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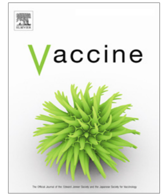

Enhanced immune responses to E2 protein and DNA formulated with ISA 61 VG administered as a DNA prime–protein boost regimen against bovine viral diarrhea virus

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ABSTRACT

The aim of this study was to develop and test an optimal vaccination strategy against bovine viral diarrhea virus (BVDV) based on the E2 glycoprotein of the BJ1305 strain. To achieve higher E2-specific antibody titers and to broaden the cellular immune response, a plasmid encoding the E2 protein (pcDNA3.1-E2) was constructed and a purified recombinant E2 protein was generated. The E2 protein was emulsified in the adjuvant ISA 61 VG prior to administration. We immunized mice three times with pcDNA3.1-E2 or the recombinant E2 protein or primed twice with pcDNA3.1-E2 and boosted once with the E2 protein. To evaluate the protection against BVDV conferred by the vaccines, the mice were challenged with BVDV strain Oregon C24V after the third immunization. Although all immunized mice developed humoral and cellular immune responses, the E2-specific antibody titers in the DNA prime–protein boost group were significantly higher than those elicited by either the DNA or the protein vaccine. In addition, vaccination with the E2 DNA vaccine induced higher percentages of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}IFN\text{-}\gamma^{+}$ T cells among total $CD3^{+}$ T cells than the other regimens. The predominant antibody subclass in the vaccinated mice was IgG1. Serum tumor necrosis factor alpha (TNF-α) levels in the DNA prime–protein boost group were significantly higher after the third immunization than in the other groups. Moreover, the mice treated with the DNA prime–protein boost vaccination regimen acquired protection against BVDV challenge, as shown by a significant reduction of viremia, only minor pathological changes, and a lower viral antigen burden than in the control and solo vaccinated mice. These results demonstrate the potential advantage of a DNA prime–protein boost vaccination approach over a solo vaccination for the prevention of BVDV. The ability of this vaccine strategy to control and eradicate BVD in herds warrants further investigation.

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1. Introduction

Bovine viral diarrhea (BVD) or mucosal disease is a prevalent cattle infection with a substantial economic impact on the beef and dairy industries in several countries [1]. It is caused by bovine viral diarrhea virus (BVDV), which is responsible for a wide spectrum of clinical syndromes in cattle, including respiratory disease, reproductive dysfunction, immunosuppression, persistent infection, and mucosal disease [2]. Seroepidemiological studies have shown that most cattle are infected with BVDV during their lifetimes [3]. Estimations of yearly losses due to BVDV reach approximately US$88 per animal [1,4]. The current strategies to reduce the losses caused by BVD are combination vaccination and the elimination of persistently infected (PI) animals [3,5].

A number of killed virus (KV) and modified live virus (MLV) vaccines are commercially available for the protection of cattle from BVDV infection. However, both of types of vaccines have drawbacks and risks. KV vaccines are expensive to produce and susceptible to the loss of important immunogenicity during inactivation. MLV vaccines carry a limited antigen mass and pose potential risks of *in utero* infection and/or immunosuppression. Furthermore, a major shortcoming of the existing vaccines is that they do not allow for the differentiation of vaccinated from naturally infected animals [6–8].

Abbreviations: BVD, bovine viral diarrhea; BVDV, bovine viral diarrhea virus; PI, persistently infected; KV, killed virus; MLV, modified live virus; PBS, phosphate-buffered saline; FBS, fetal bovine serum; HRP, horseradish peroxidase; H&E, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay; RT, room temperature; TNF-α, tumor necrosis factor alpha; IL-4, interleukin 4; DAPI, 4′,6-diamidino-2-phenylindole; SEM, standard error of the mean; T helper, Th; CTL, cytotoxic T lymphocyte.

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BVDV is an enveloped single-strand RNA virus classified as a member of the genus *Pestivirus* within the Flaviviridae family. Its genome is approximately 12.5 Kb in length and contains a single large open reading frame that is translated into a polyprotein that is processed by viral and cellular proteases to produce the mature structural and non-structural proteins of the virus [9]. The structural proteins originate from the N-terminal end of the polyprotein: the nucleocapsid protein C and the surface glycoproteins E^{rns}, E1, and E2 [10]. The structural envelope glycoprotein, E2, is the major immunogenic determinant of the BVDV virion, which makes it a potential target for new prophylactic vaccines against the virus [7,10]. Monoclonal antibodies specific to E2 demonstrate virus neutralizing ability against both BVDV-1 and BVDV-2 [11]. These findings supported the design of a BVDV vaccine based on E2 to distinguish infected from vaccinated animals. Thus, the E2 protein is a prime candidate for a subunit vaccine; the E2 gene has also been used in DNA immunizations [12–14].

Subunit vaccines offer advantages, such as increased stability and better safety profiles, over conventional vaccines. E2 subunit vaccines have been used to immunize animals to induce protection against BVDV [15–17]. However, a limiting factor of subunit vaccines is their inability to elicit complete T cell-mediated immunity [7,15]. Fortunately, plasmid DNA vaccines provide several advantages over classical vaccines. Numerous studies have demonstrated that humoral and cellular immunity can be induced after DNA plasmid injection [13,18–21]. Interestingly, a DNA prime–protein boost regimen has been proven more effective at eliciting an immune response than either a solo DNA or subunit vaccine immunization approach [22,23]. Montanide™ ISA (SEPPIC, Paris, France) is a well-established brand of vaccine adjuvants that have already been applied in combination with diverse antigens in a variety of animal models at an industrial scale. In particular, the Montanide™ ISA series of oil adjuvants confers superior efficacy to inactivated vaccines in various susceptible animals than other conventional adjuvants [24,25]. Montanide™ ISA 61 VG has been used as an adjuvant to stimulate strong immune responses [26–28].

In the present study, the E2 gene of the BVDV isolate BJ1305, which has the 1d genotype that is prevalent in Beijing, China [29,30], was amplified and expressed using molecular cloning technology. We vaccinated mice with an E2 DNA vaccine or E2 subunit vaccine or primed mice with an E2-expressing plasmid and boosted with E2 protein emulsified in ISA 61 VG with the aim of optimizing the vaccination strategy against BVDV. Then, we evaluated the immunogenicity and protective efficacy against BVDV infection that were elicited by the various strategies. The aim of the research was to evaluate if the DNA prime–protein boost regimen is a suitable immune strategy for controlling BVDV infection. Our work may identify a novel, safe, and effective approach for controlling BVD.

