes and sequences of lentiviral shRNAs.

Target gene	Site	Target sequences (NJ2008)
E1	1555–1573	5′-GGAGTGGACTGAACACTGA-3′
E2	1681–1699	5′-ACAGAGAACTCCTCATGGA-3′
NS3	6085–6103	5′-AGATCATGTTAGACAACAT-3′
NS4b	7352–7370	5′-GAATGCCGTTGTTGACGGA-3′
NC	Nonsense	5′-TTCTCCGAACGTGTCACGT-3′

2.5. $TCID_{50}$ measure

The supernatants of the N2a and SK-N-SH cells were harvested at different times post challenge (48, 72, 96 or 120 h), and the $TCID_{50}$ of BHK-21 cells was determined using the Reed–Muench method. The supernatants of the other cells were harvested at 48 hpc, and the $TCID_{50}$ was determined using the same procedure.

2.6. Real-time PCR assay

The total RNA of N2a and SK-N-SH cells was isolated with TRIzol (Invitrogen) following the manufacturer's instructions. Reverse transcription was carried out using MLV-RT (Takara) in 10 μl reaction mixtures containing 3 μl RNA. SYBR Green I real-time PCR was performed using Taqman probe quantitative real-time PCR (TaKaRa) following the manufacturer's protocols. The specific primers were based on the sequence of the JEV 3′ noncoding region (3′ N-F: 5′-CCCTCAGAACCGTCTCG GAA-3′, 3′ N-R: 5'-CTATTCCCAGGTGTCAATATGCTG-3′). Beta-actin served as an internal reference (N2a-actin-F: 5′-TATCCTGACCCTGAAGTA-3′; N2a-actin-R: 5′-TCATTGTAGAAGGTGTGG-3′; SK-actin-F: 5′-GGACTTCGAGCAAGAGAT GG-3′; SK-actin-R: 5′-AGCACTGTGTTGGCGTACAG-3′). The real-time PCR was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s and analyzed with ABI PRISM 7300_{SDS} software (Applied Biosystems). The relative RNA levels were calculated using the ΔΔCt method.

2.7. Flow cytometry assay (FCA) in vitro and in vivo

At 120 hpc, the N2a cells were washed, trypsinized and resuspended in PBS and then incubated with an anti-JEV-E protein mAb, followed by incubation with Alexa Fluor 645 goat anti-mouse IgG (Invitrogen). The positive cells were evaluated by flow cytometry using the FACS Calibur Flow Cytometry System (BD Biosciences).

At 5 days post-challenge (dpc), the brain homogenates of BALB/c mice were inoculated for 1.5 h on N2a cells. At 72 hpc, the N2a cells were assayed by FCA.

2.8. Western blotting analysis

At 96 hpc, the N2a and SK-N-SH cells were harvested, washed twice with PBS and boiled for 10 min. An equal volume of the samples was analyzed by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in PBS containing 5% skim milk overnight and then incubated with anti-JEV-E protein or anti-beta-actin mAb (Boster). The samples were then incubated with goat anti-mouse IgG conjugated with HRP (Boster). The proteins were visualized by enhanced chemiluminescence. Beta-actin and E protein served as the internal reference and target protein, respectively.

2.9. ELISA for cytokine analysis in neuroblastoma cells and BALB/c mice

At 48 h post-lentivirus-injection, the cell supernatants were collected and measured by a Mouse/Human IFN-beta ELISA Kit (R&D Systems). Cells treated with poly(I-C) (Sigma) were the positive control (Vigne et al., 2009). At 96 h post-JEV-challenge, the cell supernatants were collected and measured by a Mouse/Human TNF-α/IL-6/IL-8/MCP-1 Quantikine ELISA Kit (R&D Systems).

At 5 days post-JEV-challenge, serum from BALB/c mice was analyzed using a Mouse TNF-α/IL-6 Quantikine ELISA Kit (R&D Systems).

2.10. Immunohistochemistry analysis

Suckling mice were divided into four groups of four mice and then inoculated intracranially (IC) twice (the first inoculation was at 3 days, and the second inoculation was at 1 day before the JEV challenge) with 5×10^5 TU of LV-E1, LV-E2 or LV-NC in each group through the bregma (4 mm deep vertically into the brain using a Hamilton syringe fitted with a 30-gauge needle) (Kumar et al., 2006). The mice were challenged IC with 5 LD_{50} NJ2008 through the bregma at the same location. After 5 days, the brains were removed and placed in 10% formalin, embedded in paraffin, processed with an anti-JEV-E protein mAb followed with peroxidase-conjugated SPA (Boster), and stained with DAB (Boster). The sections were stained with hematoxylin for immunohistochemistry analyses. The mice challenged with PBS were used as a blank control.

