Identification of E2 strain with improved secretion and immunogenicity against CSFV in piglets

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Abstract
Background: Outbreaks of CSFV cause significant economic losses in the swine industry. Subunit vaccines present advantages in DIVA immunogenicity and safety. The technology was limited due to the low yield and the high cost with multiple and large doses.

Results: A novel E2 sequence (E2ZJ) was identified from an epidemic strain of Zhejiang for outstanding secretion in baculovirus and enhanced immunogenicity. E2 secretion induced with the selected signal peptide, SPZJ, increase at least 50% as compared to any other signal peptides tested. Besides, unique antigenic features were identified in E2ZJ. E2ZJ elicited CSFV antibody at the earlier stage than other E2 types tested in mice. Meantime, higher level of neutralization antibody with E2ZJ was detected than other E2s at the same dosage. Further, in piglets, E2ZJ successfully elicited neutralizing immunity. A single dose of 5 μg of E2ZJ was sufficient to induce protective antibody against CSFV in piglets and provided 100% protection against lethal virus challenge.

Conclusions: In this study, with advantages in secretion and immunogenicity over counterparts, SPZJ-E2ZJ is a cost-effective and efficacious vaccine candidate against CSFV. Keywords: Classical swine fever virus; novel signal peptide; SPZJ-E2ZJ; subunit vaccine; protective immunity

Background
Classical Swine Fever (CSF) is a highly contagious viral disease which is economically important to the pig industry worldwide [1]. It is endemic in Asia, some areas of Central and South America and in many Eastern European countries with sporadic occurrence in Western Europe [2, 3]. CSFV has three major genetic groups, namely, Groups 1, 2, and 3. Groups 1 and 2 are further divided into three subgroups, genotypes 1.1-3 and 2.1-3 [4, 5]. The disease can cause several clinical signs, including fever, loss of appetite, weakness and conjunctivitis, which were often accompanied by death within 10–20 days of infection [6]. The etiological agent, CSF virus (CSFV), belongs to the Pestivirus genus within the family Flaviviridae [7]. CSFV genome contains a single open reading frame that encodes a polyprotein which is processed into 12 proteins, including Npro, C, Erns, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [8, 9]. The structural protein E2 is a key determinant for viral entry and immunity [10]. Besides, previous studies revealed that a critical area in E2 is required for CSFV
replication in SK6 cells between protein residues 136–156 [11]. It has been well established that CSFV E2 protein is the major protective antigen which elicits neutralizing antibodies that make it important for producing subunit vaccines against CSFV [12, 13].

Currently, vaccination is the widely used strategy to prevent CSF. Most vaccines available against CSFV are live attenuated vaccines (LAVs). So far, commercially available vaccines mainly consist of live attenuated CSFV of cell line origin or rabbit tissue origin, derived from the commonly used C-strain. These live attenuated vaccines have outstanding efficacy and safety but lack a serological concept of differentiating infected from vaccinated animals (DIVA) thus hampering CSF eradication and cause concern in animal welfare when live rabbits are exploited in vaccine production [14, 15].

Besides, current commercial CSFV E2 vaccines fail to elicit complete protection against heterologous strains of genotype 2.1, which may be caused by antigenic variations among different genotypes [16, 17]. E2 subunit vaccines have been confirmed to induce sufficient CSFV specific antibodies and provide complete protection against homologous CSFV in rabbits [17] and pigs [18, 19]. However, these subunit vaccines usually require large multiple doses to induce the comparable CSFV-specific protective immunity as C-strain LAVs [20]. And there is a delay in the induction of the protective antibody response upon vaccination [6, 21]. Besides, due to the low yield of E2 in a soluble and correct-folding form, the wide application of E2 in CSFV vaccine market is limited [22]. Therefore, the enhancement of soluble E2 expression with correct immunogenicity is the corner stone for this CSFV vaccine to be one of the most efficacious and practical strategies against CSFV in future.

Compared to prokaryotic expression system, recombinant baculovirus expression system produces target proteins with correct protein structure and post-translational modifications such as protein glycosylation and disulfide bonds formation as their natural conformation [23, 24], which leads to efficacious immunogenicity of vaccines. With a proper signal peptide, baculovirus expressed E2 secretes in culture supernatant [25]. However, the secretion efficiency and the immunogenicity of E2 have not been well optimized to meet the needs in vaccine production.