2. Materials and methods

2.1. Ethics statement

The animal care procedures for this study were in strict accordance with the Guidelines for the National Institutes of Health guide for the use of Laboratory Animals and care of the Chinese Center for Disease Control and Prevention and the Rules for Medical Laboratory Animals of the Chinese Ministry of Health. The work was covered under protocol CAU20160627-1, which was approved by the Animal Ethics Committee of China Agricultural University. All mice were sacrificed under sodium phenobarbital anesthesia and every effort was made to minimize animal suffering.

2.2. Plasmid construction, purification, and expression

All restriction enzymes were purchased from New England BioLabs (Ipswich, MA, USA). The E2 gene (~1020 bp) of the BJ1305 isolate of BVDV (accession number KT951840) was amplified by PCR using the sense primer 5′-CGCGGATCCATGCACTTG GATTGCAAACCTGAA-3′ and anti-sense primer 5′-CCGCTCGAGTTA GAAGTAATCCCGGTGATGGTCA-3′. The PCR product was then inserted into the vector pMD19T. The E2 gene of pMD19T-E2 was digested with *Bam H*I and *Xho* I, then cloned into pET32a(+) and pcNDA3.1(+) to generate pET32a-E2 and pcNDA3.1-E2, respectively. The constructs were confirmed by restriction enzyme digestion and agarose gel electrophoresis, then sequenced to establish cloning accuracy. The plasmid used for the immunization of the mice was grown in *Escherichia coli* DH5α cells and purified with an Endo-Free Plasmid Maxi Kit (Omega Bio-Tek, Norcross, GA, USA). The expression of E2 by the plasmid was confirmed by transiently transfecting COS-7 cells and analyzing the cell lysates by western blotting.

2.3. Purification of recombinant E2 protein from E. coli BL21 (DE3) cells expressing pET32a-E2

We induced the expression of the BVDV E2 protein in prokaryotic expression vector pET28a-E2-positive Rosetta (DE3) cells by treating with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C. The cells were harvested 6 h post-induction by centrifugation at 4000 rpm for 15 min at 4 °C, then resuspended in phosphate-buffered saline (PBS; pH 8.0) for sonication. Immediately after centrifugation, cell pellets were lysed in lysis buffer (10 mM NaH_2PO_4, 10 mM Tris–HCl, and 8 M urea; pH 8.0). The soluble fractions were harvested and applied to a His-Tagged Protein Purification Kit (CWbiotech, Beijing, China). E2 protein was eluted, dialyzed using a dialysis bag (molecular weight cutoff: 8000–14,000; USA) at 4 °C for further purification, then concentrated in polyethylene glycol 6000. The concentration of the purified E2 was determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's instructions. The purity and antigenicity of the protein were evaluated by SDS-PAGE and western blot analysis using an E2-specific mouse monoclonal antibody (Veterinary Medical Research & Development-VMRD, Pullman, WA, USA).

2.4. Cells and virus

COS-7 cells, which were used for transfection, were grown in Dulbecco's minimum essential medium (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco™) at 37 °C in a 5%-CO_2 atmosphere. MDBK cells were purchased from CVCC. We confirmed that the cells were BVDV-free by reverse transcription-PCR and immunofluorescence. The MDBK cells were cultured in Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture (pH 7.2; Gibco™) supplemented with 10% FBS and penicillin and streptomycin (100 U/ml, Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5%-CO_2 atmosphere. The FBS used in the cultures was BVDV-free as assessed with a BVDV Antigen Test Kit (IDEXX, Westbrook, ME, USA).

MDBK cells were infected with BVDV (BJ1305 or Oregon C24V) at a multiplicity of infection of 0.1 for 1 h at 37 °C. After 72 h, the cells were subjected to three freeze–thaw cycles and centrifugation at 4000 rpm for 15 min at 4 °C (Xiangyi, China). Then, the supernatants were harvested and subjected to centrifugation at 20,000*g* for 2 h (Beckman Coulter, Brea, CA, USA). The pellets were resuspended in PBS (HyClone™, GE Healthcare, South Logan, UT, USA). The viral suspensions were titrated and frozen at −70 °C until further study.

2.5. Transfection of COS-7 cells

To determine the expression of E2 *in vitro*, COS-7 cells were transfected with pcDNA3.1-E2 using Lipofectamine® with Plus™ Reagent (Invitrogen) according to the manufacturer's instructions. The COS-7 cells were incubated with the transfection mixtures for 5 h and cultured in Opti-MEM™ (2 mL; Invitrogen). After 48 h, the cells and supernatants were collected separately. The cells were lysed in cold radio-immunoprecipitation assay buffer (1 mL) containing protease inhibitor cocktail (5 μL) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) on ice for 10 min, then centrifuged at 13,000 rpm for 10 min at 4 °C to pellet the insoluble material. The supernatants were subjected to western blot analysis. Protein concentrations were determined with a BCA Protein Assay kit (Pierce Chemical) according to the manufacturer's instructions.

2.6. Western blotting

The purified recombinant E2 protein of prokaryotic origin and the lysates of the pcDNA3.1-E2-transfected COS-7 cells were boiled in SDS-PAGE Loading Buffer (Reducing, 5×; CWbiotech) for 5 min, then separated on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated with an E2-specific mouse monoclonal antibody (VMRD), followed by the secondary horseradish peroxidase (HRP)-conjugated Affinipure Goat Anti-Mouse IgG(H + L) (Proteintech).

2.7. Emulsification of candidate vaccines in ISA 61 VG

ISA 61 VG is a mineral oil-based water-in-oil adjuvant. One day prior to vaccination, DNA or protein was prepared in ISA 61 VG (1:1.5 [w/w]) as previously reported [25]. The DNA or protein was emulsified in ISA 61 VG using a two-way syringe. The antigen and ISA 61 VG were first processed by 20 cycles of low-speed emulsification for approximately 8 s/cycle, followed by 60 cycles of high-speed emulsification for approximately 0.5 s/cycle. After emulsification for 24 h, the quality of the complete vaccines was evaluated by placing a drop of each emulsion on the surface of water. The vaccine preparation was considered successful if the droplet floated on the surface of the water and maintained its water-in-oil emulsified state.

