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A novel alphavirus replicon-vectored vaccine delivered by adenovirus induces sterile immunity against classical swine fever

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ABSTRACT

Low efficacy of gene-based vaccines due to inefficient gene delivery and expression has been major bottleneck of their applications. Efforts have been made to improve the efficacy, such as gene gun and electroporation, but the strategies are difficult to put into practical use. In this study, we developed and evaluated an adenovirus-delivered, alphavirus replicon-vectored vaccine (chimeric vector-based vaccine) expressing the E2 gene of classical swine fever virus (CSFV) (rAdV-SFV-E2). Rabbits immunized with rAdV-SFV-E2 developed CSFV-specific antibodies as early as 9 days and as long as 189 days and completely protected from challenge with C-strain. Pigs immunized with rAdV-SFV-E2 ($n = 5$) developed robust humoral and cell-mediated responses to CSFV and were completely protected from subsequent lethal CSFV infection clinically and virologically. The level of immunity and protection induced by rAdV-SFV-E2 was comparable to that provided by the currently used live attenuated vaccine, C-strain. In contrast, both the conventional alphavirus replicon-vectored vaccine pSFV1CS-E2 and conventional adenovirus-vectored vaccine rAdV-E2 provided incomplete protection. The chimeric vector-based vaccine represents the first gene-based vaccine that is able to confer sterile immunity and complete protection against CSFV. The new-concept vaccination strategy may also be valuable in vaccine development against other pathogens.

1. Introduction

Classical swine fever (CSF), which is caused by infection with classical swine fever virus (CSFV), results in significant losses in the pig industry in many countries. CSFV belongs to the *Pestivirus* genus within the *Flaviviridae* family [1]). It is an enveloped virus with single-stranded, positive-sense RNA encoding a 3898 amino acid polyprotein, which undergoes co- and post-translational processing by cellular and viral proteases to yield 11–12 cleavage products (NH2–N^{pro}–C–E^{rns}–E1–E2–p7–NS2–NS3-NS4A–NS4B–NS5A–NS5B–COOH) [2]. The E2 glycoprotein is highly immunogenic [3,4] and induces neutralizing antibodies (NAbs), providing protection against virulent CSFV challenge.

The two main strategies for the control of CSF are prophylactic vaccination and stamping out of exposed animals in the event of an outbreak. Vaccination is effective in protecting pigs against the disease. However, the countries free of CSF do not recommend the use of the currently available live attenuated vaccines (LAVs) to control disease outbreaks, due to the difficulties in differentiation of infected from vaccinated animals (DIVA) [5]. For this reason, the alternative vaccination strategies that allow DIVA are urgently necessary. Several alternative strategies have been extensively studied, including viral vectors, recombinant proteins, virus-like particles and DNA vaccines based on the E2 protein [6–11]. Among them, replication-defective recombinant adenoviral vector (rAdV)-based vaccines, which do not require bio-safety level 3 containment facilities and are much easy to stockpile, have been used as vaccines since the vectors have been widely used in preclinical and clinical studies and proven to be safe and effective [12–16]. Previously, we showed that the rAdV-based CSFV vaccine rAdV-E2 was effective in pigs [17], suggesting that this approach is a promising alternative to traditional LAVs vaccines. However, the vaccine induced incomplete protection (short-term fever and viremia upon challenge) and delayed antibody responses (occurring 3 weeks post-vaccination with rAdV-E2), which limit its potential application [17].

Alphavirus replicons (self-replicating RNAs) have potential to be vaccine vectors due to their ability to support high-level protein expression. We previously reported that a Semliki Forest virus (SFV) replicon-vectored plasmid DNA vaccine (pSFV1CS-E2) encoding the E2 gene of CSFV induced specific immunity and protection against CSFV in pigs. However, the titers of CSFV-specific serum NAbs induced by the vaccine were relatively low and a few pigs

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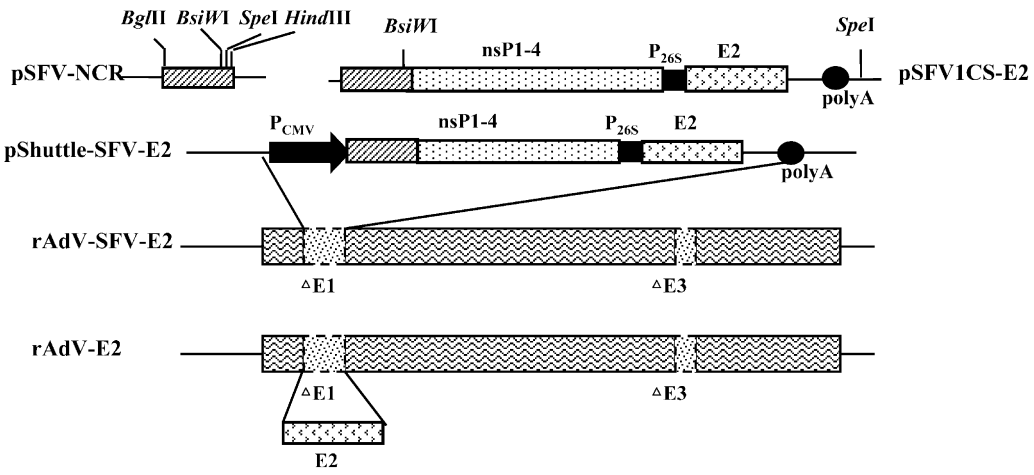

Fig. 1. Schematic diagram of recombinant transfer vector and adenovirus. The Semliki Forest virus (SFV) replicon consists of noncoding regions (NCR) and nonstructural proteins (nsP1–4) and its structural protein genes are replaced by the classical swine fever virus E2 gene under the control of the subgenomic 26S promoter (P_{26S}) of SFV. nsP1–4-E2 sequences are flanked by the immediate-early promoter of cytomegalovirus (P_{CMV}) and the polyadenylation signal of Simian virus 40 (polyA). The expression cassette was cloned into the locus of the E1 coding region (deleted) of human adenovirus type 5 (AdV). Cloning sites are indicated.

showed short-term fever and pathological changes upon virulent challenge [18,19]. The biggest challenge for this vector system may be inefficient introduction of the plasmid DNA into tissues [20].