To facilitate the efficacy and wide application of E2 vaccine against CSFV, E2s from different CSFV strains were expressed and compared in this study. A novel signal peptide SPZJ successfully induces
higher E2 expression in cell culture supernatant than any other constructs tested. The protective immune response of E2ZJ protein is superior to E2HZ and E2C protein as shown in IFA and neutralization test. Furthermore, it was shown that reliable protection and immunogenicity is provided as early as 7 days after a single dose vaccination against lethal CSFV challenge. Thus, SPZJ-E2ZJ is a promising candidate subunit vaccine against CSFV.

Results

Identification of a novel signal peptide SPZJ for robust E2 production

To identify the efficient signal peptide for E2 secretion, E2 protein was fused to a series of truncated candidates (SP13, SP18, SP21, SP23, SP25, SP28 or SP33) individually (Fig. 1A). SP23 induced higher level of E2 secretion than any other signal peptides tested (Fig. 1B). Sequence variation was found in SP23s among different CSFV strains, including ZJ01, HZ08 and C strain (Fig. 2A). SP23 of ZJ01 (SPZJ) was able to induce the expression of other E2s (E2C and E2HZ) as well as its native E2ZJ, indicating SPZJ is a common signal peptide for variable E2 production. To identify the best peptide for E2 secretion, the expression of E2 under different SP23s in baculovirus were compared. SPZJ was revealed to induce higher level of E2ZJ secretion than the signal peptides from HZ08 (SPHZ) and C strain (SPC). Finally, SPZJ was compared with SPHZ and SPC individually to induce the secretion of E2HZ and E2C. The secretion level of either E2HZ or E2C elicited with SPZJ is higher than the one with their native signal peptides (Fig. 2B), confirming SPZJ is a strong signal peptide for different E2s to secret in baculovirus. Finally, SPZJ induced at least 50% increase in E2 secretion as compared to conventional signal peptides, such as honeybee melittin signal peptide and SPC from C strain (Fig. 2C). In the small-scale production with shaking flasks (50 ml), E2 yield with SPZJ is up to 65 µg/ml. The yield could be further enhanced when SPZJ is applied to industrial production.

E2ZJ elicits efficient antibody response against CSFV

E2ZJ was classified to group 2.1 based on phylogenetic tree (Fig. 3A). There are three signature mutations, T107S, F155Y and 90T, identified in the antigenic regions of E2ZJ. Meanwhile, E2ZJ, as a strain of group 2, presents sequence features of group 1 as well, such as 20P and 179P (Fig. 3B). This epitope-chimera-like characteristic potentially improve the cross immunogenicity [26]. Thus, the immunogenicity of three recombinant E2s (E2ZJ, E2HZ, E2C) was primarily tested in mice. As shown in
IFA with CSFV infected cells, homologous antibody was observed in the group vaccinated with E2ZJ as early as 14 dpi while no antibody was detected in groups with other E2 types (Fig. 4A). The other E2 vaccinated groups shown positive CSFV antibody response until 21 dpi while E2ZJ elicited significant heterologous immunogenicity (Fig. 4B). Further, in ELISA, E2 antibody level in all vaccinated groups started to rise up at 14 dpi and peaked at 28 dpi. However, higher E2 level against both two genotypes was observed in the group of E2ZJ as compared to other groups from 14 dpi to 28 dpi (Fig. 4C). Similarly, E2ZJ elicited significantly higher level of CSFV neutralizing antibody than other E2 types. At 28 dpi, the neutralization titer of E2ZJ was up to 2000 while the titers from other E2 groups were less than 500. Neither CSFV-specific antibody nor neutralizing antibody was detected in PBS group (Fig. 4D). These findings indicated that E2ZJ presents improved protective immunogenicity than other candidates against different E2s.