2.8. Animals and experimental design

BVDV-negative, 6-to-8-week-old BALB/c mice (n = 100) were purchased from Beijing Vital River Laboratory (Beijing, China). The mice were randomly allocated to five groups of 20 animals each and immunized subcutaneously as follows: (1) three times with PBS; (2) three times with pcDNA3.1 (100 μg) in ISA 61 VG (150 μg); (3) three times with pcDNA3.1-E2 (100 μg) in ISA 61 VG (150 μg); (4) three times with E2 (40 μg) in ISA 61 VG (60 μg); or (5) twice with pcDNA3.1-E2 (100 μg) in ISA 61 VG (150 μg), followed by one injection of E2 (40 μg) in ISA 61 VG (60 μg). Vaccination took place on days 0, 14, and 28. In addition, sera were collected for serology on days 0, 7, 14, 21, 28, 35, 42, and 49. The serum was separated from the blood collected from the posterior venous plexus and subjected to centrifugation at 3000 rpm for 20 min. On days 35 and 49, six mice per group were euthanized for flow cytometric analysis and their spleens were collected aseptically for lymphocyte isolation. Two weeks after the third immunization, the mice were inoculated with the median tissue culture infective dose (6×10^6) of BVDV strain Oregon C24V via intraperitoneal injection. On day 7 post-infection, the mice were euthanized and their lungs, spleens, and kidneys were immediately fixed in 4% paraformaldehyde. The tissues were routinely processed and embedded in paraffin. The paraffin-embedded tissues were then sectioned (3 μm) and stained with hematoxylin and eosin (H&E) (Beyotime, Beijing, China). The histopathological changes in the mice after BVDV challenge were investigated. All evaluations were performed by two pathologists who were blinded to the treatment regimens. The severity of the histopathological changes was scored as previously described [31].

2.9. Serology

We performed enzyme-linked immunosorbent assays (ELISAs) to determine the serum concentrations of anti-BVDV antibodies in the mice. The wells of 96-well polystyrene microtiter plates were coated with 0.5 μg purified E2 protein/well overnight at 4 °C (Immulon™ 2; Thermo Fisher Scientific). The plates were incubated with the mouse sera (diluted 1:4000, 100 μL/well) for 2 h at 37 °C. After washing three times, the plates were incubated with the HRP-conjugated ProteinFind® Goat Anti-Mouse IgG(H + L) (diluted 1:4000 in PBS; TransGen Biotech, Beijing, China).

An indirect ELISA was used to detect the BVDV E2-specific IgG1, IgG2a, IgG2b, and IgG3 antibodies in the mouse sera. The wells of 96-well Nunc Maxisorp™ Immunoplates (Sigma-Aldrich, Poole, UK) were coated overnight with purified BVDV E2 antigen (5 μg/mL). Mouse serum samples (100 μL) obtained two weeks after the third immunization were added to the wells and incubated for 2 h at room temperature (RT). After a further wash step, antibodies against IgG1, IgG2a, IgG2a, and IgG3 (diluted 1:1000 in PBS; Mouse Monoclonal Antibody Isotyping Reagents, Sigma-Aldrich, Poole, UK) were added to the wells. Subsequently, the plates were incubated with the HRP-conjugated rabbit anti-goat IgG(H + L) (diluted 1:5000; Golden Bridge Biological Technology, Beijing, China) for 15 min at RT.

2.10. Detection of cytokines

On days 35 and 49, the serum concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 4 (IL-4) were determined by direct high-sensitivity sandwich ELISAs for mouse TNF-α (R&D Systems, Minneapolis, MN, USA) and IL-4 (LifeSpan Biosciences, Seattle, WA, USA), respectively. The experimental procedures were based on the manufacturers' instructions with minimal modifications.

2.11. Flow cytometric analysis

Splenic lymphocytes were incubated with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich) and 5 mM calcium ionophore A23187 (Sigma-Aldrich) in the presence of BD GolgiStop™ (BD Biosciences, San Jose, CA, USA) in complete RPMI 1640 medium for 4 h at 37 °C. The cells were stained with monoclonal mouse anti-mouse CD3 (PE-Cy5-conjugated; eBioscience, San Diego, CA, USA), anti-CD4 (PE-conjugated; BD Biosciences), and anti-CD8a (FITC-conjugated; BD Biosciences) for 30 min at 4 °C. Then, the cells were washed with staining buffer, fixed in BD Cytofix/Cytoperm™, permeabilized with Perm/Wash buffer (BD Biosciences), and incubated with Alexa Fluor® 647-conjugated anti-IFN-γ (BD Biosciences) or the appropriate isotype control antibody for 30 min at 4 °C. The antibody-stained cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, USA).

2.12. Immunofluorescence detection

Serum from each mouse (100 μL; collected on day 49) was incubated on a monolayer of MDBK cells for 2 h at 37 °C. The MDBK

cells were grown at 37 °C in a 5%-CO_2 atmosphere. After three days, the cells were fixed with acetone at RT, permeabilized, and incubated with mouse anti-BVDV E2 antibodies diluted in PBS. We incubated the cells with the second antibody Cy™3-Conjugated AffiniPure Goat Anti-Mouse IgG(H + L) (Zhongshan Golden Bridge Biotechnology) and 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to detect nuclear staining. The stained MDBK cells were visualized and photographed using a Nikon Eclipse Ti-U inverted fluorescence microscope equipped with a Nikon DS cooled camera head (Nikon, Tokyo, Japan).

