



# Co-administration of a plasmid encoding CD40 or CD63 enhances the immune responses to a DNA vaccine against bovine viral diarrhea virus in mice

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**ABSTRACT.** Bovine viral diarrhea virus (BVDV) causes substantial economic losses in the livestock industry worldwide. Plasmids encoding the BVDV E2 protein are potential DNA vaccines against BVDV, but their immunogenicity has been insufficient. Here, we investigated the adjuvant effect of CD40 and CD63 plasmids on the immune responses to a BVDV E2 DNA vaccine in mice. We constructed pUMVC4a-based plasmids encoding the BVDV E2 protein (pE2), mouse CD40 (pCD40), or mouse CD63 (pCD63). Protein expression by each plasmid was confirmed through Western blot analysis and immunofluorescence staining of cultured cell lines. BALB/c mice were immunized intradermally twice with pE2 in combination with, or without, pCD40 or pCD63, with 3 weeks between the two doses. pE2 with pCD40 induced significantly higher neutralizing antibody titers against BVDV than pE2 alone. pE2 with pCD63 induced significantly higher anti-E2 IgG<sub>2a</sub> antibody titers than pE2 alone. Furthermore, pE2 with pCD40 or pCD63 induced significantly increased lymphocyte proliferation and interferon (IFN)- $\gamma$  production in response to BVDV, compared with E2 alone. These results suggest that a plasmid encoding CD40 or CD63 can be used as an adjuvant to enhance immune responses to DNA vaccines against BVDV.

**KEYWORDS:** bovine viral diarrhea virus, CD40, CD63, DNA vaccine, E2

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Bovine viral diarrhea virus (BVDV), which belongs to the *Flaviviridae* family and genus *Pestivirus*, is an enveloped single-stranded positive-sensed RNA virus [45, 46]. BVDV is mainly a pathogen of cattle, but it also infects buffaloes, deer, pigs, sheep, goats, yaks, and wild ruminants [48]. The clinical manifestations of BVDV are acute infection, fetal infection, and mucosal disease. Fetal infections are particularly associated with congenital malformations and abortion. BVDV can be transmitted to newborn calves through the placenta, potentially resulting in persistent infection in calves that can transmit the virus throughout their lives [21, 23, 50]. BVDV infection has caused huge economic losses to the global animal husbandry industry through reduced milk production, abortions, and the decreased lifespan of infected animals [17]. Fatality losses due to BVDV have been estimated at USD5.26 per infected animal, with performance losses at USD88.26 per infected animal [14].

Vaccination is used to control BVDV infection [42]. However, several problems exist in the current BVDV vaccines. Although modified live virus (MLV) vaccines induce greater immune responses and longer duration of protection than killed virus (KV) vaccines, MLV vaccines are at risk of generating a pathogenic revertant and vertical transmission in pregnant cattle. KV vaccines do not carry such a risk, but they requires multiple immunizations to achieve protection against infection [50]. Furthermore, unlike MLV vaccines, KV vaccines do not induce cell-mediated immune responses efficiently [34–36].

DNA vaccines are plasmids that encode protective antigens. Compared with conventional MLV and KV vaccines, DNA vaccines are more stable, easier to manufacture, and safer during handling [37]. DNA vaccines induce not only humoral immune responses but also cell-mediated immune responses by directly expressing antigens on antigen-presenting cells [15]. DNA vaccines have been investigated for various applications, including therapy of cancer [11], allergies [40], autoimmunity, [52] and infectious diseases [22]. Some DNA vaccines have been approved for veterinary use, including those against West Nile virus in horses, canine melanoma, growth hormone releasing hormone in pigs, and infectious hematopoietic necrosis virus in fish [1, 6–8, 20]. DNA vaccines encoding the BVDV E2 protein have been shown to induce virus neutralizing antibodies [16, 44, 49]. To enhance their efficacy, BVDV E2

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DNA vaccines have been tested in combination with adjuvants [28].