2.11. Plaque formation assay

At 5 dpc, the brain homogenates of suckling mice were inoculated for 1.5 h on N2a cells in six-well plates. Overlay medium (2% low melting-point agarose with DMEM medium containing 2% FBS) was added to each well, and the plates were further incubated at 37 °C with 5% CO_2 for 5 days. The cells were then stained with 0.5% crystal violet.

2.12. Survival analysis

To determine the prophylactic effect, two different immune programs were performed in BALB/c mice. In one program, the mice (6 per group) were inoculated IC singly with 10^6 TU of LV-E1 or LV-E2 through the bregma. After 24 h, the mice were challenge with 100 LD_{50} NJ2008. In the other program, the mice were injected IC twice (the first injection was at 3 days, and the second injection was at 1 day before the 100 LD_{50} JEV challenge) with 5×10^5 TU of LV-E1 or LV-E2 each time. To determine the therapeutic effect, BALB/c mice were challenged IC with 20 or 100 LD_{50} of the JEV virulent NJ2008 stain and then inoculated IC with 10^6 TU of LV-E1 or LV-E2 through the bregma. The mice were observed for their survival and morbidity for 21 days. Mice treated with LV-NC or PBS served as the positive control and the blank control, respectively.

2.13. Analysis of JEV intradermal infection model

BALB/c mice were divided in four groups (6 mice per group). The mice were injected subcutaneously (s.c.) in the scruff of the neck with 10^6 TU of lentiviral shRNAs and then challenged s.c. in the same site with 100 LD_{50} of JEV. The mice treated with LV-NC or PBS served as the positive control and the blank control, respectively. At 5 dpc, three hundred microliters of peripheral blood was collected into EDTA tubes by mandibular vein puncture or cardiac puncture (Lim et al., 2011). The supernatants were determined by $TCID_{50}$ and RT-PCR as described above.

2.14. Statistical analysis

All of the assays described were repeated at least three times, and all of the measurements were made in triplicate. The statistical analysis was performed by one-way analysis of variance, and values were considered significant when $P < 0.05$. The figures were created using GraphPad™ Prism 5.0.

3. Results

3.1. Lentiviral shRNAs inhibit JEV infection in neuroblastoma cells

The titers of N2a and SK-N-SH cells treated with four lentiviral shRNAs were reduced significantly by $>10^3$-fold compared with the group LV-NC, and the $TCID_{50}$ values of the LV-E1 and LV-E2 groups were decreased by nearly 10^4-fold (Fig. 1AA). The results of real-time PCR showed that the relative expression of JEV mRNA was 4.8% or 6.1% in N2a cells, and 9.7% or 12.5% in SK-N-SH cells treated with LV-E1 or LV-E2. LV-NS3 and LV-NS4b suppressed JEV mRNA expression by >83% or 79% in N2a and SK-N-SH cells (Fig. 1B). No significant band was observed in the cells treated with LV-E1. A faint band was observed in the cells treated with LV-E2, LV-NS3 and LV-NS4b compared with the LV-NC group (Fig. 1C). The results of the western blot showed that the 4 lentiviral shRNAs suppressed JEV replication markedly at the protein level (Fig. 1C).

These results indicated that the 4 lentiviral shRNAs inhibited JEV replication effectively in N2a and SK-N-SH cells, especially LV-E1 and LV-E2.

3.2. Lentiviral shRNAs suppress JEV replication long-term in N2a cells

The supernatants and RNA of the cells were collected at 48, 72, 96 and 120 hpc to determine the $TCID_{50}$ values and the relative expression of JEV. The data demonstrated that the viral titers of the LV-E1 and LV-E2 groups were lower than the LV-NV group by $>10^3$-fold during 120 hpc; the viral titers of the LV-NS3 and LV-NS4b groups were lower than the LV-NV group by $>10^3$-fold during 96 hpc (Fig. 2A). The data of the real-time PCR indicated that the inhibitory effect was time-dependent but stably for at least 96 hpc. LV-E1 suppressed 95.5% of the viral mRNA expression at 96 hpc. A MOI of 10 of LV-E1, LV-E2 or LV-NS4b silenced >85% of JEV mRNA at 120 hpc (Fig. 2B). The data of the flow cytometry experiments displayed that the extent of E protein downregulation by LV-E1 or LV-E2 was greater 90% by assessing the number of fluorescence-positive cells relative to the LV-NC group. LV-NS3 or LV-NS4b suppressed JEV replication by >80% (Fig. 2C). The lentiviral shRNAs suppress JEV replication long-term in N2a cells for a minimum of 120 hpc, especially LV-E1 and LV-E2.