2.13. Immunohistochemistry

Immunohistochemistry was performed with the highly sensitive non-avidin-biotin complex technique (EnVision polymer system, BioGenex Laboratories, San Ramon, CA, USA). Briefly, splenic tissue sections were boiled in 10 mM citrate buffer (pH 6.0) to retrieve antigens. The slides were quenched with 3% hydrogen peroxide solution for 10 min at RT. After they had been washed with PBS, the slides were incubated with mouse anti-BVDV E2 monoclonal primary antibody (1:1000 dilution, VMRD) overnight in a humidified chamber at 4 °C. After three rinses in PBS, the slides were incubated with secondary HRP goat anti-mouse IgG (nonbiotinylated antibodies, EnVision System, DakoCytomation, Agilent) for 20 min at 37 °C. Tissue staining was visualized with 3,3′-diaminobenzidine (Zhongshan Golden Bridge Biotechnology). Spleens from unchallenged mice were used as negative controls. Images were captured using an Olympus BX41 microscope (Olympus, Tokyo, Japan) equipped with a Canon EOS 550D camera head (Canon, Tokyo, Japan).

2.14. Statistical analysis

All data were compared between groups via analysis of variance and Tukey's honestly significant difference *post hoc* test. All tests were performed in GraphPad Prism 6.0 software (San Diego, CA, USA). The data are presented as the mean ± standard error of the mean (SEM). The differences were considered statistically significant where $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

3.1. Expression and purification of E2

We assessed the expression and purification of recombinant BVDV E2 protein from the plasmid pET32a-E2 by western blotting (Fig. S1A in Supplementary material). We found that the antigenicity of the recombinant E2 protein was optimal. The miscellaneous low-molecular-weight proteins were removed by purification. We transfected COS-7 cells with pcDNA3.1-E2 to facilitate the analysis of E2 expression *in vitro* by western blotting with an E2-specific antibody. As shown in Fig. S1B, a band with an apparent molecular mass of 60 kDa was observed in the pcDNA3.1-E2-transfected cells, but not in the pcDNA3.1-transfected cells.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.07.054.

3.2. Serology

To evaluate humoral responses after immunization, we measured the concentrations of E2-specific antibodies in the sera of challenged mice (Fig. 1A). There were no obvious differences in antibody levels among the groups after the first immunization. Notably, the antibody levels continued to rise in the vaccinated groups following the final two immunizations. On day 28 (two weeks after the second immunization), the DNA, protein, and DNA prime–protein boost groups had higher antibody levels than the two control groups ($P < 0.001$). Furthermore, higher IgG titers were observed in the protein group than in the DNA and DNA prime–protein boost groups ($P < 0.001$). No significant differences were found between the DNA and DNA prime–protein boost groups. On day 42 (2 weeks after the third immunization), all three vaccinated groups developed strong humoral immune responses with antibody levels exceeding those of the two control groups ($P < 0.001$). The DNA prime–protein boost group had higher antibody levels than the other two vaccination groups. Importantly, the IgG titers of the DNA prime–protein boost group were significantly higher than those of the DNA group ($P < 0.001$). In addition, the protein vaccination elicited higher antibody levels than the DNA only vaccination ($P < 0.001$).

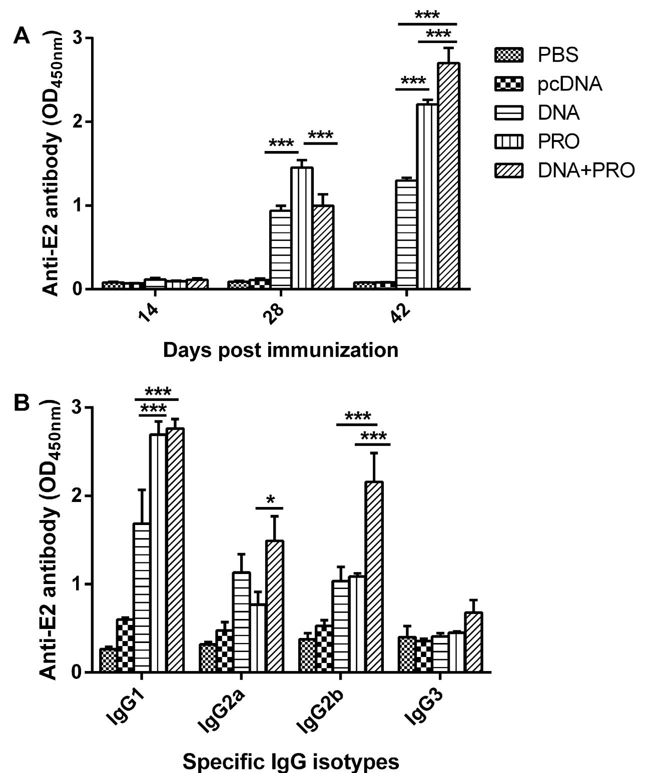

Fig. 1. Relative levels of total IgG antibodies and specific IgG1, IgG2a, IgG2b, and IgG3 isotypes after vaccination. (A) Relative levels of E2-specific serum total IgG antibodies two weeks after the third vaccination in mice. Sera were collected from experimental mice and total antibody levels were evaluated via optimized indirect ELISA. (B) Levels of specific IgG1, IgG2a, IgG2b, and IgG3 isotypes in the immunized groups. Sera were collected from experimental mice two weeks after the final booster and specific IgG isotypes were evaluated via indirect ELISA. The data from each group are presented as the mean ± SEM (n = 6). OD_{450nm}, optical density at 450 nm; PRO, protein. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

We evaluated the isotypes of the serum IgG antibodies in the vaccinated mice on day 42 (Fig. 1B). IgG1, IgG2a, and IgG2b were the main IgG isotypes in the sera of the vaccinated mice (Fig. 1B). The vaccinated groups had higher IgG1 titers than the control groups. Furthermore, the DNA prime–protein boost group had higher IgG2a titers and higher IgG2b titers than the control groups (the same $P < 0.001$ to these comparisons). The IgG2a titers in the DNA prime–protein boost group were also higher than those in the protein alone group ($P = 0.02$).