The co-stimulatory molecule CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily and is constitutively expressed on the surfaces of diverse immune and non-immune cell types, including B cells, dendritic cells, macrophages, and endothelial cells [31]. CD40 directly binds TNF receptor-associated factor 2 (TRAF2), TRAF3, TRAF5, and TRAF6 and indirectly associates with TRAF1 [3]. These interactions result in transcription factor activation, activation of mitogen and stress-activated protein kinase cascades, cytokine secretion, proliferation, differentiation of B cells into Ig-secreting plasma cells, and formation of humoral memory [9]. Once administered, agonistic anti-CD40 antibodies bind to CD40 on B cells and dendritic cells, activate the CD40/CD40L signaling pathway in these cells, and promote enhanced immune responses to DNA or synthetic peptide vaccines in mouse models [2, 4, 26, 47]. The co-administration of CD40-encoding plasmids with DNA vaccines enhances humoral immune responses to these vaccines [5, 51]. Although the precise mechanisms are not well understood, this effect is, at least in part, mediated by agonistic anti-CD40 antibodies induced by CD40-encoding plasmids [51].

CD63 is a member of the tetraspan family and is expressed on various cellular membranes, including extracellular vesicles. Also known as lysosomal-associated membrane protein 3, CD63 is involved in antigen presentation [10, 25, 32]. CD63 represents an activation-induced reinforcing element, the triggering of which promotes sustained and efficient T-cell activation and expansion [33]. A plasmid encoding ovalbumin (OVA)-CD63 fusion protein produced extracellular vesicles containing OVA at the site of administration and efficiently delivered OVA to dendritic cells. As a result, administration of the plasmid encoding the OVA-CD63 fusion protein enhanced cellular immune responses to OVA [18]. However, it is still unclear whether the co-administration of CD63-encoding plasmids with DNA vaccines enhances immune responses to these vaccines.

In this study, we used mice to investigate whether co-administration of a plasmid encoding CD40 or CD63 enhanced immune responses to a BVDV E2 DNA vaccine.

## MATERIALS AND METHODS

### Cell lines and virus

Madin-Darby bovine kidney (MDBK) cells and mouse myeloma P3U1 cells were kindly provided by the National Institute of Animal Health (Tsukuba, Japan). Human embryonic kidney (HEK) 293T cells were generously provided by Prof. Kei-ichiro Kizai (Iwate University, Morioka, Japan). Chinese hamster ovary (CHO)-K1 cells were purchased from ATCC (catalog no. CCL-61; Manassas, VA, USA). MDBK and HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan). Primary bovine fetal muscle (BFM) cells were prepared as described previously [43] and stored in liquid nitrogen before use. BFM cells within 20 passages were cultured in Eagle's Minimal Essential Medium (Nissui) supplemented with 10% tryptose phosphate broth. CHO-K1 and P3U1 cells were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan). These three media were supplemented with 5% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 units/mL penicillin (Nacalai Tesque), 100 µg/mL streptomycin (Nacalai Tesque), and 2 mM L-glutamine (Thermo Fisher Scientific). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air. The FBS used was confirmed to be free of anti-BVDV antibodies by using a virus neutralizing assay (described below).

BVDV 1 (Nose strain [19]) was propagated in MDBK cells. At 72 to 96 hr after infection at an MOI of 0.1 to 0.01, the cells underwent three freeze-thaw cycles; cell debris was removed by centrifugation at 1,710 × g for 15 min at 4°C. The virus titer was determined according to the 50% tissue culture infective dose (TCID<sub>50</sub>) for BFM cells in 96-well flat culture plates. The virus stock was stored at -70°C until use.

### Construction of DNA plasmids

For the construction of plasmids encoding mouse CD40 (pCD40) or CD63 (pCD63), total RNA was extracted from splenocytes or kidney, respectively, of BALB/c mice by using TRIzol reagent (Thermo Fisher Scientific). For construction of a plasmid encoding BVDV E2 protein (pE2), TRIzol-LS reagent (Thermo Fisher Scientific) was used to extract total RNA from the culture supernatant of MDBK cells infected with BVDV 1 Nose strain. A PrimeScript RT Reagent Kit (Takara Bio, Kusatsu, Japan) was used for reverse transcriptions of mRNA or the viral RNA genome. cDNA was synthesized by incubation at 37°C for 15 min and 85°C for 5 sec in a T100 Thermal Cycler (BioRad, Hercules, CA, USA).