3.3. Broad-spectrum antiviral activity of LV-E1 and LV-E2

JEV of genotypes I and III are mainly epidemical around the world, especially in Southeast and East Asia. Five different cell lines pretreated with LV-E1 or LV-E2 were infected with three JEV strains. The titers of the N2a or SK-N-SH cells were reduced nearly 10^3-fold compared with the LV-NC group. The $TCID_{50}$ values of Vero, PK-15 and A549 cells treated with LV-E1 or LV-E2 were decreased notably compared with the LV-NC group. The results showed that LV-E1 and LV-E2 inhibited NJ2008 replication effectively in these five cell lines (Fig. 3A). LV-E1 and LV-E2 inhibited the HEN0701 of JEV genotype I in various cell lines (Fig. 3B). The JEV classic strain SA14 of genotype III was effectively suppressed in different cell lines by LV-E1 and LV-E2 (Fig. 3C). LV-E1 and LV-E2 have broad-spectrum antiviral activity against different JEV genotypes in various cells.

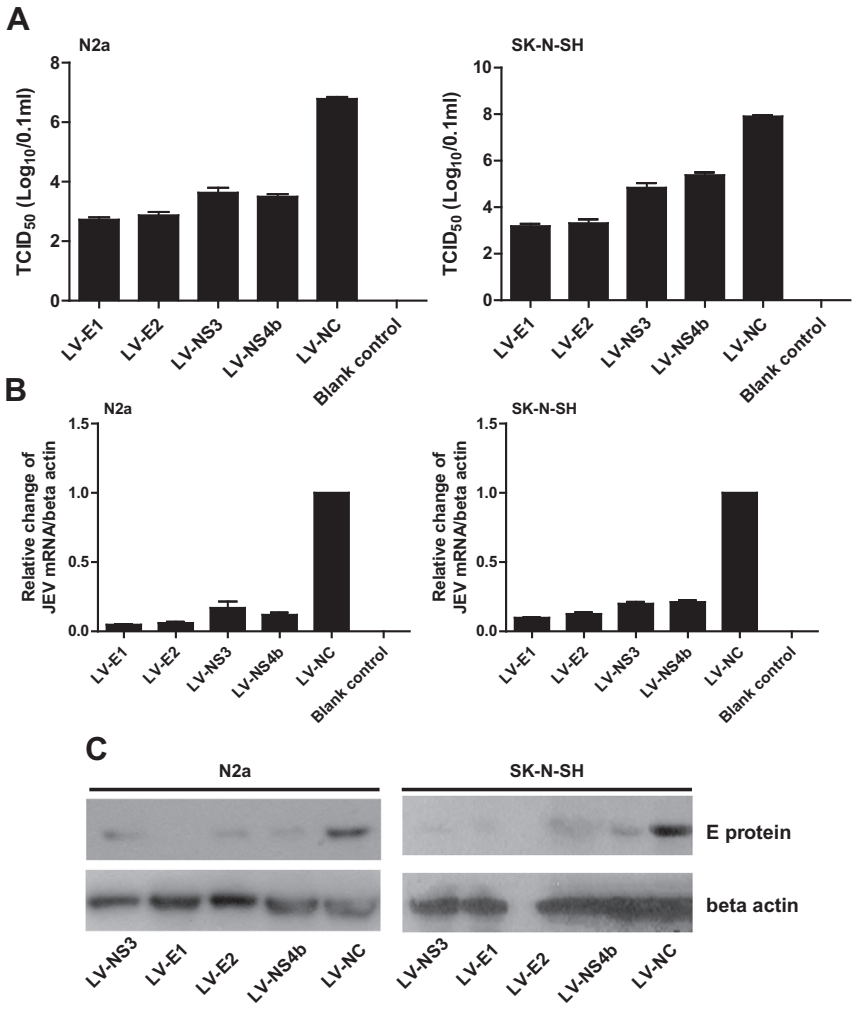

Fig. 1. Lentiviral shRNAs inhibit JEV replication in N2a and SK-N-SH cells. (A) $TCID_{50}$ assay. The cells were inoculated with LV-E1, LV-E2, LV-NS3, LV-NS4b and LV-NC for 24 h and then challenged with the JEV. The culture supernatants were collected at 96 hpc, and the virus yields were measured on BHK-21 cells by $TCID_{50}$. The left panel shows the N2a cells, and the right panel shows the SK-N-SH cells. (B) Real-time PCR assay. The cells were inoculated with lentiviruses for 24 h and then challenged with the JEV. The total RNA was collected at 96 hpc, and the virus yields were measured by real-time PCR. The left panel shows the N2a cells, and the right panel shows the SK-N-SH cells. (C) Western blot assay. The cells were inoculated with lentiviruses for 24 h and then challenged with the JEV. At 96 hpc, cells were harvested and separated by 12% SDS–PAGE. The beta actin protein served as the loading control. The reductions of the JEV-E protein levels in N2a (left) and SK-N-SH (right) cells are shown. The data are from a representative experiment.