3.3. Cytokine responses in vaccinated mice

The cytokine concentrations in the peripheral blood of the mice are shown in Fig. 2. The concentrations of TNF-α and IL-4 in the

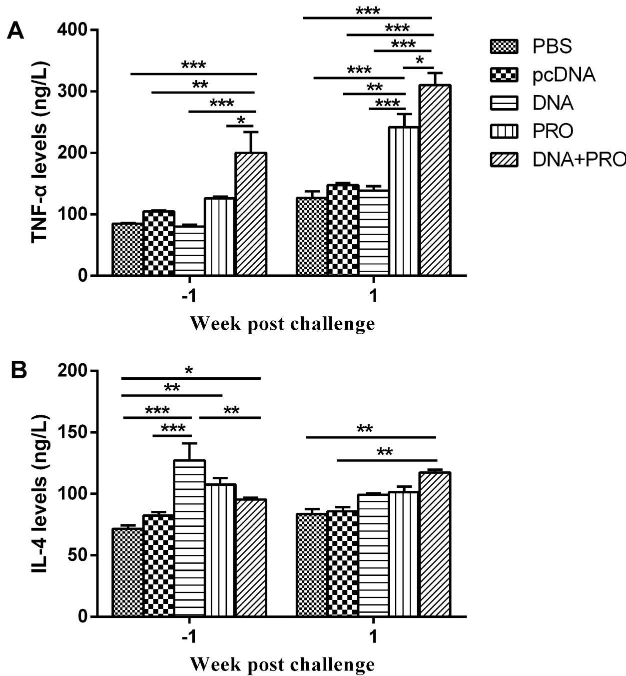

Fig. 2. TNF-α and IL-4 concentrations in mouse sera one week prior to and one week after challenge. The levels of TNF-α and IL-4 were detected by direct high-sensitivity sandwich ELISAs for mouse TNF-α (R&D Systems, Minneapolis, MN, USA) and porcine IL-4 (LifeSpan Biosciences, Seattle, WA, USA), respectively. The data from each group are presented as the mean ± SEM (n = 3 per group). PRO, protein. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

sera of mice before and after BVDV challenge were detected by ELISA. One week prior to challenge, the serum levels of TNF-α in the mice immunized with the DNA prime–protein boost regimen were higher than those in the protein, DNA, pcDNA, and PBS groups ($P = 0.015$, $P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively; Fig. 2A). After challenge, the TNF-α levels appeared to be slightly elevated in the sera and the concentration of TNF-α in the DNA prime–protein boost group was higher than those in the protein, DNA, pcDNA, and PBS groups ($P = 0.027$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The E2 subunit vaccine group had significantly higher levels than those in the DNA, pcDNA, and PBS groups ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively).

One week prior to challenge, the concentrations of IL-4 in the sera of the mice in the DNA, protein, and DNA prime–protein boost immunization groups were higher than that in the PBS group ($P < 0.001$, $P < 0.01$, and $P = 0.038$, respectively; Fig. 2B). One week post-challenge, the concentrations of IL-4 in the sera of the mice immunized with the DNA prime–protein boost strategy were higher than those in the sera of the mice in the PBS and pcDNA groups ($P = 0.002$ and $P = 0.004$, respectively).

3.4. The proportions of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}IFN\text{-}\gamma^{+}$ T cells among splenic lymphocytes

To examine the effect of the candidate vaccines on cellular immunity in mice before and after BVDV challenge, we analyzed the percentages of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}IFN\text{-}\gamma^{+}$ T cells among splenic lymphocytes by flow cytometry (Fig. 3). One week prior to challenge, the proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ cells among splenic T cells in the DNA prime–protein boost group was significantly higher than that in the PBS control group ($P < 0.001$). The proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ cells was also higher in the pcDNA3.1-immunized mice than in the PBS control group ($P = 0.001$). The percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells in the protein vaccination group was lower than those in the DNA and DNA prime–protein boost groups (the same $P < 0.001$ to these comparisons). The proportions of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells were lower in all groups after BVDV challenge. Notably, the mice in the DNA prime–protein boost group had a higher post-challenge proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells than the protein, DNA, pcDNA, and PBS groups ($P = 0.006$, $P = 0.02$, $P = 0.005$, and $P < 0.001$, respectively).

The pre-challenge proportions of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells in the DNA, protein, and DNA prime–protein boost groups were higher than that in the PBS group ($P = 0.006$, $P < 0.001$, and $P < 0.001$, respectively). After challenge with BVDV, the mice immunized with the candidate vaccines had higher proportions of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells than those in the PBS control group (the same $P < 0.001$ to these comparisons). In addition, the proportions of $CD8^{+}IFN\text{-}\gamma^{+}$ T in the DNA, protein, and DNA prime–protein boost groups were higher than that in the pcDNA group ($P < 0.001$, $P = 0.001$, and $P < 0.001$,