The coding sequences of the mouse CD40 and CD63 genes were PCR-amplified by using specific primers. The primer sequences were (restriction enzyme sites are underlined): CD40 forward primer (ApaI), 5'-GTAGGGCCCGCGATGGTGTCTTTGCCTCGGCTGTG-3'; CD40 reverse primer (XbaI), 5'-GCTCTAGAGCTCAGACCAGGGGCCTCAAGG-3'. CD63 forward primer (EcoRI), 5'-GCGGAATTCACCACCATGGCGGTGGAAGGAGGAAT-3'; and CD63 reverse primer (ApaI), 5'-ATGGGGCCCCCCTACATTACTTCATAGCCAC-3'. Reaction mixtures consisted of 1 µL of 40 ng/µL cDNA (40 ng/10 µL of reaction mixture), 1 µL each of 10 µM forward and reverse primers, 5 µL of KOD One (Toyobo, Osaka, Japan), and 2 µL of sterilized ultrapure water. Amplification was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 60°C or 55°C for 5 sec (for CD40 or CD63, respectively), and extension at 68°C for 25 sec.

The BVDV E2 gene was PCR-amplified by using specific primers (E1 forward primer [ApaI + E1], 5'-GTGGGCCCACCATGGTACAGGGCATCTATGGCTA-3'; E2 reverse primer [KpnI + E2], 5'-ACGGTACCTCAAGCGAAGTAATCCCGGT-3') that were designed to amplify a partial E2 fragment that lacked the transmembrane region located at the 3' end (33 amino acids) to be expressed as a secreted form of E2 protein [27, 28]. Furthermore, the forward primer

was located in the C-terminal hydrophobic region of glycoprotein E1 so that the construct contained 16 amino-acid residues as a leader peptide. PCR amplifications were performed by using KOD One PCR Master Mix (Toyobo). Reaction mixtures consisted of 1  $\mu$ L of 40 ng/ $\mu$ L cDNA (40 ng/10  $\mu$ L of reaction mixture), 1  $\mu$ L each of 10  $\mu$ M forward and reverse primers, 5  $\mu$ L of KOD One (Toyobo), and 2  $\mu$ L of sterilized ultrapure water. Amplifications were performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 63°C for 5 sec, and extension at 68°C for 25 sec.

Each amplified DNA fragment was enzyme-digested and inserted into pUMVC4a expression vector (Aldevron, South Fargo, ND, USA) to yield pE2, pCD40, or pCD63. The plasmid constructs were verified by DNA sequencing (3500 Genetic Analyzer, Applied Biosystems, Carlsbad, CA, USA). The plasmids were amplified in *E. coli* DH5 $\alpha$  cells and purified by using a GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Plasmids were eluted by using nuclease-free ultrapure water and diluted to a concentration of 3  $\mu$ g/ $\mu$ L.

#### *Expression of recombinant BVDV E2 protein in E. coli*

The partial E2 gene (492 bp corresponding to the N-terminal 164 amino acids) of BVDV 1 Nose strain with N-terminal and C-terminal 6 $\times$  histidine tags was synthesized by using codon optimization (Eurofins Genomics, Tokyo, Japan). This partial E2 fragment was restriction digested and cloned into pET-41a expression vector (Merck, Darmstadt, Germany). The resulting plasmid, pET-41a/E2, was used to transform *E. coli* BL21 (DE3) cells (Merck). To induce the expression of recombinant BVDV E2 protein (rE2), isopropyl- $\beta$ -D-thiogalactopyranoside (final concentration, 1.0 mM) was added to the culture, which was then incubated for 6 hr at 30°C. Inclusion bodies containing rE2 were sonicated and solubilized in phosphate-buffered saline (PBS) containing protein denaturants (8 M urea and 10 mM  $\beta$ -mercaptoethanol). rE2 was affinity purified on Co<sup>2+</sup>-charged TALON resin (Takara Bio) under denaturing conditions, according to the manufacturer's instructions. This was followed by buffer exchange to remove denaturants. The resulting rE2 protein was used as the antigen for ELISA and the production of monoclonal antibodies (mAbs).