Inefficient gene delivery is the major bottleneck of the DNA vaccines. Many efforts have been made to improve the efficacy, such as gene gun and electroporation. However, these strategies are impractical for veterinary clinical applications due to inconvenience and high costs. We propose that the replicon-based vaccine could be substantially improved by increasing the efficiency of delivery with the multipurpose adenovirus vector. In this study, we developed an adenovirus-delivered, alphavirus replicon-vectored vaccine (chimeric vector-based vaccine) against CSF (rAdV-SFV-E2) that induces CSFV-specific NAbs and confers sterile immunity and complete protection against lethal challenge.

2. Materials and methods

2.1. Construction and production of adenoviral vectors

PCR was used to amplify a 518-bp SFV 5′-non-coding region (NCR) fragment from pSFV1CS-E2 [18] using primers P5NCRB2C1S (5′-GAC AGA TCT ATC GAT GGC GGA TGT GTG ACA TAC-3′) and PH3Sp1Su1R (5′-ATT AAG CTT ACT AGT GGA TCC GTA CGA CAC GTG ACG TC-3′), which incorporated restriction enzyme sites *Bgl*II, *Hind*III, *Spe*I and *Bsiw*I (underlined). The amplified product was digested with *Bgl*II and *Hind*III and ligated into the adenovirus transfer vector pShuttle-CMV (Stratagene, USA) digested with *Bgl*II and *Hind*III, resulting in pSFV-NCR. pSFV-NCR and pSFV1CS-E2 both digested with *Bsiw*I and *Spe*I were ligated, resulting in pShuttle-SFV-E2 (Fig. 1). All plasmids were confirmed by sequencing. pShuttle-SFV-E2 was transformed into BJ5183 cells (Stratagene, USA) harboring the pAdEasy-1 vector which contains most of the human adenovirus serotype 5 except E1 and E3 genes to generate adenovirus plasmid (admid) pAdV-SFV-E2. The recombinant adenovirus rAdV-SFV-E2 was rescued by transfecting the *Pac*I-linearized pAdV-SFV-E2 into HEK 293 cells. An empty recombinant adenovirus rAdV-SFV-empty, which is everything identical to rAdV-SFV-E2 but E2 insertion, was also generated. Infectivity and titer of the recombinant adenovirus were determined on HEK 293 cells.

2.2. Immunofluorescence assay (IFA)

To analyze the CSFV E2 protein expression, HEK 293 cells were infected with rAdV-SFV-E2, rAdV-E2 [17] or rAdV-SFV-empty at a multiplicity of infection (MOI) of 20. Cells were collected 48 h post-infection, smeared onto glass slides, and fixed with cold acetone for 10 min. The fixed cells were then incubated with the anti-E2 monoclonal antibody (mAb) HQ06 [21] for 1 h at 37 °C in a humidified chamber followed by three washes with PBS. The cells were then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Sigma–Aldrich, USA) for 45 min at 37 °C, followed by three washes with PBS. The cells were covered with 90% alkaline glycerine and examined under a fluorescence microscope (Nikon TE200, Japan).

2.3. Western blot analysis

HEK 293 cells were seeded into 75-cm^2 tissue culture flasks and cultured for 24 h to 80% confluence. The cells were infected with rAdV-SFV-E2, rAdV-E2 or rAdV-SFV-empty at an MOI of 20 and incubated for 48 h. The expression of E2 protein in the cells was identified by Western blot with anti-E2 mAb HQ06 [21]. Cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. HEK 293 cells infected with rAdV-E2 or rAdV-SFV-empty were used as positive and negative controls, respectively. After transfer, the membrane was blocked in blocking solution (5% skimmed milk in PBS, PBS-M) at 4 °C overnight and incubated with mAb HQ06 in PBS-M for 2 h at 37 °C, followed by incubation for 45 min with goat anti-mouse IgG antibody (whole molecule) conjugated with horseradish peroxidase (Sigma–Aldrich, USA), diluted at 1:5000 in PBS-M. Then, DAB (3,3′-diaminobenzidine) was added for color development.

2.4. Immunization and challenge in rabbits

Thirty-five 14-week-old New Zealand white rabbits were randomly assigned to seven groups of five rabbits each and immunized intramuscularly (i.m). One-dose of rAdV-SFV-E2 of different titers (10, 5, 2.5, 1.25, 1 or 0.1×10^5 $TCID_{50}$ rAdV-SFV-E2) or control virus rAdV-SFV-empty, and challenged intravenously with 2000 RID_{50} (50% rabbit infection dose) C-strain 4 weeks post-immunization. Serum samples were collected and tested for anti-CSFV antibodies by the IDEXX HerdChek* CSFV Antibody Test Kit (a blocking ELISA based on competition between peroxidase-labeled anti-E2 neutralizing mAb and serum NAbs for binding coated CFSV antigens). Rectal temperatures were recorded every 6-h post-challenge, and the spleen was collected and tested for the viral RNA copies by the real-time RT-PCR as described previously [22] (see below). Fever

was recognized with elevated rectal temperature of ≥1 °C and lasting for at least 18 h.

Ten 14-week-old New Zealand white rabbits were randomly assigned to two groups of five rabbits each and immunized i.m. with 10^6 $TCID_{50}$ rAdV-SFV-E2 or rAdV-SFV-empty, and serum samples were collected essentially weekly and tested for the persistence of anti-CSFV antibodies using the IDEXX HerdChek* CSFV Antibody Test Kit.

2.5. Immunization and challenge in pigs

Thirty 5-week-old healthy pigs from a CSF-free swine herd were randomly allocated into six groups of five animals each. Pigs in Group A were inoculated i.m. with 10^7 $TCID_{50}$ rAdV-SFV-E2. Group B pigs were immunized with one-dose C-strain vaccine. Group C were immunized with 10^7 $TCID_{50}$ rAdV-E2. Group D and E were immunized with 100 μg of pSFV1CS-E2 or pSFV1CS, respectively. Group F were immunized with 10^7 $TCID_{50}$ rAdV-SFV-empty. The pigs were boosted 3 weeks later with the same vaccine and dose. Seven weeks after the booster immunization, all pigs were challenged with 100 LD_{50} CSFV Shimen strain. After challenge, clinical signs and body temperature were recorded daily throughout the experiment as described previously [17].