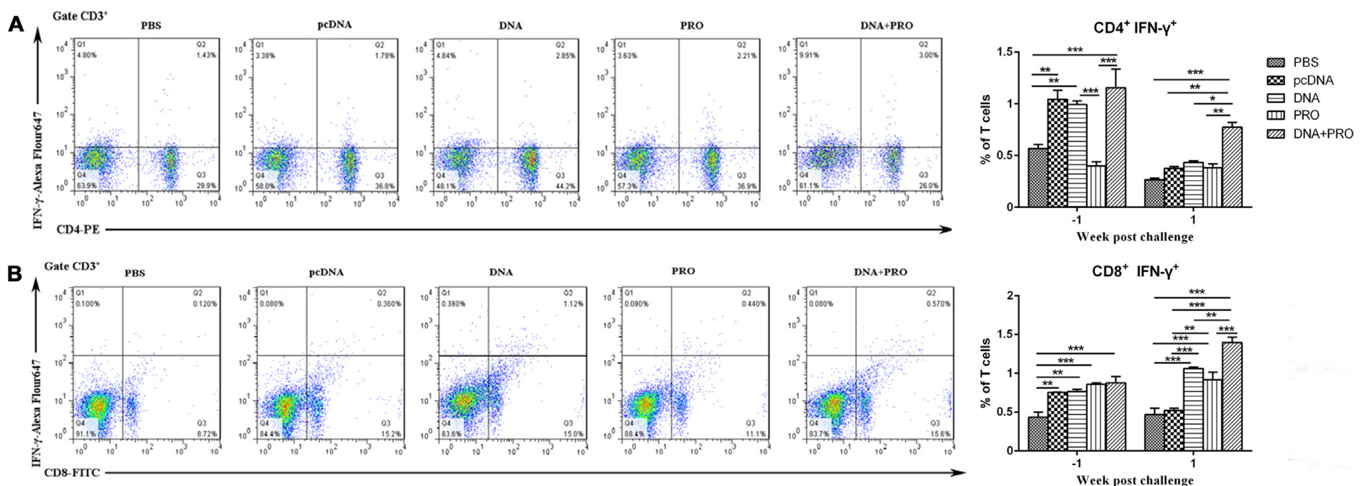

Fig. 3. Vaccination induced the expansion of splenic $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}IFN\text{-}\gamma^{+}$ T cells. On days 35 and 49, spleens were collected aseptically for lymphocyte isolation from mice in each group. (A) Flow cytometric analysis of the percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ cells among $CD3^{+}$ T cells. Left: representative flow cytometry dot plot shows the gating strategy for CD4 and IFN-γ expression in peripheral $CD3^{+}$ T cells. (B) Flow cytometric analysis of the percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells among $CD3^{+}$ T cells. Left: representative flow cytometry dot plot shows the gating strategy for CD8 and IFN-γ expression in peripheral $CD3^{+}$ T cells. The data from each group are presented as the mean ± SEM (n = 6 per group). PRO, protein. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

respectively). The proportion of CD8$^+$IFN-γ^+ T cells in the mice immunized with the DNA prime–protein boost strategy was also higher than that in the mice immunized with E2 protein alone ($P < 0.001$).

3.5. Virus determination in serum and spleen

To assess the immune protective effects of the candidate BVDV vaccines, we isolated virus from the sera of mice one week after challenge. MDBK cells were co-cultured with sera from the five groups for three days, then immunofluorescence detection was used to detect BVDV (Fig. 4). The protection ratios determined on week 1 post-challenge were 87.5% (1/8 with detectable virus) in the DNA prime–protein boost group, 75% (2/8 with detectable virus) in the protein group, 75% (2/8 with detectable virus) in the DNA group, and 100% in the control groups. We also assessed viral antigen burden in the spleens of the mice at day 7 post-BVDV challenge by immunohistochemistry (Fig. 5). BVDV antigen was detected in splenic lymphocytes. The detection rate for BVDV antigen was 75% (2/8) in the DNA prime–protein boost group, 37.5% (3/8) in the protein group, 37.5% (3/8) in the DNA group, and 100% in the control groups. No BVDV antigen was detected in the negative controls.

3.6. Histopathology of lung, spleen, and kidney

To evaluate the immune protection against BVDV conferred by the vaccines, histopathological examinations were performed on the lungs, spleen, and kidneys at day 7 post-challenge (Fig. 6). The lungs of the mice in the PBS group exhibited marked lesions, including some lymphocyte and macrophage infiltration, mild alveolar expansion, and alveolar wall thickening. The lungs of the mice in the pcDNA3.1 group showed bronchial dilatation and other mild pathological lesions. Although copious mucus appeared in the lungs of the protein vaccine group, the lungs of the DNA and DNA prime–protein boost groups exhibited no severe pathological changes.

The spleens of the PBS group displayed more intense lymphocyte necrosis within the lymphatic nodule than those of the vaccinated groups. We observed phagocytosis of giant cells in the spleens of the DNA and DNA prime–protein boost groups. While the spleens of the protein and DNA prime–protein boost group had lymphatic cells empty.

The kidneys of the two control groups exhibited homogeneous staining for renal tubular red dye, epithelial cell degeneration, and luminal loss. The empty pcDNA carrier group showed epithelial injury. However, the kidneys of the vaccinated groups had normal renal structures.

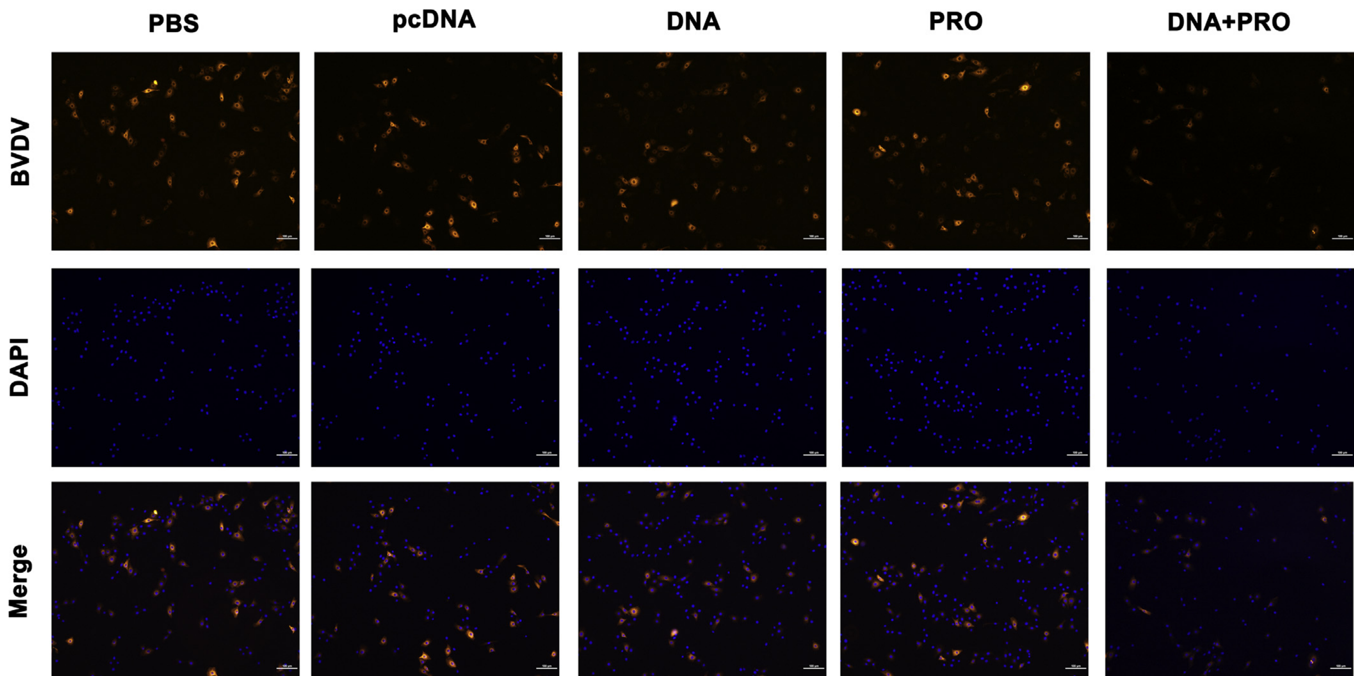

Fig. 4. Immunofluorescence analysis of viremia in mice after BVDV challenge. BVDV was detected with a monoclonal primary anti-E2 antibody and the secondary Cy™3-Conjugated AffiniPure Goat Anti-Mouse IgG(H + L) antibody (orange). Nuclei were stained with DAPI (blue). Scale bars, 100 μm. PRO, protein.