Single dose of E2ZJ stimulates protective antibody response in piglets against CSFV

The efficacy of E2ZJ to elicit the protective antibody response was further evaluated in piglets. 25 piglets were individually vaccinated with different dosages of E2ZJ (5 µg, 15 µg or 30 µg) to determine the minimal effective dose for the complete protection against CSFV. In competitive ELISA for CSFV antibody with a commercial kit, at 14 dpi, the mean antibody blocking rates of piglets vaccinated with 15 µg and 30 µg of E2ZJ were higher than the cut-off value of 40% though the group of 5 µg was less than 25%. Meanwhile, in the group of non-ZJ E2 at the dose of 60 µg, average antibody blocking rate was less than 40%. Non-ZJ E2 was a CSFV E2 vaccine candidate expressed in eukaryotic system other than baculovirus. At 21 dpi, the blocking rates were higher than 40% as positive in all vaccinated groups, including the groups of 5 µg of E2ZJ. The antibody response of all E2ZJ groups peaked at 28 dpi at the similar level, which exceeded the group of non-ZJ E2. No CSFV-specific antibody was detected in PBS group (Fig. 5A). CSFV neutralizing antibody response with E2ZJ was evaluated against either the challenge strain Shimen (Fig. 5D) or a pandemic strain HZ08 (Fig. 5C). At 7 dpi, CSFV neutralizing antibody was initially detected in all E2 vaccinated groups, including the group of 5 µg, but not in PBS group. Meanwhile, the neutralizing antibodies titers in 5 µg, 15 µg or 30 µg groups peaked at 28 dpi, with 1:1286, 1:1564, 1:1089 against HZ08 and 1:533, 1:746, 1:533 against Shimen
strain respectively. Neutralization antibody titers against HZ08 strain were higher than those against Shimen strain. PBS group showed no neutralizing antibodies all the time.

Low-dose immunization with E2ZJ protected piglets from CSFV lethal challenge

Further, the protection with E2ZJ against CSFV lethal challenge was studied based on clinical symptom and pathologic presentation. From 3 days after challenge infection, the control PBS group presented cute febrile response (41-41.8 °C) and other clinical symptoms including diarrhea and anorexia. 4 of 5 infected piglets in the PBS group died at 15 dpc (days post challenge). All piglets immunized with E2, including the group of 5 µg E2ZJ, did not develop any symptoms during the whole experiment, except that one pig from the group with 30 µg showed temporary mild fever response (40.3–41.5 °C) at 4 dpc and recovered at 6 dpc (Fig. 5B).

At 16 dpc, all the piglets were euthanized and subjected to pathological and histopathological examination. Non-vaccinated PBS group appeared severe clinical lesions in different organs, including defusing hemorrhage in the kidney and bladder and necrosis in the tonsils. Besides, splenic infarcts and petechia in submaxillary and inguinal lymph nodes were observed in the PBS group. In contrast, no lesion was detected in any organ tested of all E2ZJ vaccinated groups (Fig. 6). Similar severe histopathological lesion in the control group were shown in H&E (Fig. 7A), including depleted lymphoid follicles and hemorrhages (arrow) in the lymph nodes, diffusing hemorrhages (arrow) throughout splenic parenchyma and interstitial spaces of kidney.

CSFV viral antigens in tissues were examined by immunohistochemistry with anti-CSFV polyserum. As shown in Fig. 7B, CSFV positive signals (arrow) were observed in tissues of PBS group but not in the groups vaccinated with any dose of E2ZJ, not even with the dose of 5 µg. These results indicated that a single dose of 5 µg of E2ZJ in piglets successfully prevented CSFV infection replying on the efficient elicitation of protective antibody response against CSFV.

Discussion

Outbreaks of CSFV cause significant economic losses in the swine industry. E2 based subunit vaccines have been shown to be a promising strategy for the control and eradication of this disease, as live attenuated vaccines fail to elicit differentiable immunity between infected and vaccinated animals.
In contrast to prokaryotic systems, baculovirus infected insect cells produce recombinant proteins with correct 3D structure and glycosylation as the original eukaryotic system, which leads to the proper biological function and immunogenicity in protein products, making them functional vaccine candidates [29]. As the main immunogenic protein inducing neutralizing antibodies against CSFV, E2 protein is widely used for vaccine production [30]. Secretion of E2 protein is preferred for the large-scale protein production and purification. Studies performed here demonstrated an efficient E2 secretion system in baculovirus relying on the newly identified signal peptide SPZJ. Together with the selected E2 antigen, SPZJ-E2ZJ presents improved immunogenicity and protective immunity against CSFV infection.