3.4. Lentiviral shRNAs do not induce interferon and inflammatory mediators expression in neuroblastoma cells

It was previously reported that siRNA could produce innate immune stimulation in a sequence-specific manner (Malathi et al., 2007; Robbins et al., 2009). To rule out the possibility that the stimulation of interferon production contributed to the antiviral activity observed in this study, we examined whether lentiviral shRNAs were able to induce interferon production in N2a and SK-N-SH cells. The results showed that the supernatants had no apparent differences at the level of IFN-β between the lentivirus-treated and non-treated cells (blank control), and poly(I-C) induced a strong IFN response (Fig. 4A).

To substantiate the potential of clinical anti-encephalitis, we determined whether lentiviral shRNAs were able to control the induction of inflammatory mediators, because JEV infection results in increased levels of inflammatory mediators that bear a direct correlation with the mortality rate of the infected animals. The results showed that the levels of TNF-α, IL-6, IL-8 and MCP-1 were notably decreased (Fig. 4B–E). The results indicated that LV-E1 and LV-E2 effectively inhibit JEV-induced inflammation in N2a and SK-N-SH cells.

3.5. Lentiviral shRNAs protect suckling mice against JEV-induced encephalitis

Many virus particles were present in the brain sections, staining dark brown with DAB. The brain sections from the mice pretreated with LV-E1 or LV-E2 showed no virus particles, similar to the blank control (Fig. 5A). The results of the plaque assays indicated that the brain homogenates from the mice treated with LV-NC had extremely high levels of virion, and the titers of the homogenates from the mice treated with LV-E1 or LV-E2 were decreased significantly by at least 10^3-fold (Fig. 5B).

According to these results, the suckling mice pretreated twice by lentiviral shRNAs at a suitable dose resisted JEV-induced encephalitis and survived.

3.6. Lentiviral shRNAs protect BALB/c mice against JEV-induced encephalitis

With a single injection of 10^6 TU of LV-E1 or LV-E2, the survival of the mice was 83.3% against 100 LD_{50} JEV (Fig. 6A). The mice inoculated with LV-NC showed typical symptoms of viral encephalitis, including ruffling of the fur, hunching and hind limb

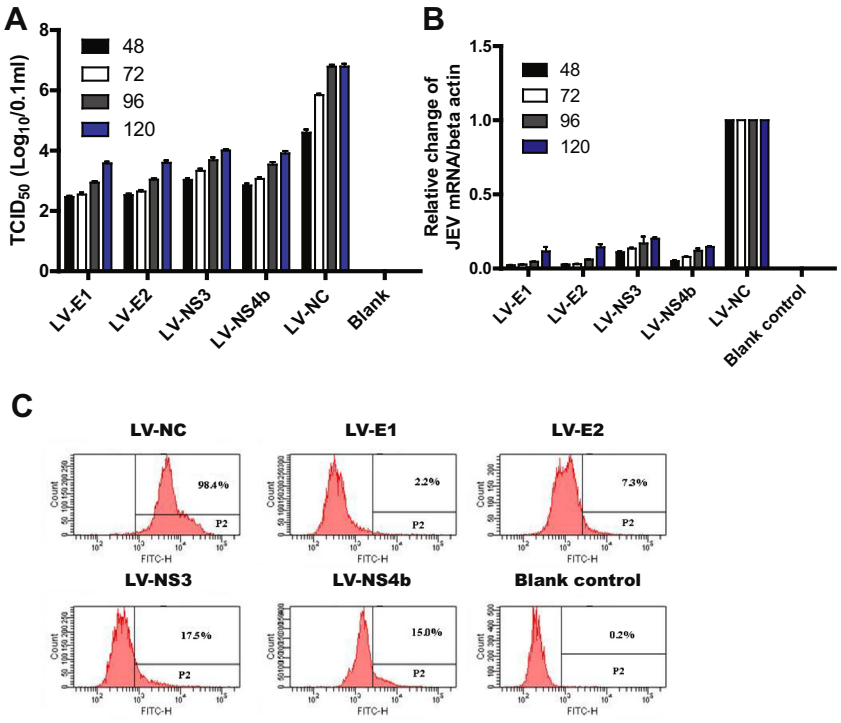

Fig. 2. Lentiviral shRNAs inhibit JEV replication long-term in N2a cells. (A) and (B) $TCID_{50}$ and real-time PCR assay. The cells were inoculated with LV-E1, LV-E2, LV-NS3, LV-NS4b and LV-NC for 24 h and then challenged with JEV. The culture supernatants or cells were collected at different time points (48, 72, 96 and 120 hpc) for virus detection. The relative expression of JEV mRNA in N2a cells treated with LV-NC was normalized as 1 at various time points. (C) Flow cytometry assay of the antiviral effect of LV-E1, LV-E2, LV-NS3 and LV-NS4b. N2a cells were collected at 120 hpc to calculate the number of fluorescent-positive cells by the FACS Calibur Flow Cytometry System.