#### *Hybridoma production*

BALB/c mice were injected intraperitoneally with rE2 (100  $\mu$ g/dose); Imject Alum (Thermo Fisher Scientific) was used as an adjuvant. After three booster immunizations at 1-week intervals, splenocytes were harvested and then fused with P3U1 cells by using PEG1500 (Roche Diagnostics, Indianapolis, IN, USA). The resulting hybridomas were grown in RPMI 1640 medium supplemented with 10% FBS and hypoxanthine–aminopterin–thymidine selection medium supplement (Thermo Fisher Scientific). Hybridomas were chosen on the basis of the reactivity of their culture supernatants against rE2 in ELISAs. We established two hybridoma clones: E2-1a-1 and E2-1a-6. The reactivity of these mAbs to the antigen was confirmed by Western blot analysis and immunofluorescence staining. Protein G Sepharose 4 Fast Flow (Cytiva, Marlborough, MA, USA) was used to purify the mAbs from the supernatants of hybridomas cultured in Hybridoma-SFM medium (Thermo Fisher Scientific).

#### *Western blot analysis*

At 48 hr after transfection of plasmid pE2, pCD40, or pCD63 into HEK 293T cells, the cell lysates and culture supernatants were harvested; aliquots of each were boiled in SDS sample buffer (BioRad) and then electrophoresed on 10% polyacrylamide gels. The separated proteins were electrotransferred onto polyvinylidene difluoride membranes (Merck). After being blocked in PBS containing 5% skim milk, the membranes were incubated overnight at 4°C with 0.1  $\mu$ g/mL of anti-BVDV E2 mAb (clone E2-1a-1; this study), 0.1  $\mu$ g/mL of anti-CD40 mAb (clone G-12; Santa Cruz Biotechnology, Dallas, TX, USA), 0.1  $\mu$ g/mL of anti-CD63 mAb (clone: R5G2, MBL, Nagoya, Japan), or 0.1  $\mu$ g/mL of anti- $\beta$ -actin mAb (clone AC-15; Sigma-Aldrich) in the 1st antibody diluent (Can Get Signal Immunoreaction Enhancer Solution 1, Toyobo). The overnight incubation was followed by incubation for 1 hr at room temperature with peroxidase-conjugated anti-mouse IgG or anti-rat IgG antibodies (dilution, 1:10,000; Jackson ImmunoResearch, West Grove, ME, USA) in the 2nd antibody diluent (Can Get Signal Immunoreaction Enhancer Solution 2, Toyobo). Antibody binding was visualized by using ECL Prime Western Blotting Detection Reagent (Cytiva) and a ChemiDoc XRS+ Gel Imaging System (BioRad).

#### *Immunofluorescence staining*

pE2, pCD40, or pCD63 was transfected into CHO-K1 cells by using Polyethylenimine Max (Polysciences). At 24 hr after transfection, cell monolayers in Lab-Tek Chamber Slides (Thermo Fisher Scientific) were fixed with ice-cold methanol at -30°C for 20 min. After they had been blocked in PBS containing 3% bovine serum albumin, cells were incubated with 1  $\mu$ g/mL of anti-BVDV E2 mAb (clone E2-1a-6; this study), 1  $\mu$ g/mL of anti-CD40 mAb (clone G-12; Santa Cruz Biotechnology), or 1  $\mu$ g/mL of anti-CD63 mAb (clone R5G2; MBL) overnight at 4°C in PBS containing 3% bovine serum albumin and then incubated with FITC-conjugated anti-mouse IgG antibodies (dilution, 1:200; Jackson ImmunoResearch). Cell nuclei were stained by incubation for 15 min at room temperature in the dark in 1  $\mu$ g/mL 4–6-diamidino-2-phenylindole in PBS containing 3% bovine serum albumin. Slides were mounted by using buffered glycerol and then examined by fluorescence microscopy (IX73, Olympus, Tokyo, Japan).