Currently E2 subunit vaccines produced in insect cells have not been widely available yet due to the limited yield [30]. Many strategies have been applied to improve E2 expression, including codon optimization of E2 gene in yeast [32] and promoter selection, such as a polyhedrin, p10, and a minimal Drosophila melanogaster Hsp70 promoter, for the modulated expression [33]. Besides, efforts have been made to signal peptide engineering. Previously, with the deletion of the transmembrane region [34] and the fusion with a certain signal peptide, such as honeybee melittin signal peptide [25] or immunoglobulin kappa (Igκ) signal peptide [35], E2 was adapted to secretion. In this study, compared with a series of truncated signal peptides, results showed that SP23 was important for E2 efficient secretion (Fig. 1B). SPZJ was identified as the most efficient one for E2 secretion in baculovirus system of candidates tested. Besides E2ZJ, SPZJ increase the expression of other E2 types as compared to their native signal peptides (Fig. 2B). E2 yield with SPZJ has been significantly enhanced in comparison to conventional methods. In protein alignment of multiple signal peptides, results indicated that SPZJ shared 86.9% homology with SPC and 95.6% homology with SPHZ (Fig. 2A). 3 amino acids were involved in sequence variations of signal peptides. It was believed that these amino acids be key points in the guidance of protein secretion.

Antigen immunogenicity is mainly determined by epitopes. In antigenic regions of E2, E2ZJ of group 2.1 presents several unique mutations which are not found in other strains within the same group. These substitutions in identified or predicted epitopes should contribute to the enhancement in the
immunogenicity of E2ZJ. E2ZJ displays antigenic features of both genotype I and II CSFV regarding to E2, which may help to improve the cross immunogenicity against different E2 strains. Besides, E2ZJ carries epitopes glycosylation in envelope proteins contributes to the regulation of the immunogenicity as well. E2 glycoprotein protein contains one putative O-linked glycosylation site (O1) and six N-linked glycosylation sites (N1, N2, N3, N4, N5 and N6) [36]. Among them, O1, N1, N2, N3 and N4 are involved in structural unit assembling, which affects the induction of neutralizing antibody [37]. In this study, E2ZJ protein induced the higher level of CSFV specific antibody at the earlier stage than other E2 types tested, confirming the excellent immunogenicity of E2ZJ. The level of glycosylation of E2ZJ detected is between E2C and E2HZ, suggesting the moderate level of glycosylation are favorable for the good immunogenicity of E2 proteins. Although all the identified neutralizing epitopes are conserved among variable strains, including E2ZJ [38], the improved immunogenicity of E2ZJ may result from other neighboring variation related to epitope folding for eases in immune recognition. These findings confirmed that with the good immunogenicity, E2ZJ protein serves as a potential candidate vaccine against CSFV infection.

Rational vaccine dosage is critical to determine the vaccine efficacy. Currently, multiple doses are required for most veterinary subunit vaccines and a large amount of antigen in each dose was used to achieve the sufficient efficacy [39]. Due to the incorrect protein folding or modification as the original viral antigens, subunit vaccines produced in prokaryotic systems usually exploit significantly high effective dose. For example, two doses of *E.coli* expressed CAP of 200 µg were required for piglets against PCV2 challenge [40]. 300 µg of E2 from yeast was used to induce neutralizing antibodies after booster immunization against CSFV infection [20]. For other E2 types, booster dose at 40 µg of baculovirus expressed genotype 1.1 and 2.1 E2 was recommended to elicit immune responses against CSFV challenge [17]. However, excessive antigen immunization may cause the tolerance against the target antigen in hosts, leading to the inefficient antibody induction. Besides, the risks of side effects will be much lifted with overdose from other unpredicted ingredients in the vaccine formulation, such as toxin from *E.coli*. Meanwhile, multiple doses at large amounts of antigen will raise the vaccine cost, causing extra economic burden to pig farms. Encouragingly, as shown in the
vaccine trials here, a single dose of 5 µg of E2ZJ in piglets is sufficient to elicit complete protection against CSFV lethal challenge. The group of 5 µg-E2ZJ developed sufficient CSFV specific antibody at 21 dpi and neutralizing antibody at 7 dpi. Previous studies indicated that CSFV specific antibody response from single dose of 32 µg of E2 last for 6 to 13 month after immunization in pigs [19, 41]. In this study, the immunogenic durability of single dose of 5 µg of E2ZJ in pigs was evaluated until 28 dpi, and the protective durability was monitored until 44 dpi (16 dpc, days post challenge). No CSFV related clinical symptoms were observed in any E2ZJ vaccinated piglets upon CSFV challenge, indicating the sufficient durability of the efficacy of the single dose E2ZJ. Besides, neutralization antibody against both homologous and heterologous strains were detected in all E2ZJ vaccinated animals, confirming the cross protective immunogenicity of E2ZJ. Meantime, for another E2 vaccine candidate (non-ZJE2), which was expressed in non-baculovirus system, one dose of 60 µg failed to induce comparable antibody response as E2ZJ, confirming the advantage of E2ZJ in immunogenicity and vaccine efficacy. Therefore, the single dose of 5 µg of E2ZJ in pigs will be recommended as CSFV vaccine for the complete protection against the disease, which will significantly reduce the cost in vaccination and enhance the vaccine quality.