Serum samples were collected weekly post-immunization. Whole blood samples were collected at 0, 1, 3, 6, 9 and 12 days post-challenge (DPC) and subjected to detection of viral RNA copies by a real-time PCR as described previously [22]. At 15 DPC, all pigs were euthanized and subjected to pathological and immunohistochemical (IHC) examinations.

2.6. Blocking enzyme-linked immunosorbent assay (ELISA) and serum–virus neutralizing test (SNT)

Serum samples were collected at weekly intervals after immunization and at 2-day intervals after challenge (days 0–12) and tested for the presence of the CSFV-specific NAbs by using the IDEXX HerdChek* CSFV Antibody Test Kit, according to the manufacturer's instructions.

Serum samples from each group were collected at 0, 3, 5 weeks following the prime immunization and 0, 6, 9 DPC and were heat-inactivated at 56 °C for 30 min before testing. The SNT was carried out in 96-well flat bottom nonpyrogenic polystyrene culture plates (Corning, USA) as described elsewhere [24]. Briefly, two-fold diluted sera were mixed with equal volume of 200 $TCID_{50}$ CSFV Shimen strain and incubated for 60 min at 37 °C. The serum–virus mixtures were inoculated to the confluent PK-15 cells cultured in 96-well plates and incubated for 60 min at 37 °C. The inoculated cells were then cultured for 72 h at 37 °C. IFA was conducted as follows. Inoculated PK-15 cells were washed with PBS for three times and fixed with cold dehydrated alcohol for 20 min. The fixed cells were then incubated with anti-E2 mAb HQ06 [21] for 1 h at 37 °C in a moist chamber followed by three times washing with PBS. The cells were then incubated with FITC-labeled goat anti-mouse IgG (Sigma–Aldrich, St. Louis, MO) for 45 min at 37 °C, followed by washing three times with PBS. The cells were covered with 90% alkaline glycerine and examined under a fluorescence microscope (Nikon TE200, Japan), and the titers of CSFV-specific NAbs were determined and expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells.

2.7. Lymphocyte proliferation and cytokine assay

Cell-mediated immunity was assessed by measuring lymphocyte proliferation. Four weeks after the booster immunization, lymphocytes were isolated from anticoagulated blood of the immunized pigs using the swine lymphocyte isolation reagent (TBD, Tianjin, China). An aliquot of 2×10^7 aseptically isolated cells was dispensed into 96-well plates and stimulated with purified CSFV virions (10 μg/mL, 200 μL each well), concanavalin A (ConA), or medium alone (mock). The lymphocyte proliferation was detected with Cell Counting Kit-8 (CCK-8) after a 4-day incubation. The OD values were measured at 450 nm using a microtiter plate reader. Each sample was analyzed in triplicate. The stimulation index (SI) was calculated by: SI = (the mean of OD_{450nm} for CSFV-stimulated cells)/(the mean of OD_{450nm} for mock-treated cells). Culture supernatants were collected after 4-day incubation at 37 °C, 5% CO_2, and the interferon (IFN)-γ concentrations in the supernatants were measured with a commercially available ELISA kit (R&D, USA).

2.8. Immunohistochemistry

At 15 DPC, all pigs were euthanized and subjected to pathological IHC examinations [23]. Tissues (tonsils, spleen, kidney and lymph nodes) were collected from the animals. Frozen sections were prepared and fixed with cold acetone for 10 min. The tissue sections underwent E2 immunostaining, as described in Section 2.2.

2.9. Real-time RT-PCR

Whole blood samples were collected at 0, 1, 3, 6, 9 and 12 DPC. Latent CSFV RNA was quantified by real-time RT-PCR using the TaqMan System, ABI 7700 Sequence Detector (PE Applied Biosystem, USA) with primers and probes specific for CSFV, as described previously [22]. Briefly, Viral RNA was extracted from 140 μL of whole blood samples using an RNA isolation kit (Qiagen, USA) according to the manufacturer's instructions. RNA was eluted using 40 μL of DEPC-treated water. Reverse transcription was performed in a final volume of 30 μL containing 20 μL of RNA solutions, 6 μL of 5 × RT buffer, 1 μL of dNTP (10 mM each), 50 pmol of 9-random hexamers, 20 U of reverse transcriptase XL (AMV), and 20 U of RNase inhibitor (TaKaRa, Dalian, China), and incubated at 42 °C for 1 h and at 70 °C for 15 min. The reaction products were used for real-time PCR. The real-time PCR was performed in a total volume of 25 μL containing 2 μL of cDNA, 2.5 μL of 10× Ex Taq buffer, 2 μL of dNTP (2.5 mM each), 7 μL of $MgCl_2$ (25 mM), 1 μL of CSFV-F (5′-GAA CTG GGC TAG CCA TG-3′) and CSFV-R (5′-ACT GTC CTG TAC TCA GGAC-3′) (10 mM), 0.5 μL of FAM-CSFVv111 (5′-FAM-AGG ACT AGCAA ACG GAG GGA CTA GCC G-TAMARA-3′), and 1 U of Hot Start EX Taq polymerase (TaKaRa). Cycle times were: pre-denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 45 s. Fluorescent signal measurements were carried out during the elongation step. Data were calculated as the geometric mean from three independent experiments.

2.10. Statistical analysis

Data were analyzed using the SPSS 14.0 software. One-way ANOVA followed by Duncan's multiple range test was used to compare the parameters among the different groups.

3. Results

3.1. Expression of the E2 protein in rAdV-SFV-E2-infected HEK 293 cells

Cytopathogenic effects (CPE) appeared in the HEK 293 cells at 24 h following infection with rAdV-SFV-E2. A high titer of rAdV-SFV-E2 was obtained after five passages (10^8 $TCID_{50}$/mL). IFA was used to determine whether E2 protein was expressed in the HEK