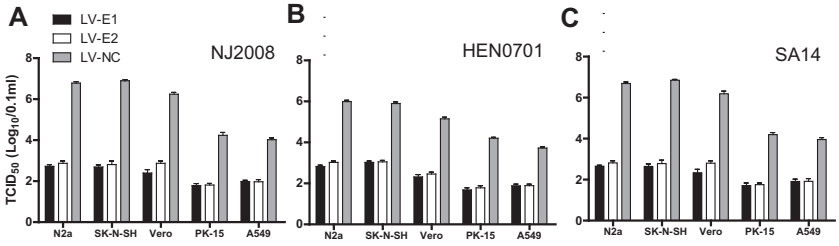

Fig. 3. LV-E1 and LV-E2 inhibit the replication of different JEV strains in various cell lines. N2a, SK-N-SH, Vero, PK-15 and A549 cells were inoculated with LV-E1, LV-E2 and LV-NC for 24 h, and then these five different cell types were challenged with NJ2008 (A), HEN0701 (B) or SA14 (C). The culture supernatants were collected at 96 or 48 hpc, and the virus yields were measured by $TCID_{50}$. The culture supernatants of N2a and SK-N-SH cells were collected at 96 hpc. The culture supernatants of Vero, PK-15 and A549 cells were collected at 48 hpc.

weakness beginning at 3 dpc, which rapidly progressed to paralysis, marked ataxia and death. The other immune program had good results. With two treatments of 5×10^5 TU of lentivirus, the survival of the mice was elevated to 100% (Fig. 6B). The protective ability of the same lentivirus at a low dose with two injections were superior to a single injection of a large dose.

Furthermore, LV-E1 and LV-E2 have therapeutic abilities. The survival results showed that 66.7% or 83.3% of the mice were protected against 20 LD_{50} JEV (Fig. 6C), and 67.7% or 50% of the mice challenged with 100 LD_{50} JEV survived within the 21-d-observation period (Fig. 6D).

The $TCID_{50}$ and real-time PCR results of an intradermal infection model in mice showed that LV-E1 and LV-E2 effectively inhibited JEV infection. Thus, LV-E1 and LV-E2 inhibited JEV infection at the periphery to prevent neurological damage (Fig. 5C and D).

The flow cytometry results showed that the brain sections of the mice treated with lentiviral shRNAs revealed a significant reduction in viral particles (Fig. 5E).

TNF-α and IL-6 are key mediators of severe JEV disease in mice. Measurements taken at 5 dpc showed that the PBS- and LV-NC-treated mice had significantly elevated serum TNF-α and IL-6 levels compared with the mice treated with LV-E1 or LV-E2 (Fig. 5F and G). This result indicated that lentiviral shRNA treatment was associated with reduced levels of inflammatory mediators known to be related to the encephalitis symptom.

4. Discussion

JE is a serious public health threat worldwide. Vaccination is the major strategy to control JE infections, but inoculation failures and emerging infections often lead to disease. There is no specific treatment for JEV. We previously performed shRNAs screening targeting the JEV entire genome and constructed shRNA-expressing plasmids to determine the antiviral activity. In this study, we developed a new strategy of anti-JEV infection by lentivirus-mediated RNAi, which inhibited JEV infection