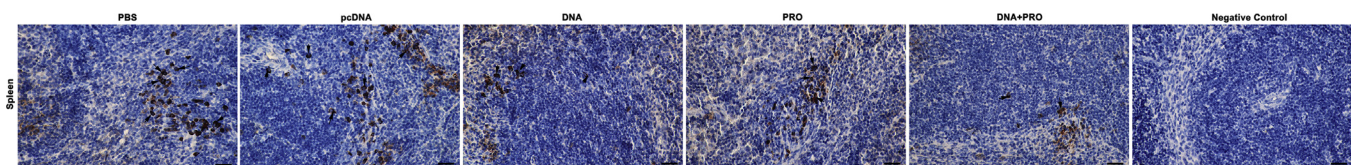

Fig. 5. Detection of BVDV antigen in the spleen by immunohistochemistry at day 7 post-intraperitoneal administration. BVDV was detected with a primary monoclonal anti-E2 antibody and the secondary HRP-conjugated goat anti-mouse IgG. Viral antigen was detected in splenic lymphocytes. Scale bars, 100 μm. The black arrows indicate viral antigen in the spleen. PRO, protein.

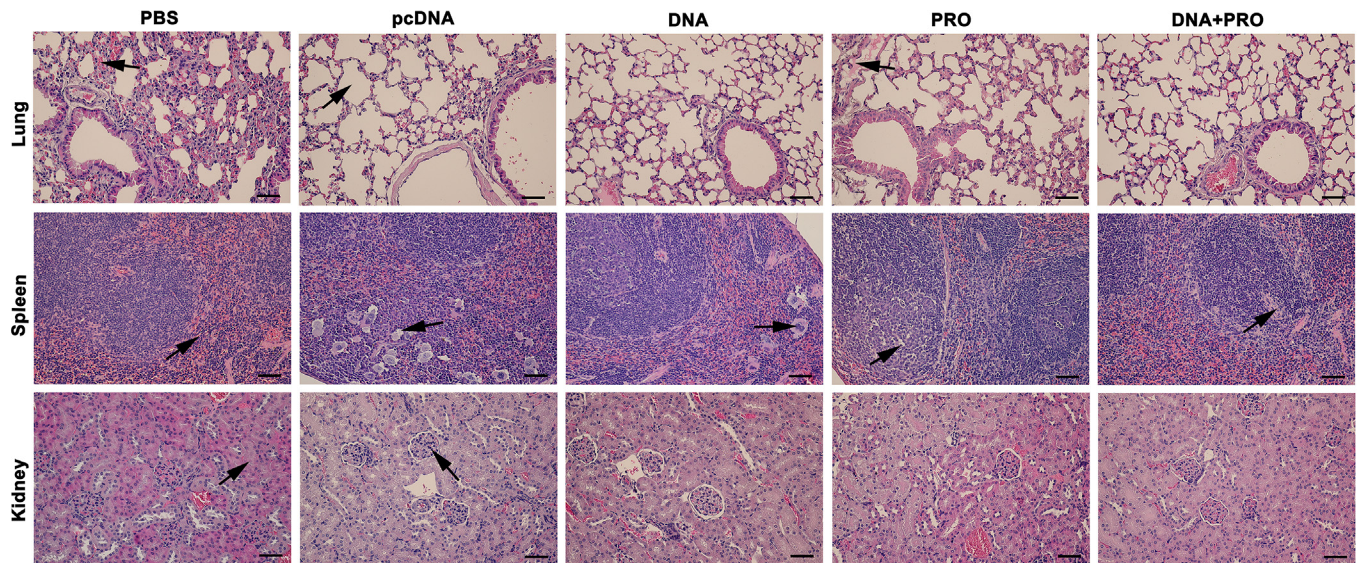

Fig. 6. Representative photomicrographs of H&E-stained lung, spleen, and kidney sections. On day 7 post-infection, mice were euthanized and their lungs, spleens, and kidneys were immediately fixed in 4% paraformaldehyde. Paraffin-embedded tissue samples were then sectioned (3 μm) and stained with H&E (Beyotime, Beijing, China). Scale bars, 100 μm. The black arrows indicate histopathological changes in the lungs, spleens, and kidneys. PRO, protein.

4. Discussion

BVD is prevalent throughout the world and its diverse symptoms and related immunosuppression result in heavy yearly losses for the dairy and beef cattle industries [1,2,4]. Controlling and eradicating BVDV are challenging due to the wide distribution of the virus in the dairy industry. Our results illustrate that an E2 DNA prime–protein boost strategy could enhance humoral and cellular immunity to provide protection against BVDV infection.

Vaccination with the E2 subunit produced in recombinant baculovirus-infected insect cells induced neutralizing antibody production and limited protection from BVDV challenge in cattle [32]. The vaccination of cattle with an E2 DNA vaccine elicited moderate immune responses and partial protection against BVDV challenge [15]. We found that the DNA prime–protein boost strategy elicited stronger immune responses and greater protection against BVDV in mice than solo vaccination strategies. In previous studies, priming with DNA and boosting with protein improved neutralizing antibody titers [20,33,34]. On day 42, we detected statistically significant differences in the antibody titers of the protein group and the DNA or DNA prime–protein boost groups because the humoral immunity elicited by the DNA vaccine is weaker than that induced by the subunit vaccine in the early stages of the immune response. On day 42, the mice of the DNA prime–protein boost group had statistically higher antibody titers than the mice of the solo DNA and protein groups. One of the key features of vaccine efficacy is the induction of a sufficient humoral immune response to afford protection; the levels of serum antibodies are likely to positively correlate, to an extent, with the level of protection [7].