In summary, the study presented a novel efficient E2 signal peptide for E2 secretion and the lowest effective dose of E2 reported against CSFV lethal challenge in pigs. A single dose of 5 µg of E2ZJ developed a complete protective immune response in pigs and conferred broad protection against the homologous and heterologous CSFV strains. This replies on robust SPZJ-E2ZJ secretion and efficient immunogenicity against CSFV. Hence, serving as a promising vaccine platform, baculovirus expressed SPZJ-E2ZJ is an economical and effective vaccine candidate. It will also be a useful tool for CSF eradication in China, together with other strategies, including differential diagnosis, regional or national-wide eradication campaigns, serological surveillance, and biosecurity procedures.

Conclusions
The study identified SPZJ (SP23) was characterized to promote strong E2s secretion and significantly improved immunogenicity over conventional counterparts. Besides, a single dose of 5 µg E2ZJ was sufficient to induce protective antibody against CSFV in piglets and conferred broad protection
against the homologous and heterologous CSFV strains. The study confirmed that SPZJ-E2ZJ is a cost-effective and efficacious vaccine candidate against CSFV.

Methods
Viruses and cells
CSFV strains Shimen (GenBank: FJ598612.1) and HZ08 (GenBank: EF683627) were received from CATG lab, Zhejiang University, China. C-strain was obtained from China Animal Husbandry Industry Co. Ltd. (GenBank: HM175885, Beijing, China). All CSFV strains were propagated in porcine kidney cells (PK-15, ATCC) in Dulbecco’s minimal essential media (DMEM, Hyclone, Thermo Scientific, USA) with 6% fetal bovine serum (FBS, Invitrogen, USA) at 37 °C with 5% CO₂. High Five and Sf9 insect cells were used to propagate recombinant baculoviruses in SF900 III SFM (Invitrogen, USA) at 27.5 °C.

Construction of recombinant baculoviruses
The transmembrane domain was deleted from E2. Truncated E2 fragments with a signal peptide (SP) were inserted into the EcoRI and Xhol sites of the transfer vector pFBD, generating plasmids as pFBD-E2 and pFBD-SP-E2. All plasmids were verified by sequencing analysis. Recombinant baculoviruses Ac-E2 and Ac-SP-E2 were subsequently generated using the Bac-to-Bac system (Invitrogen) according to the manufacturer’s instructions.

Expression and Purification of E2 protein
High Five cells were cultured in SF900 III SFM at 27.5 °C with shaking (115 rpm) and then inoculated with recombinant baculoviruses at a multiplicity of infection (MOI) of 1. After 96 hours post infection, the culture was centrifuged at 12,000 rpm for 30 min, and supernatant was collected and loaded onto 2 ml Ni-NTA Agarose (Novagen, USA). After washed with 30 ml PBS containing 5 mM imidazole, E2 protein was eluted with 3 ml PBS containing 400 mM imidazole. Purified E2 protein was stored at -20 °C.