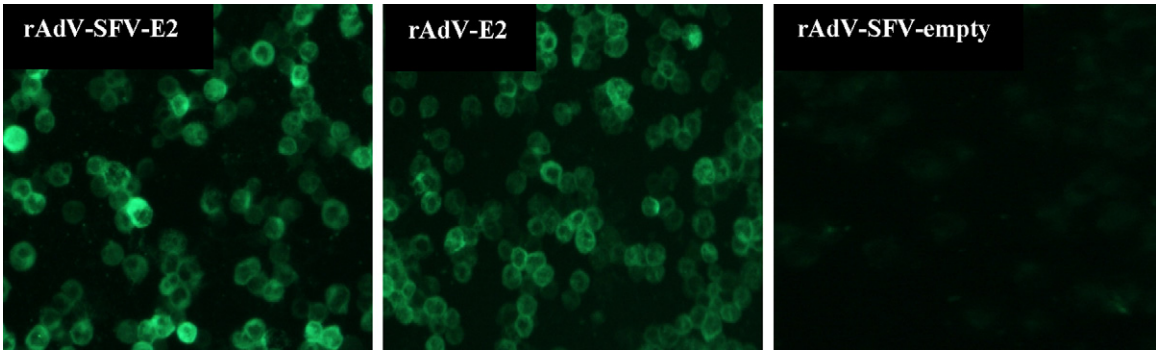

Fig. 2. Immunofluorescence assay (IFA) analysis of the E2 expression in HEK 293 cells infected with the recombinant adenovirus rAdV-SFV-E2. HEK 293 cells infected with rAdV-E2 or rAdV-SFV-empty were used as positive or negative control, respectively. The cells were fixed 48 h post-infection and were analyzed by IFA using the anti-E2 mAb HQ06 as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody.

293 cells infected with rAdV-SFV-E2, rAdV-E2, or rAdV-SFV-empty. Specific E2 immunostaining was demonstrated in the HEK 293 cells infected with rAdV-SFV-E2 or rAdV-E2, but not with rAdV-SFV-empty, and the E2 protein was detected predominantly on the cell membrane, as shown in Fig. 2.

Western blot results showed that a ~50 kDa protein band, consistent with the predicted size of the E2 protein, was identified in the HEK 293 cells infected with rAdV-SFV-E2 or rAdV-E2-infected (lanes 1 and 2, Fig. 3), but not in the HEK 293 cells infected with rAdV-SFV-empty (lane 3, Fig. 3).

3.2. Antibody production and protection in rabbits induced by rAdV-SFV-E2

To evaluate the immunogenicity of the chimeric vector vaccine, an immunization and challenge experiment was conducted in the rabbit, which is a nice and convenient model for the efficacy evaluation of vaccines against CSF [17], based on protection of rabbits from fever induced by C-strain, a lapinized live vaccine against CSF. As low as 2.5×10^5 $TCID_{50}$ rAdV-SFV-E2 was able to confer complete protection of rabbits from challenge with C-strain, and even

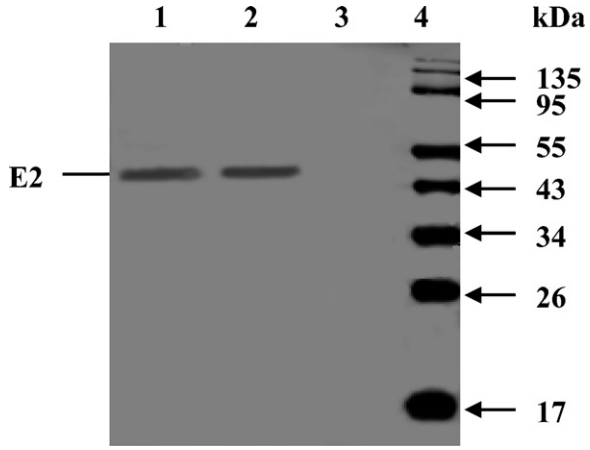

Fig. 3. Western blot analysis of the E2 protein expression in rAdV-SFV-E2-infected HEK 293 cells. Lane 1, cell lysates of rAdV-SFV-E2; Lane 2, cell lysates of rAdV-E2; Lane 3, cell lysates of rAdV-SFV-empty; Lane 4, prestained protein marker (MBI Fermentas, USA). HEK 293 cells were infected with respective recombinant adenoviruses for 48 h and collected and lyzed. The cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with the anti-E2 mAb HQ06, followed by incubation with goat anti-mouse IgG conjugated with horseradish peroxidase. The arrows indicate the bands of proteins standards.

10^5 $TCID_{50}$ rAdV-SFV-E2 provided at least half protection (Table 1). The anti-CSF antibodies developed as early as 9 days and lasted at long as 189 days in rabbits immunized with rAdV-SFV-E2 (Fig. 4).

3.3. Antibodies induced by rAdV-SFV-E2 in pigs

The level of E2-specific antibodies was measured by blocking ELISA and SNT following vaccination. E2-specific antibodies were first detected at 2 weeks post-immunization with rAdV-SFV-E2 or C-strain, with the mean antibody blocking rate of 50.9% or 46.77% (the cutoff of the assay is 40%), respectively. Four weeks post-immunization (1 week after the second immunization), all rAdV-SFV-E2- or C-strain-vaccinated pigs and some of the rAdV-E2-vaccinated pigs seroconverted. The pigs of other three groups (pSFV1CS-E2, pSFV1CS, and rAdV-SFV-empty) did not seroconvert. The CSFV-specific antibodies of the rAdV-SFV-E2, rAdV-E2 and C-strain groups increased thereafter and reached a peak at 5 weeks post-immunization. After virulent challenge, the antibody levels in groups rAdV-SFV-E2, rAdV-E2, C-strain or pSFV1CS-E2 increased markedly after a short-time slight decrease, and the antibody blocking rates of the four groups increased to about 80% at 9 DPC, indicating an anamnestic response upon challenge. There is no significant difference in antibody level between the rAdV-SFV-E2 group and the C-strain group ($p > 0.05$), whereas there is a highly significant difference between the rAdV-SFV-E2 group and the pSFV1CS-E2 or rAdV-E2 group in most time points prior to challenge ($p < 0.01$). None of the pigs in the rAdV-SFV-empty or pSFV1CS groups produced measurable CSFV-specific antibodies throughout the experiment (Fig. 5).

Anti-CSFV NAbs were detected in several groups at 3 weeks post-vaccination, with mean neutralization titers of 1:16 for rAdV-SFV-E2 and rAdV-E2 groups, and 1:17 for C-strain group. The neutralization titers peaked at 8 weeks post-vaccination (5 weeks post-boost), with 1:278 for rAdV-SFV-E2 group, 1:300 for C-strain group, and 1:110 for rAdV-E2 group. Nine days after challenge, neutralization titers rose to 1:766 for C-strain group, followed by rAdV-SFV-E2 group (1:686), rAdV-E2 (1:520), and pSFV1CS-E2 (1:544) (Table 2). No neutralization titers were detected in control groups rAdV-SFV-empty and pSFV1CS.