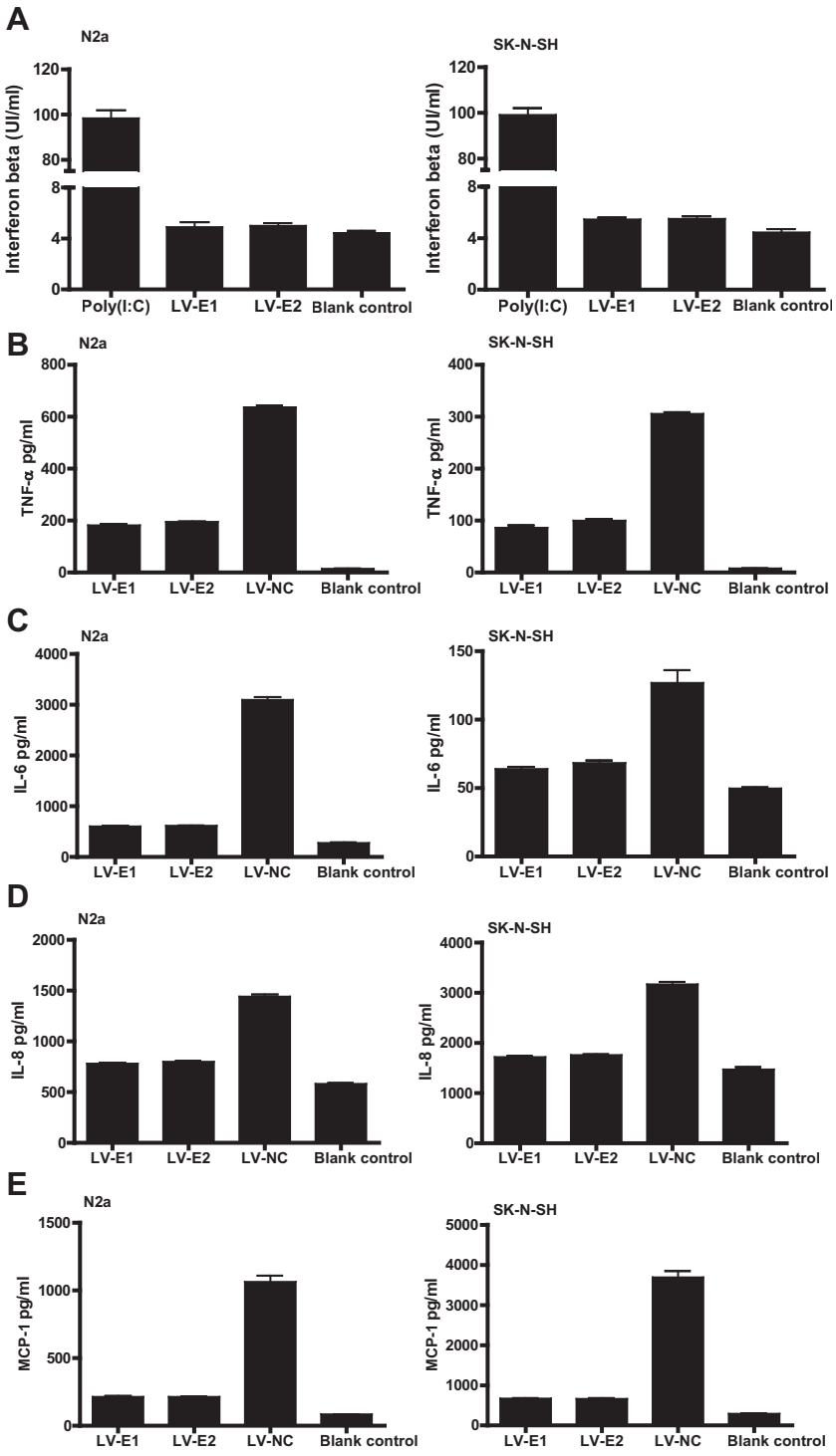

Fig. 4. Absence of interferon and inflammatory mediators induction in lentivirus infected cells. (A) The IFN-β levels in N2a and SK-N-SH cells were determined by ELISA using the supernatants of noninfected cells (blank control) and the lentivirus infected cells ($P < 0.05$). (B)–(E) The inflammatory mediators (TNF-α, IL-6, IL-8 and MCP-1) levels in N2a and SK-N-SH cells were determined by ELISA ($P < 0.05$). At 96 h post-JEV-challenge, the cell supernatants were collected and the levels of inflammatory mediators compared with the LV-NC group were measured.

in vitro and *in vivo* more effectively, more stably and more long-term than the plasmid system. Lentiviral shRNAs reduced the levels of inflammatory mediators (TNF-α, IL-6, IL-8 and MCP-1) in neuroblastoma cells and in mice to mitigate the symptoms of JEV-induced encephalitis, which was not previously reported.

Many shRNA-expressing systems have been explored (Lares et al., 2010). Compared with other shRNA-expressing systems, lentiviruses infect host cells more efficiently and stably (Escors and Breckpot, 2010). The transfection efficiency of plasmids varies among different cells and hosts because of cell membrane features, and lentiviruses enter host cells by virion infection in a simple and