The main antibody subtypes induced by vaccination were IgG1, IgG2a, and IgG2b. Similar results have been observed in other studies of immune responses to BVDV subunit and DNA vaccines [13,19,21]. The titers of IgG1 and IgG2b in the sera of the DNA prime–protein boost group were higher than those of the other groups. All vaccinated mice produced IgG1 antibodies; in a previous study, IgG1 antibodies were capable of binding to a receptor on mammary gland secretory epithelium, which selectively captured and concentrated this isotype when colostrum was produced [7].

The serum concentrations of TNF-α in the DNA prime–protein boost mice were significantly higher than those in the other groups both before and after challenge. As an adjuvant for a foot-and-mouth disease virus DNA vaccine, TNF-α can enhance the expression of co-stimulatory molecules and MHC class II on dendritic cells and boost cell-mediated responses [35]. In this study, the DNA prime–protein boost strategy stimulated the production of high levels of TNF-α, suggesting that the DNA prime–protein boost scheme successfully strengthened the CD4 T helper (Th) 1 immune response against BVDV infection. Studies have shown that the expression of TNF-α mRNA increases at 1 h post-cytopathic-type BVDV infection [36]. The DNA prime–protein boost strategy significantly promoted the secretion of IL-4 post-challenge in the peripheral blood compared to the other vaccination methods, indicating that the immune system produced better immune memory in response to the DNA prime–protein boost regimen. In a natural exposure model using a type 1b PI calf, serum IL-4 levels increased along with pro-inflammatory cytokine levels in steers exposed to BVDV [37]. Taken together, our findings suggest that the DNA prime–protein boost vaccine can promote Th1 immune responses and improve humoral and Th2 responses.

In the present study, the percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells, which are known to play a major role in viral clearance [21], significantly increased in the DNA prime–protein boost mice after the third immunization. The development of $CD8^{+}IFN\text{-}\gamma^{+}$ T cell responses in vaccinated mice indicated that ISA 61 VG promoted cross-presentation of the antigen by MHC class I to induce $CD8^{+}$ T cell responses. The proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells decreased after challenge in the DNA prime–protein boost mice, but remained higher than in the other groups. This may indicate viral clearance by $CD4^{+}IFN\text{-}\gamma^{+}$ T cells and lower rates of viral infectivity, apoptosis, or necrosis of $CD4^{+}$ T cells due to the protection provided by the vaccine before and after challenge. Furthermore, $CD4^{+}$ T cells play an important role in coordinating the cellular immune response in the early stage of viral infection [38]. Expression of the BVDV E2 protein on the surface of *Saccharomyces cerevisiae* can promote the production of the chemokine CXCL-8 in macrophages and stimulate $CD4^{+}$ T cell proliferation [39]. The failure of the BVDV E2 protein alone to alter the proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells in this study may be due to differences in E2 expression between the systems.

$CD8^{\pm}$ cytotoxic T lymphocytes (CTLs) participate in the immune response to acute BVDV infection [9,37]. Our results demonstrated that BVDV upregulates the proportion of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells in the

mouse spleen. Increased CD4 $^{+}$ T cell numbers aid CD8^{+} T lymphocyte secondary expansion and memory formation [38,40,41]. A subunit vaccine administered with poly(I:C) as an adjuvant also demonstrated the ability of the E2 protein to induce CTL responses in cattle [16]. Our results indicated that the DNA prime–protein boost strategy induces larger Th1 and CTL responses than solo protein vaccination.

The protective effects of the vaccine against BVDV infection were analyzed by assessing the rate of viremia and changes in histopathology after viral challenge. BVDV antigen was detected in the sera and spleens of each group after challenge. However, the virus detection rate was lower in the DNA prime–protein boost group than in the other treatment groups, indicating that the combined strategy partially inhibited virus replication *in vivo*. In this study, vaccination with an E2 DNA vaccine elicited moderate antibody responses and partial protection against BVDV challenge. An E2 subunit vaccine developed in a baculovirus expression system failed to confer total protection in cattle [42]. In another study, virus was isolated from the three calves in the DNA prime–protein boost group on day 6 or day 7 after challenge [19]. We found that the serum of only 1 of 8 mice was BVDV-positive on day 7 post-challenge in the DNA prime–protein boost group, whereas the spleens of 2 of 8 mice were BVDV antigen-positive. These viral detection rates were lower than those in the solo DNA and protein vaccination groups. The low level of detection may be attributable to our isolation of the virus at a single time point, which was insufficient to fully evaluate the efficacy of the different vaccination protocols. Our future vaccination-challenge experiments in cattle will include serial sampling for viremia detection to comprehensively address the induction of protection against BVDV infection. Although the protective efficiency of the vaccine was assessed at only a single time point, our results suggested that the vaccines can protect mice against BVDV.

The lungs, spleens, and kidneys of the mice in the five groups were damaged to differing degrees by BVDV infection; the DNA prime–protein boost mice displayed the minor damage among the groups. Compared to the lesions observed in the present study, another study reported that BVDV infection in mice caused more extramedullary hematogenesis, more intense lymphocyte necrosis within the lymphatic nodule in the spleen, marked atrophy of the glomerulus in the kidney, hyperplasia of the alveoli wall, interstitial thickening, and alveolar macrophage infiltration in the alveolar lumen in the lung [31]. We conclude that the vaccines in our study minimized the damage caused by BVDV.

In conclusion, we prepared ISA 61 adjuvant-coated vaccines containing recombinant E2 protein and DNA encoding E2. We found that the DNA prime–protein boost strategy induced greater humoral and cellular immunity, which provided strong protective effects against BVDV in mice, than the solo vaccinations. Although this work was not conducted in cattle, both DNA and subunit E2 vaccines have previously been tested in cattle. Despite the limitations of the study, our results are encouraging and warrant vaccination-challenge experiments in cattle. Our results indicate that E2 protein subunit and DNA vaccines have great potential for use in BVDV control and eradication programs.

Conflict of interest

None.

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Authors' contributions

DJC and QJS participated in the study design, performed the experiments, analyzed the data, and wrote the manuscript; CD performed the immunofluorescence assay; SHW performed the flow cytometry analyses; YHZ and JFW conceived and designed the study. All authors attest they meet the ICMJE criteria for authorship.

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