3.4. Lymphoproliferation induced by rAdV-SFV-E2 in pigs

To evaluate cell-mediated immunity in immunized pigs, CSFV-specific lymphocyte proliferation was measured four weeks after the booster immunization. Proliferative responses were observed

Table 1
Protection of rabbits from challenge with C-strain conferred by different dosages of rAdV-SFV-E2.

Groups	Doses ($\times 10^5$ $TCID_{50}$)	Seroconvertion 10 days post-immunization	Seroconvertion 4 weeks post-immunization (before challenge)	Mean antibody blocking rates before challenge (%)	Fever reaction post-challenge	Viral RNA in the spleen
rAdV-SFV-E2	10	5/5	5/5	72.46	0/5	0/5
	5	5/5	5/5	72.15	0/5	0/5
	2.5	3/5	4/5	54.11	0/5	0/5
	1.25	2/5	5/5	56.89	0/5	1/5
	1	1/5	3/5	35.28	2/5	2/5
	0.1	0/5	1/5	14.41	5/5	5/5
rAdV-SFV-empty	10	0/5	0/5	7.97	5/5	5/5

Thirty-five 14-week-old New Zealand white rabbits were randomly assigned to seven groups of five rabbits each and immunized intramuscularly with one-dose of rAdV-SFV-E2 of different titers or control virus rAdV-SFV-empty, and challenged intravenously with 2000 RID_{50} (50% rabbit infection dose) C-strain 4 weeks post-immunization. Serum samples were collected and tested for anti-CSFV antibodies by the IDEXX HerdChek* CSFV Antibody Test Kit (cutoff: 40%). Rectal temperatures were recorded every 6-h post-challenge, and the spleen was collected and tested for the viral RNA copies by the real-time RT-PCR as described previously (22). Fever was recognized with elevated rectal temperature of $\geq 1\,^{\circ}C$ and lasting for at least 18 h.

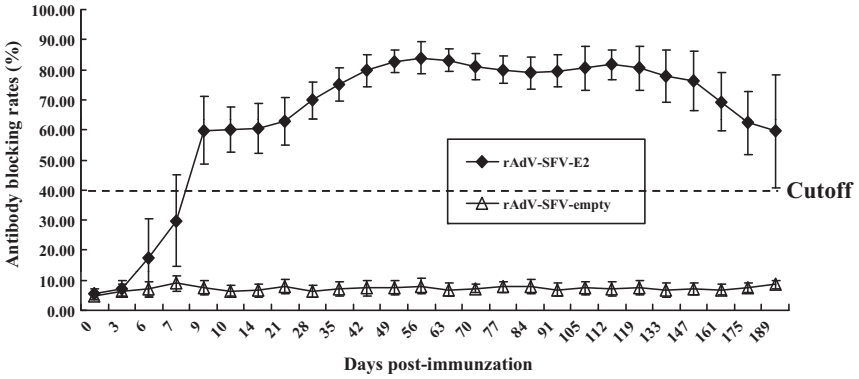

Fig. 4. Serum antibody responses to rAdV-SFV-E2 in rabbits. Ten 14-week-old New Zealand white rabbits were randomly divided into two groups of five rabbits each and immunized intramuscularly with 10^6 $TCID_{50}$ rAdV-SFV-E2 or rAdV-SFV-empty, and serum samples were collected weekly and tested the anti-CSFV antibodies with the IDEXX HerdChek* CSFV Antibody Test Kit.

in all groups except the rAdV-SFV-empty and pSFV1CS groups. Lymphocyte proliferation was the highest in the C-strain group, followed by the group rAdV-SFV-E2, then groups pSFV1CS-E2 and rAdV-E2. No CSFV-specific proliferation was observed in the control groups rAdV-SFV-empty and pSFV1CS. There was no significant difference in SI between the rAdV-SFV-E2 group and the C-strain group ($p > 0.05$), whereas there is a significant difference in SI between the rAdV-SFV-E2 group and the pSFV1CS-E2 or rAdV-E2 group ($p < 0.05$), and a highly significant difference in SI between the rAdV-SFV-E2 group and the rAdV-SFV-empty or pSFV1CS group ($p < 0.01$) (Fig. 6).

IFN-γ contents in the supernatants of lymphocytes were measured using an ELISA kit. The highest IFN-γ was detected in rAdV-SFV-E2 group, followed by C-strain group, pSFV1CS-E2 group and rAdV-E2 group, and the lowest was seen in pSFV1CS and rAdV-SFV-empty groups. There was no significant difference between the rAdV-SFV-E2 group and the C-strain group ($p > 0.05$). In contrast, there was a significant difference between the rAdV-SFV-E2 group

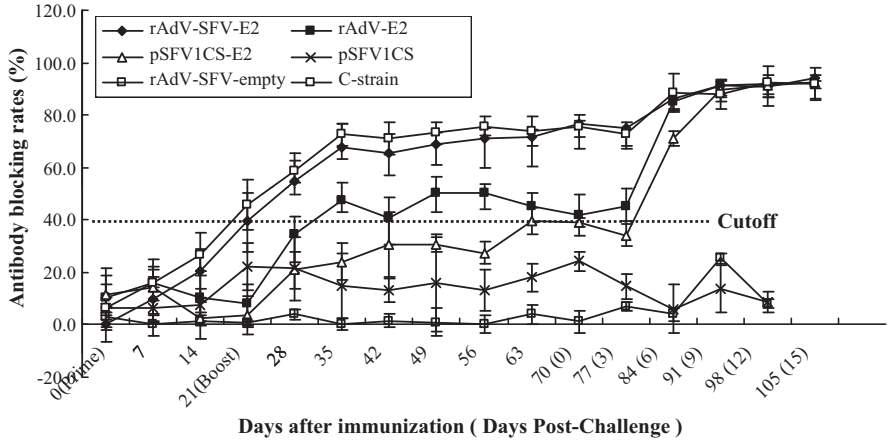

Fig. 5. Detection of serum antibodies in the immunized pigs using blocking ELISA. Pigs were immunized with respective vaccines at weeks 0 and 3 and challenged with CSFV Shimen strain at week 10. Serum samples were collected at weekly intervals after immunization and at two-day intervals after challenge (days 0–12) and tested for the presence of the CSFV-specific NAbs using the IDEXX HerdChek* CSFV Antibody Test Kit (a blocking ELISA based on competition between peroxidase-labeled anti-E2 neutralizing mAb and serum NAbs for binding CFSV antigens), according to the manufacturer's instructions. Standard deviations are shown as error bars.