Protein samples were separated on 12% SDS–PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, USA) and blocked with 5% (w/v) nonfat milk in PBS containing 0.05% Tween (PBST) for 1 h at 37 °C. Membranes were incubated with anti-E2 monoclonal antibody (6D10) (in-house preparation) or GP64 monoclonal antibody (1: 5000 in PBS, Abcam, USA) at 37 °C for 1 h, rinsed with PBST, and incubated with HRP-conjugated goat anti-mouse IgG (1: 5000 in PBS, Sungene,
China) at 37 °C for 1 h. After washed with PBST for three times, immunoreactive bands were visualized by Super Signal West Pico/Femto Chemiluminescent Substrate (Thermo Scientific, USA) and images were captured with a Gel 3100 chemiluminescent imaging system (Sage Creation Science).

Immunofluorescence assays
PK-15 cells were seeded in 96-well plates one day before infection and infected with CSFV strains at MOI of 1. At 48 h post-infection, cells were permeabilized with 80% ice-cold acetone in PBS for 30 min at -20 °C, blocked with 5% (w/v) non-fat milk in PBS for 1 h, and washed once with PBS. Fixed cells were incubated with diluted mouse serum samples (1: 200 in PBS) for 1 h at 37 °C. After washing with PBS for three times, the cells were incubated with FITC-conjugated goat anti-mouse IgG (H + L) (1: 1000 in PBS, Thermo Scientific) as secondary antibody at 37 °C for 1 h. Finally, the labeled cells were treated with the nuclear dye 4’,6’-diamidino-2-phenylindole dihydrochloride (DAPI, 1:2000 dilution in PBS, Beyotime, China). Cells were observed by an inverted fluorescence microscope (Olympus, Corporation, Tokyo, Japan).

Antigen preparation
The concentration of proteins was measured by BCA protein assay kit (Beyotime Biotechnology, China) and the percentage of purified proteins was determined using the layer chromatography scanner (Biotek, USA). The final concentration of target proteins was calculated based on the readings. Antigens were emulsified with ISA-206 adjuvant (Seppic, France) at a ratio of 50:50 (w/w) according to the manufacturer’s instructions.

Animal immunization and challenge trial
6-week-old female BALB/c mice were purchased from Zhejiang Chinese Medical University Laboratory Animal Research Center (Hangzhou, China). The mice were randomly divided into 4 groups (A, B, C and D, n = 5). Groups A to C were respectively immunized twice with a 2-week interval by the subcutaneous injection of 50 µg of E2 (E2ZJ, E2HZ or E2C). Group D was injected with PBS as control. Serum samples were immediately collected on 0, 7, 14, 21 and 28 days post immunization (dpi) for examination of the levels of CSFV-specific antibodies with indirect ELISA and IFA.

25 piglets were purchased from commercial farm (Hangzhou, China) and were randomly divided into 5 groups (E, F, G, H and L, n = 5) which were free of CSFV before the experiment. Each group was
housed individually. Groups E to G were respectively intramuscularly immunized with a single dose of E2ZJ at 5 µg, 15 µg or 30 µg. Group H was intramuscularly immunized with a single dose of 60 µg of Non-ZJ E2. Group L was intramuscularly injected with PBS as control. Serum samples were immediately collected at 0, 7, 21 and 28 days post immunization (dpi) to detect CSFV specific antibodies by commercial ELISA (IDEXX Laboratories, Shiphol-Rijk, The Netherlands) and the neutralizing antibodies against CSFV. At 28 dpi, piglets were intravenously challenged with CSFV Shimen strain (10^{5.5} TCID_{50} in 2 ml PBS), and piglets were checked daily for clinical signs and rectal temperature [42]. All survival animals were euthanized at 16 days post challenge (dpc). Spleens, kidneys, tonsils, lymph nodes and bladders were collected and subjected to pathological examinations [43–45].

Virus neutralization
Serum samples were twofold serially diluted (starting from 1/4) with DMEM after heat-inactivated for 30 min at 56 °C. Diluted samples were mixed with the equal volume of 100 TCID_{50} of CSFV strain (Shimen or HZ08) and incubated at 37 °C for 1 h. The antibody-virus mixtures were then added to the 96-well plates containing PK-15 cells for 1 h at 37 °C. The highest dilution of serum samples that inhibited virus growth was considered as the neutralization antibody titer and was determined by IFA after incubation in DMEM with 6% FBS at 37 °C for 72 h. IFA was performed as described above.