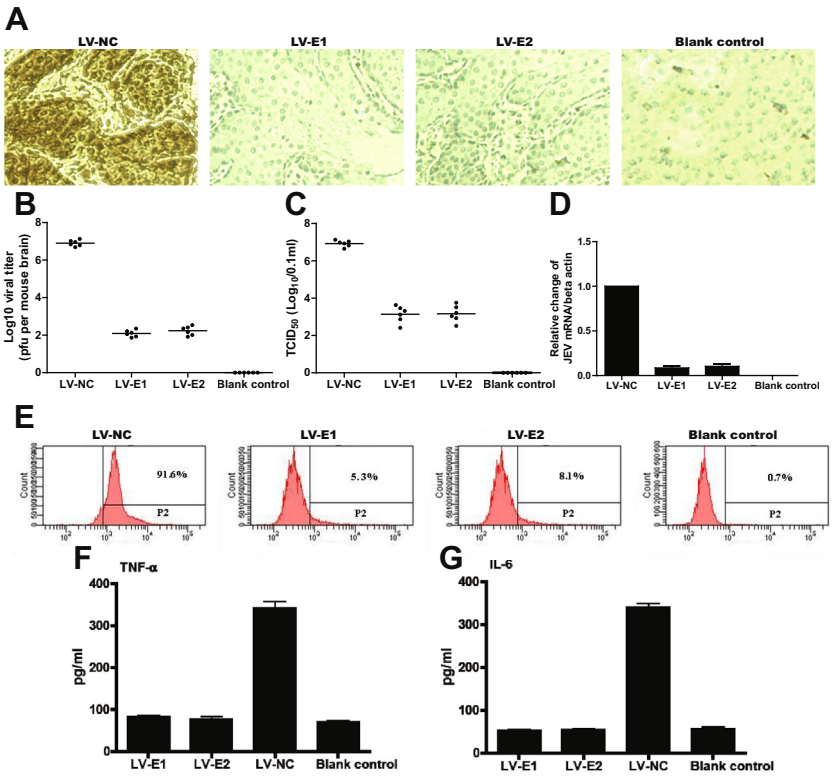

Fig. 5. Lentiviral shRNAs protect mice against JEV-induced encephalitis. (A) The immunohistochemistry analyses of brain sections from suckling mice with two treatments of lentivirus. Magnification, 400×. The data are from a representative experiment. (B) The brain viral load of suckling mice treated twice with a lentivirus was plaque-titrated on N2a cells. Each symbol represents an individual mouse. The horizontal line corresponds to the median viral titers of the group ($P < 0.05$). (C) and (D) BALB/c mice were injected s.c. with lentivirus and then challenged s.c. in the same site with JEV. The mice treated with LV-NC or PBS served as the positive control and the blank control. At 5 dpc, peripheral blood was collected. The supernatants were determined by $TCID_{50}$ and RT-PCR. (E) FCA of the JEV-positive SK-N-SH cells infected with the brain homogenates of BALB/c mice treated twice with lentivirus. The data are from a representative experiment. (F) and (G) The levels of TNF-α and IL-6 in serum from BALB/c mice were determined by ELISA at 5 days post-JEV-challenge.

economic way to highly express shRNAs. Plasmids are easy to degrade *in vitro* and *in vivo* because of their short half-life, but lentiviruses are reproducible vectors that express shRNAs stably and continuously. This difference suggests that a low-dose intracerebral injection could be used in encephalitis treatment.

JEV could cross the blood–brain barrier (BBB) in patients or infected animals and cause encephalitis (Azzouz et al., 2004a,b). The inflammatory response level of the patients is directly related to the mortality and disease sequelae. Therefore, the inhibition of JEV replication in the central nervous system (CNS) without the induction of inflammatory response is an important index to evaluate the antiviral activity and safety of lentiviruses (Azzouz et al., 2004c; Bienemann et al., 2003; Dodart et al., 2005). Meanwhile, as a core and fragile organ, the brain needs safe and mild treatments. Fortunately, lentivirus could be injected directly into the brain with low doses and few injections, and then the lentivirus flows into the CNS with the blood with less side-effects because of the low levels of inflammatory mediators, which are the important benefits compared with plasmid therapy (Kirik et al., 2004; Jakobsson et al., 2003).

Currently, lentivirus-mediated gene therapy has been used in a wild range of human diseases (Humeau et al., 2004; Dykxhoorn et al., 2008; Sumimoto and Kawakami, 2007; Farah, 2007). The results from several research teams showed that lentivirus definitely possesses multiple advantages and has the potential of clinical application. However we are still concerned about the risks and biosafety of lentivirus. In order to develop an *in vivo* system to examine lentivirus biosafety, the researchers chose a long-term mouse xenograft model, consisting of immunodeficient mice with a life span of 2 years. No vector-associated adverse events were observed, and none of the mice had detectable human HIV p24 antigen in their sera (Bauer et al., 2008). These studies demonstrate that there is very low risk from replication-competent vectors or the occurrence of adverse events when the HIV-based lentiviral vectors are used for delivering a marker gene. In view of the fact that some of the hurdles involved in the use of these vectors have been recently overcome and clinical efficacy can now be achieved in some settings with the use of lentivirus, the documentation of the safety of these vectors is reassuring. Nevertheless, many more clinical trials in human need to be done in the future.

In this study, LV-E1 and LV-E2 showed the highest antiviral activity in N2a and SK-N-SH cells. The antiviral activity was broad in various cells (N2a, SK-N-SH, Vero, PK-15 and A549 cells) against JEV genotypes I and III and lasted for at least 120 hpc. Besides, LV-E1 and LV-E2 supplied high protection for mice against lethal JEV injection throughout 21 dpc. More importantly, lentiviral shRNAs control the induction of JEV-induced inflammatory mediators *in vitro* and *in vivo*.