Table 2
Neutralizing antibody titers in the sera of the immunized pigs.

Groups	Titers					
	Weeks after the priming immunization or days post-challenge					
	0	3 (0 week post-boost)	5 (2 weeks post-boost)	8 (5 weeks post-boost)	6 days post-challenge	9 days post-challenge
rAdV-SFV-E2	<10	16 ± 3	102 ± 32	278 ± 82	435 ± 111	686 ± 111
C-strain	<10	17 ± 13	129 ± 0	300 ± 55	663 ± 111	766 ± 145
rAdV-E2	<10	16 ± 5	65 ± 0	110 ± 55	217 ± 0	520 ± 145
pSFV1CS-E2	<10	< 10	4 ± 18	82 ± 18	179 ± 65	544 ± 111
pSFV1CS	<10	<10	<10	<10	<10	–
rAdV-SFV-empty	<10	<10	<10	<10	<10	–

Serum samples were collected at different times following immunization and challenge and subjected to detection of anti-CSFV neutralizing antibody titers by serum–virus neutralization test. The diluted sera were mixed with equal volume of 200 $TCID_{50}$ CSFV Shimen strain and incubated for 60 min at 37 °C. The serum–virus mixtures were inoculated to confluent PK-15 cells cultured in 96-well plates and incubated for 60 min at 37 °C. The inoculated cells were then incubated for 72 h at 37 °C. IFA was performed as described in Section 2. The cells were examined under a fluorescence microscope, and the titers of CSFV-specific NAbs were determined and expressed as the reciprocal of the highest dilution at which infection of the PK-15 cells was inhibited in 50% of the culture wells. –: Not detectable.

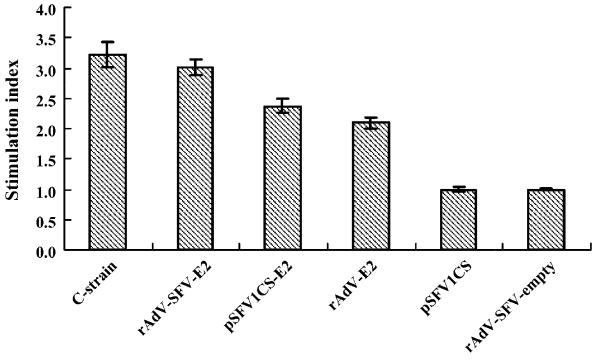

Fig. 6. Splenocyte proliferation responses in the immunized pigs measured by CCK-8 assay. Four weeks after the booster immunization, lymphocytes were isolated from anticoagulated blood of the immunized pigs and dispensed into 96-well plates and stimulated with CSFV Shimen strain antigens (10 μg/mL, 200 μL each well). After 4 days of incubation, the lymphocyte proliferation was measured by Cell Counting Kit-8 (CCK-8). The OD_{450nm} values of samples were determined and the stimulation index was calculated.

and the pSFV1CS-E2 ($p < 0.05$), and a highly significant difference between the rAdV-SFV-E2 group and the rAdV-E2, rAdV-SFV-empty or pSFV1CS groups ($p < 0.01$) (Fig. 7).

3.5. Protection of pigs immunized with rAdV-SFV-E2

No adverse clinical reactions were observed in any of the pigs after vaccination. Seven weeks after the booster immunization, pigs were challenged with 100 LD_{50} CSFV Shimen strain. No clinical symptoms were observed after challenge in pigs immunized with rAdV-SFV-E2 or C-strain. Three out of five pigs in the rAdV-E2 and pSFV1CS-E2 groups exhibited fever, but all returned to normal 2–4 days later. In the rAdV-SFV-empty and pSFV1CS groups, all pigs displayed typical CSF signs (inappetence, apathy, chill, prostration, incoordination, constipation followed by diarrhea, locomotor ataxia and posterior paresis) with high fever (40.5–42 °C) from 2 DPC until death. The fever frequencies are the highest for rAdV-SFV-empty group (29/33) and pSFV1CS group (33/43), followed by pSFV1CS-E2 group (12/75) and rAdV-E2 group (10/75) (Table 3). All the pigs in the pSFV1CS and rAdV-SFV-empty groups died 6–9 DPC, while the pigs in other groups survived.

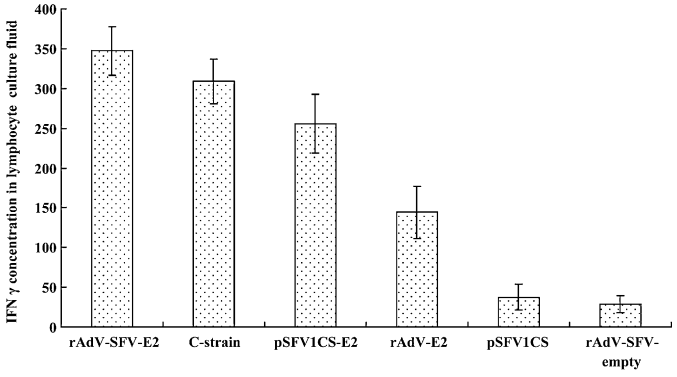

Fig. 7. Detection of IFN-γ from lymphocyte supernatants. Lymphocytes were isolated from anticoagulated blood of the immunized pigs at 4 weeks after the booster immunization and dispensed into 96-well plates and stimulated with CSFV Shimen strain antigens (10 μg/mL, 200 μL each well). After 4 days of incubation the lymphocyte supernatants were collected, and the IFN-γ contents of the supernatants were measured using commercially available ELISA kits following the manufacturer's instructions (R&D, USA).

Whole blood samples were collected at 0, 1, 3, 6, 9 and 12 DPC, and latent CSFV RNA was quantified by real-time RT-PCR. No viral RNA was detected in pigs immunized with rAdV-SFV-E2 and C-strain. Viral RNA were detected in two out five pigs in the rAdV-E2 and pSFV1CS-E2 groups at 3 and 6 DPC, with RNA loads about 10^3 copies/μL. Greater than 10^3 copies/μL were detected in 5 out of 5 pigs immunized with rAdV-SFV-empty or pSFV1CS from 3 DPC, up to a peak load of over 10^6 copies/μL (Table 4).