#### *DNA vaccination*

Specific-pathogen-free, age (6 to 8 weeks)-matched female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and kept under conventional conditions. All procedures and animals used in this study were approved by the Iwate University Animal Care and Use Committee (no. A201909).

The mice were anesthetized through intraperitoneal injection of a combination of anesthetics (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol). BALB/c mice ( $n=50$ ) were randomly allocated to 10 groups; details of the vaccinated

**Table 1.** Mouse groups used for administration of plasmid DNA

Group	Injection dose ( $\mu\text{g}$ )			Number of mice	
	Empty vector control	pE2	pCD40		
Mock	100	–	–	5	
pE2	–	100	–	6	
12.5 $\mu\text{g}$ pCD40	–	100	12.5	4	
25 $\mu\text{g}$ pCD40	–	100	25	5	
50 $\mu\text{g}$ pCD40	–	100	50	5	
100 $\mu\text{g}$ pCD40	–	100	100	5	
12.5 $\mu\text{g}$ pCD63	–	100	–	12.5	5
25 $\mu\text{g}$ pCD63	–	100	–	25	5
50 $\mu\text{g}$ pCD63	–	100	–	50	5
100 $\mu\text{g}$ pCD63	–	100	–	100	5

groups are shown in Table 1. By using a 1-mL syringe and 27-gauge needle, 100  $\mu\text{L}$  of plasmid solution containing 100  $\mu\text{g}$  of pE2 and 12.5, 25, 50, or 100  $\mu\text{g}$  of pCD40 or pCD63 was administered intradermally (50  $\mu\text{L}$  each at two different sites) 1–2 cm distal to the tail base [29, 38]. The mice received a booster immunization at 3 weeks after the initial immunization; 2 weeks after the booster immunization, blood samples were collected and serum was analyzed for humoral immune responses. Splenocytes were harvested at this same time, and BVDV E2 antigen-specific cell proliferation and interferon (IFN)- $\gamma$  production were evaluated, as described below.

#### Humoral immune responses

Virus neutralization titers were determined by using a 96-well microplate assay. Sera were inactivated for 30 min at 56°C. Two-fold serially diluted serum samples were mixed with an equal volume of a BVDV 1 (Nose strain) suspension containing 200 TCID<sub>50</sub>/25  $\mu\text{L}$ ; the resulting solutions were placed in microplate wells and the plate was incubated at 37°C for 60 min. After this incubation, bovine fetal muscle cells (10<sup>5</sup> cells/well) were added to each mixture, which was incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 5 days. Each serum dilution was titered twice. The neutralizing antibody titer was expressed as the reciprocal of the highest dilution of the test serum that completely prevented cytopathic effects.

BVDV E2 antigen-specific IgG titers were determined by ELISA. Recombinant E2 protein (1  $\mu\text{g}/\text{mL}$ ) was immobilized on the well bottoms of Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific) by incubation for 60 min. After being blocked with 5% skim milk in PBS, the plates were incubated with serum from immunized mice (dilution, 1:200), followed by horseradish peroxidase-conjugated anti-mouse IgG<sub>1</sub> (dilution, 1:5,000; Santa Cruz Biotechnology) and IgG<sub>2a</sub> antibodies (dilution, 1:5,000; Santa Cruz Biotechnology). The enzymatic reaction was conducted by using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific). Optical density was measured at a wavelength of 450 nm on an Infinite 200 PRO multimode plate reader (Tecan Group, Männedorf, Switzerland). These reactions were performed at 37°C in a total sample volume of 50  $\mu\text{L}$ .

#### Cellular immune responses

To determine BVDV-specific lymphocyte proliferation, splenocytes were cultured for 48 hr at 37°C and 5% CO<sub>2</sub> in the presence of BVDV 1 (10<sup>4.5</sup> TCID<sub>50</sub> per well). All cell cultures were grown in 96-well flat-bottom plates containing 10<sup>6</sup> splenocytes in 200  $\mu\text{L}$  RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific), 100 units/mL penicillin (Nacalai Tesque), 100  $\mu\text{g}/\text{mL}$  streptomycin (Nacalai Tesque), and 2 mM L-glutamine (Thermo Fisher Scientific). After 48 hr of viral stimulation, viable cells were counted by using a WST-8 Cell Proliferation Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

To determine BVDV-specific IFN- $\gamma$  production, splenocytes were cultured for 72 hr in the presence of BVDV 1 as described for the proliferation assay; the culture supernatants were then collected and stored at –30°C. The concentration of IFN- $\gamma$  in the supernatant was measured by using a Mouse IFN- $\gamma$  Uncoated ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Optical density was measured at a wavelength of 450 nm on an Infinite 200 PRO multimode plate reader (Tecan Group).