In conclusion, we constructed four lentiviral shRNAs targeting the E, NS3 and NS4b genes of JEV, and these lentiviral shRNAs effectively suppressed JEV replication in N2a and SK-N-SH cells. LV-E1 and LV-E2 inhibited JEV infection in various cells and in mice of different ages outstandingly with low levels of inflammatory mediators. Therefore, lentiviral shRNAs have the potential to be used as inhibitors of JEV infection. For the development of a

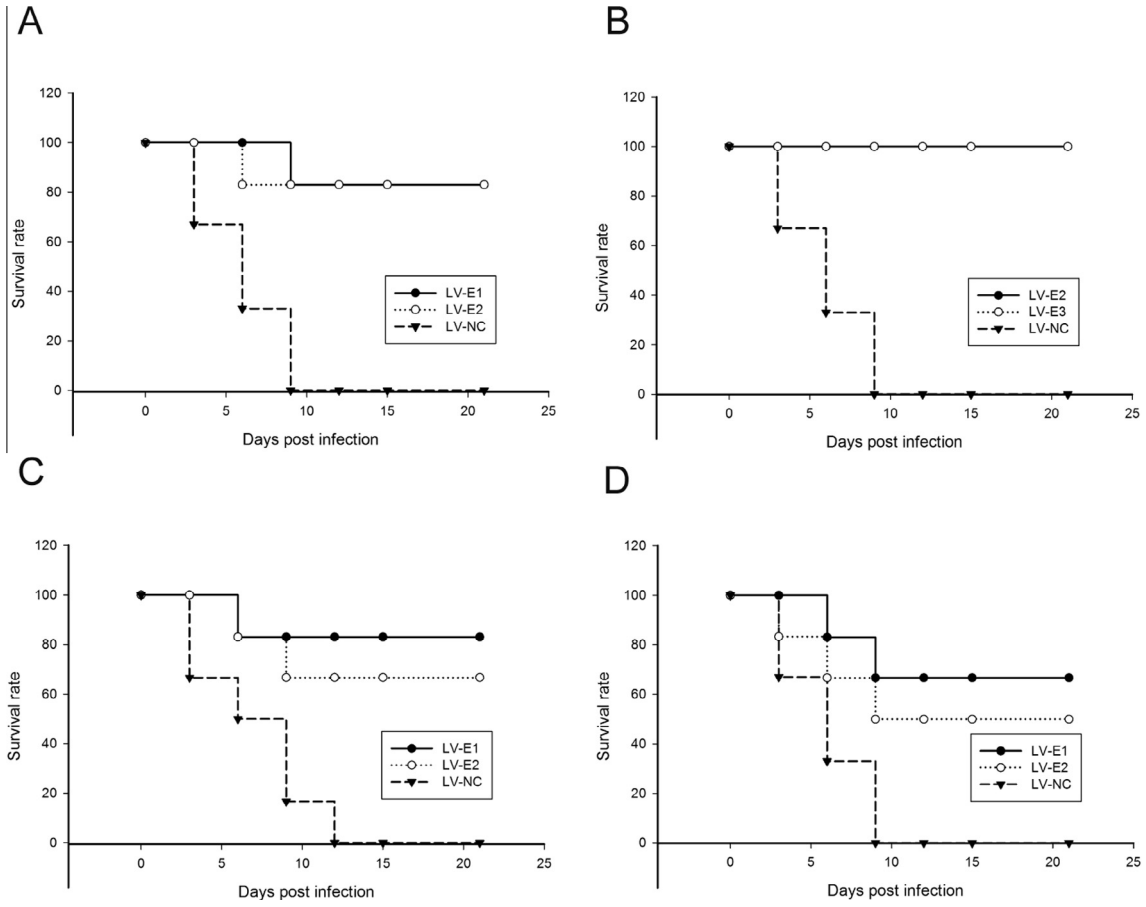

Fig. 6. Lentiviral shRNAs protect BALB/c mice from JEV challenge. (A) The survival rate of mice with a single lentivirus treatment (10^6 TU) and challenged with 100 LD_{50} NJ2008. (B) The survival rate of mice treated twice with lentivirus (5×10^5 TU/time) and challenged with 100 LD_{50} NJ2008. (C) The survival rate of mice challenged with 20 LD_{50} NJ2008 and then treated with lentivirus (10^6 TU). (D) The survival rate of mice challenged with 100 LD_{50} NJ2008 and then treated with lentivirus (10^6 TU). The observation period was 21 days.

useful antiviral drug, further clinical experiments using lentiviral shRNAs are necessary.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.008.

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