ELISA
The presence of CSFV-specific antibodies was determined by either blocking ELISA or indirect ELISA. Blocking ELISA was performed with IDEXX HerdChek® CSFV antibody test kit according to the manufacturer’s instructions. Indirect ELISA was performed according to standard protocols as described previously. Briefly, ELISA plates (Corning, USA) were coated with 100 µl of E2ZJ antigen (5 µg/ml in PBS) in each well and incubated at 4 °C overnight. The coated plates were then thoroughly washed for three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 5% nonfat milk in PBS for 2 h at 37 °C. Subsequently, Mouse serum samples diluted with PBS containing 5% nonfat milk (1: 200) were added to the plate wells and incubated for 1 h at 37 °C. The plates were washed with PBST for three times and then incubated with 100 µl of HRP-conjugated goat anti-mouse IgG (1:}
10000, Sungene, China) diluted with PBS containing 5% nonfat milk for 1 h at 37 °C. After washing with PBST for three times, plates were received 200 µl 3,3',5,5' -tetramethylbenzidine (TMB, JingQi Biological, China) at room temperature for 10 min. Finally, 50 µl 2M H₂SO₄ (Sinopharm Cemical Reagent, China) was used to stop the reaction and the absorbance of each well was read at 450 nm using the layer chromatography scanner (Biotek, USA). The samples were considered positive when P/N > 2.1 and negative when P/N < 2.1.

Histopathology and immunohistochemistry
Tissue samples (spleen, kidney, submandibular lymphatic nodes and inguinal lymph nodes) were fixed in 4% paraformaldehyde (Sangon, China) at 4 °C for 24 h and embedded in paraffin. Embedded tissues were cut in 5 µm thick sections on a microtome (KuoHai, China). Tissue sections were stained with hematoxylin and eosin (H&E) for pathological evaluation as previously described [46]. Besides, tissues sections were subjected to immunohistochemistry (IHC) using CSFV polyserum (1: 100 in PBS) [47].

Statistical analysis
Data were expressed as the mean ± SEM of the three independent experiments. Statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, USA). Differences were considered statistically significant at p < 0.05.

Abbreviations
CSFV: Classical Swine Fever Virus; SP: signal peptide (SP); HM: honeybee melittin; DMEM: Dulbecco’s minimal essential media medium; PK-15: porcine kidney; Sf9: sodoptera frugiperda 9; TCID₅₀: Tissue culture infective dose; ELISA: enzyme-linked immunosorbent assay; PVDF: polyvinylidene fluoride; FBS: fetal bovine serum; TMB: 3,3',5,5' -tetramethylbenzidine; HRP: horseradish peroxidase; FITC: Fluorescein isothiocyanate ; DAPI: 4’,6’-diamidino-2-phenylindole dihydrochloride; IFA: indirect immunofluorescence assays; IgG: immunoglobulin G; dpc: days post challenge; dpi: days post immunization; IHC: immunohistochemistry; H&E: hematoxylin and eosin; mAbs: monoclonal antibodies; MOI: multiplicity of infection; PBS: phosphate buffered saline; PBST: PBS containing Tween 20; SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis; BCA: bicinchoninic acid; ATCC: American Type Culture Collection
Declarations

**Ethics approval and consent to participate**

All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of the National Institute of Infectious Diseases (NIID). Experimental protocols were reviewed and approved by the Animal Welfare and Ethics Committee at Laboratory animal center of Zhejiang University (10622). Animals were housed in individually ventilated cages, provided with water and standard chow, and monitored daily for health and clinical signs. More than 25% body weight loss was used as the criterion for early euthanasia. The animals were euthanized by CO2 inhalation for five minutes. Written informed consent was obtained to use the animals in study from the owner of the animals.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article.

**Competing interests:**

The authors declare no conflict of interests.

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**Author Contributions**

HLX was responsible for sample collection, experiment performance and manuscript drafting. YLW draft and proofread the manuscript. GWH helped with animal experiments. WHF provided part of experimental materials and suggestions for the manuscript. FH designed the study, analyzed the data and proofread the manuscript. All the authors have read and approved the manuscript.

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