#### Statistical analysis

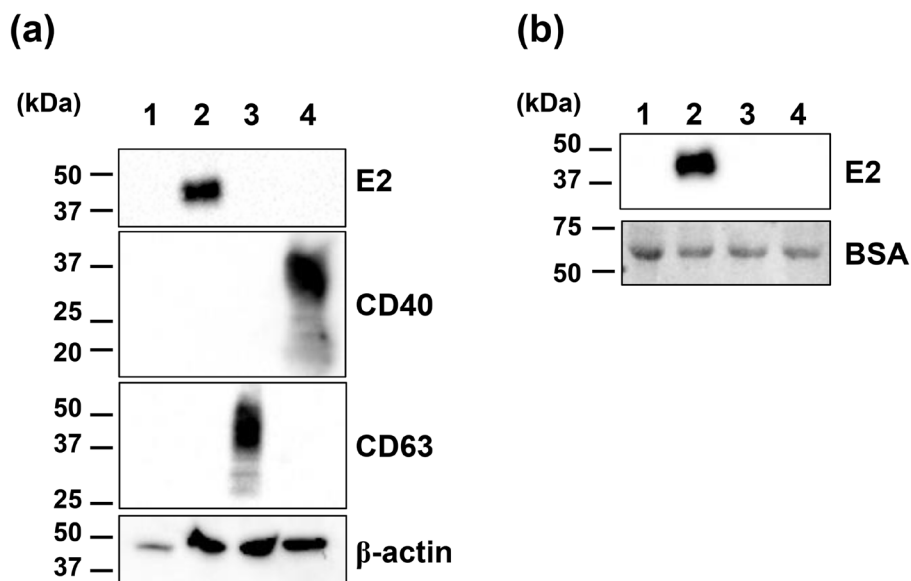
Serum neutralization antibody titers are presented as geometric means. E2-specific ELISA antibody titers are presented as means. All data were analyzed by using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) to perform one-way analysis of variance followed by Bonferroni's multiple comparison test or the Kruskal–Wallis test followed by Dunn's multiple comparison test. Statistically significant differences were defined as  $P < 0.05$ .

## RESULTS

#### Construction of expression plasmids

The coding sequences of a partial E2 (1,023 bp) with the C-terminal 16 amino acids of E1, CD40 (870 bp), and CD63 (717 bp) were PCR-amplified and individually cloned into the pUMVC4a expression vector. The recombinant protein expressed by pE2,





**Fig. 1.** *In vitro* expression of bovine viral diarrhea virus (BVDV) E2, mouse cluster of differentiation molecule (CD) 40, and mouse CD63 according to Western blot analysis. Human embryonic kidney (HEK) 293T cells were transfected with pUMVC4a (lane 1), pE2 (lane 2), pCD40 (lane 3), or pCD63 (lane 4). (a) Cell lysates (approximately 10 µg/lane) were electrophoresed through 10% polyacrylamide gels under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with anti-E2 (0.1 µg/mL), anti-CD40 (0.1 µg/mL), anti-CD63 (0.1 µg/mL), or anti-β-actin (0.1 µg/mL) mAb. (b) Cell culture supernatants (5 µL per lane) were electrophoresed through 10% polyacrylamide gels under reducing conditions and transferred onto a PVDF membrane. The membrane was stained with anti-E2 mAb (0.1 µg/mL) or Ponceau S. BSA, bovine serum albumin.