At 15 DPC, all the pigs were euthanized and subjected to pathological examination. All pigs in the rAdV-SFV-empty and pSFV1CS groups showed severe pathological changes, including hemorrhages with necrotic foci in the tonsils, enlargement and hemorrhage of the lymph nodes, infarcts in the spleen, extensive petechiae in the kidney and bladder, and button-like ulcers in the ileocecal valve. Some pigs in the rAdV-E2 (3/5) and pSFV1CS-E2 (2/5) groups showed mild lesions (such as slight hemorrhages in the lymph nodes and spleen) and the remaining pigs in these groups were free of pathology. None of the pigs immunized with rAdV-SFV-E2 and C-strain showed pathological changes. Virus antigens were readily detected by immunohistochemistry

Table 3
Clinical outcome of the immunized pigs following virulent challenge.

Groups	Fever (≥40.5 °C)	Days to fever onset	Fever frequency	Survival
rAdV-SFV-E2	0/5	–	0/75[a]	5/5
C-strain	0/5	–	0/75	5/5
rAdV-E2	3/5	3	10/75	5/5
pSFV1CS-E2	3/5	2	12/75	5/5
pSFV1CS	5/5	1	33/43	0/5
rAdV-SFV-empty	5/5	1	29/33	0/5

Note: animals were immunized with respective vaccines at week 0 and 3 and challenged with CSFV Shimen strain at week 10. After challenge, clinical signs and rectal temperatures were recorded daily throughout the experiment. Fever, days to fever onset, fever frequency and survival were also calculated. Fever is defined as rectal temperature ≥40.5 °C.

[a] days with fever/total days observed.

Table 4
Detection of viral RNA in the whole blood samples from the immunized pigs after virulent challenge by real-time RT-PCR.

Groups	Pig no.	Days post-challenge					
		0	1	3	6	9	12
r AdV-SFV-E2	A1	–	–	–	–	–	–
	A2	–	–	–	–	–	–
	A3	–	–	–	–	–	–
	A4	–	–	–	–	–	–
	A5	–	–	–	–	–	–
C-strain	C1	–	–	–	–	–	–
	C2	–	–	–	–	–	–
	C3	–	–	–	–	–	–
	C4	–	–	–	–	–	–
	C5	–	–	–	–	–	–
rAdV-E2	B1	–	–	–	–	–	–
	B2	–	–	–	–	–	–
	B3	–	–	1.10×10^2	1.10×10^3	–	–
	B4	–	–	1.13×10^3	1.67×10^3	–	–
	B5	–	–	–	–	–	–
pSFV1CS-E2	D1	–	–	1.07×10^2	–	–	–
	D2	–	–	1.59×10^2	–	–	–
	D3	–	–	–	–	–	–
	D4	–	–	–	–	–	–
	D5	–	–	1.37×10^2	9.55×10^2	–	–
pSFV1CS	E1	–	–	2.05×10^3	3.96×10^5	/	/
	E2	–	–	1.61×10^3	3.34×10^5	3.14×10^5	/
	E3	–	–	6.66×10^3	1.05×10^6	3.83×10^5	/
	E4	–	–	4.52×10^3	9.13×10^5	3.08×10^5	/
	E5	–	–	7.41×10^2	2.63×10^5	1.78×10^4	/
rAdV-SFV-empty	F1	–	–	9.6×10^3	6.7×10^6	3.79×10^5	/
	F2	–	–	2.99×10^4	3.54×10^6	7.6×10^5	/
	F3	–	–	4.27×10^4	6.2×10^6	9.3×10^3	/
	F4	–	–	2.68×10^4	1.2×10^6	9.6×10^4	/
	F5	–	–	4.43×10^4	2.04×10^6	3.75×10^5	/

Note: whole blood samples were collected at days 0, 1, 3, 6, 9 and 12 post-challenge, and RNA was extracted using the RNA isolation kit (Qiagen, USA), CSFV RNA was quantified by a real-time RT-PCR described previously. –: Not detectable;/: died.

in various organs of the pigs immunized with rAdV-SFV-empty and pSFV1CS, and to a lesser extent in the lymph nodes, kidneys and tonsils of pigs immunized with rAdV-E2 or pSFV1CS-E2; however, no CSFV antigens were detected in the pigs immunized with C-strain or rAdV-SFV-E2 (data not shown).

4. Discussion

Although attenuated CSF vaccines have been used widely to control CSF, occasional CSF outbreaks still occur in many areas. In recent years, CSF immunization failures have been reported frequently, most commonly due to interference by maternal antibodies or immune tolerance resulting from incorrect immunization schedules. Vaccine failure frequently results in atypical or chronic CSF. Moreover, it is difficult to differentiate the naturally infected from vaccinated pigs when using routine assays, which presents an additional challenge for the eradication of CSF. These issues have led to the reassessment of currently available attenuated vaccines, and efforts have been made to develop safer and more effective DIVA vaccines. It has been assumed that live attenuated vaccines are superior to non-replicating vaccines in the quality of the antiviral immune response and the level and duration of protective immunity. As the non-DIVA trait of conventional live attenuated CSFV vaccines goes against a DIVA vaccine strategy, alternative vaccine strategies are being sought that may also have improved safety and efficacy. Recent studies have demonstrated the feasibility of developing live vector and other novel DNA vaccines [25–27]. Both DNA and recombinant virus vaccines based on the CSFV E2 protein elicited varied protective antibody responses in pigs [17–19,28–31].

To overcome the low protein expression and immunogenicity of conventional DNA vaccines, a new generation of vector systems based on alphavirus replicons, including SFV, Sindbis virus (SINV), and Venezuelan equine encephalitis virus (VEEV), has been developed. These replicon-based vectors have great potential for cancer gene therapy and vaccine development. Alphavirus RNA replication induces apoptosis in most cells from vertebrate origin by mechanisms that are not yet completely understood. Besides, alphavirus vectors have a number of advantages, including a broad host cell range, tropism for dendritic and monocytic cells [7,32,33], the ability to stimulate innate immune receptors [34], high level of transgene expression, and the lack of pre-existing immunity.

In previous studies, we constructed two kinds of CSF vaccines using an SFV-replicon vector (pSFV1CS-E2) or a replication-incompetent adenovirus vector (rAdV-E2). Both vaccines provided protection against CSFV challenge [17–19]. pSFV1CS-E2, a SFV replicon-based DNA vaccine, or "suicidal" DNA vaccine, retains the advantages of alphavirus vectors and enables high level heterologous protein expression and the induction of a strong immune response. However, the high costs associated with DNA preparation and the inefficient delivery make DNA vaccination of pigs economically non-viable. Gene guns may serve as effective administration tools to enhance the immunization effect of DNA plasmids [35], but these are also unsuitable for veterinary practice due to the high cost and the need to pre-coat the DNA with gold particles. A convenient system with high efficiency gene delivery is therefore needed to maximize the potential of alphavirus replicon vectors. Replication-incompetent recombinant adenoviruses have been very widely used in gene delivery and vaccine design. They are extremely safe and stable, easily produced at high titers, can be lyophilized and administered by various routes, have a broad

host cell range. They express transgenes to high levels, activate the innate immune system, and stimulate dendritic cell maturation [36–39], leading to elicitation of strong immune responses.

In this study, we used the replication-defective Ad5 vector to develop a chimeric vector vaccine (rAdV-SFV-E2) that delivers an SFV replicon expressing the CSFV E2 gene. Ad5 vector was chosen in consideration of its high infectivity for many cell types, efficient gene delivery and powerful adjuvant activity [24] as well as the absence of pre-existing anti-Ad5 immunity in swine [40]. The alphavirus replicon was packaged correctly into Ad vector, as demonstrated by inhibition of rAdV-SFV-E2 by anti-AdV antibodies in a dose-dependant manner but no influence by anti-E2 mAb and typical AdV virions in electronic microscope (data not shown). In a rabbit vaccination-challenge model, one dose of 10^6 $TCID_{50}$ rAdV-SFV-E2 induced early (as early as 9 days) and long-lasting (up to 189 days) immune responses (Fig. 4), and one dose of rAdV-SFV-E2 as low as 2.5×10^5 $TCID_{50}$ was able to confer complete protection from C-strain challenge (Table 1). In pigs, which are the natural host of CSFV, two doses of 10^7 $TCID_{50}$ rAdV-SFV-E2 elicited robust immune responses and conferred sterile immunity against lethal CSFV challenge, and vaccinated animals showed a notable anamnestic response upon challenge. This efficacy is comparable to that of the live attenuated vaccine C-strain and is much more efficacious than that of the conventional adenovirus-vectored vaccine (rAdV-E2) or alphavirus replicon-vectored vaccine (pSFV1CS-E2). To our best knowledge, none of the previously described gene-based vaccines against CSF provided sterile protection against lethal challenge. This suggests that the "two-in-one" chimeric vector combines the advantages of the two vector systems, resulting in a substantial enhancement of immunogenicity. Therefore, the strategy represents a novel, innovative and powerful vaccine development platform, and we are currently attempting to apply this strategy to other animal diseases. It will be important to evaluate the clinical efficacy of this new vaccine strategy and establish systems for large-scale production of the vaccine. The SFV replicon containing the inserted antigen gene is approximately 11 kb, which is close to the upper limit of adenovirus vectors. Thus, cloning of the transfer vector was very difficult. The new-generation "gutless" adenovirus vectors might be superior for the large inserts that are required by our vaccine strategy.

CSF can be effectively prevented by NAbs induced by vaccination. However, T cell-mediated immunity is an important parameter for conferring early protection against CSFV challenge. There is a close correlation between IFN-γ production and the involvement of Th_1-type CD4+ T cells [12,41]. IFN-γ is the principal cytokine for activating macrophages and upregulates expression of MHC-I and MHC-II molecules, and co-stimulatory molecules. It not only promotes the differentiation of CD4+ T cells into Th_1 cells and inhibits the proliferation of Th_2 cells, but also stimulates the production of those IgG subclasses that activate the complement pathway. T cells from rAdV-SFV-E2-immunized pigs proliferated significantly and produced high-level IFN-γ upon stimulation with CSFV antigens (Figs. 6 and 7), though the stimulus indexes are relative low compared to other methods to estimate T cell proliferation (such as ^3H–T incorporation), possibly due to the less sensitivity of the CCK-8 assay. The ability of rAdV-SFV-E2 to induce a strong antigen-specific IFN-γ response may be crucial for its ability to induce solid protection against CSFV infections. It has been demonstrated that replication-defective rAd5 is the most potent recombinant vector for eliciting CD8+ T cell responses dependent on CD11c+ dendritic cells in humans [42]. It will be interesting to explore whether the CD8+ T cell response is elicited in pigs immunized with the chimeric vector vaccine. It has also been shown that Ad vectors can activate innate immunity via MyD88/TLR9-dependent and -independent mechanisms [43]. More detailed studies on the contributions of each component of immune responses including innate immunity will help us to uncover the immunological mechanisms underlying the improvement of the protection conferred by rAdV-SFV-E2 relative to that conferred by pSFV1CS-E2 or rAdV-E2.

Our novel approach will develop a vaccine with several major advantages: (1) safe and able to elicit early and long-lasting immunity; (2) low immunization dose; (3) inexpensive to manufacture. Furthermore, compared to conventional vaccines, such as C-strain-based attenuated vaccines, the rAdV-SFV-E2 vaccine would not be susceptible to interference with maternal antibodies, as there is no pre-existing immunity to Ad5 in swine [40]. In addition, the use of the E2 glycoprotein alone allows the discrimination between vaccinated and infected animals [44], and animals immunized with rAdV-SFV-E2 could be discriminated from those infected with CSFV by measuring serological responses against E^{rns}. Notably, the antibody response curve of rAdV-SFV-E2 in pigs highlights the requirement of re-immunization to induce strong immune response and confer solid protection when used as vaccine.

In summary, we have developed an adenovirus/SFV replicon chimeric vector-based vaccine, which elicited strong humoral and cellular responses, and provided sterile immunity and complete protection from lethal CSFV challenge. To our best knowledge, this represents the first gene-based vaccine that is able to confer complete protection from lethal CSF. This new-concept vaccination strategy may also be valuable in developing potent gene-based vaccines against other pathogens. In future studies, we will further optimize the inoculation dosage and frequency.

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