pCD40, or pCD63 was detected by Western blot analysis by using a BVDV E2-, CD40-, or CD63-specific mAb in the cell lysate of HEK 293T cells transfected with each expression vector (Fig. 1a). Because the partial E2 gene lacked the hydrophobic transmembrane region, recombinant E2 protein was detected as a secreted protein in the culture supernatant of pE2-transfected 293T cells (Fig. 1b). In addition, the recombinant protein expressed by pE2, pCD40, or pCD63 was detected in the cytoplasm of transfected CHO-K1 cells by immunofluorescence staining with a BVDV E2-, CD40-, or CD63-specific mAb (Fig. 2). These results indicated that pE2, pCD40, and pCD63 expressed recombinant BVDV E2 protein, CD40, and CD63, respectively, in eukaryotic cells.

#### Humoral immune responses

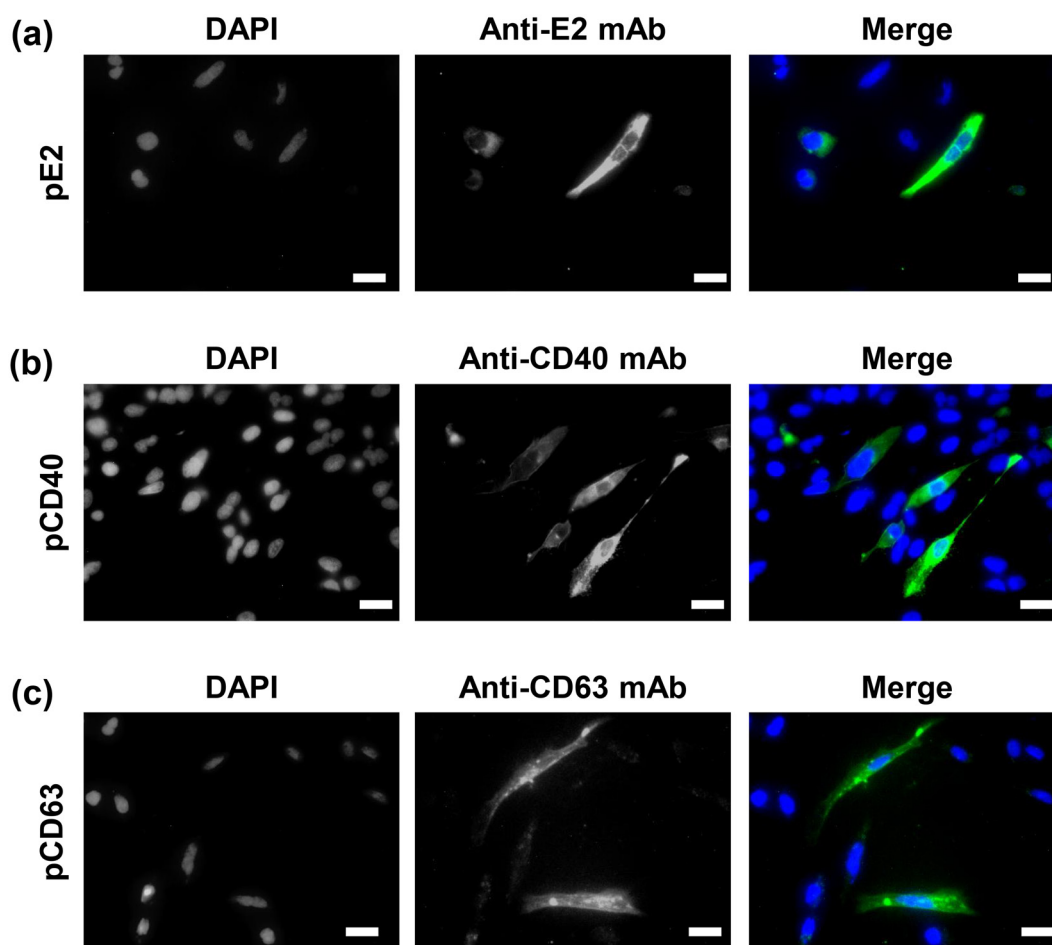
To investigate the effects of CD40 and CD63 plasmids on the humoral immune responses to a BVDV E2 DNA vaccine, virus neutralization titers were compared between the mice that received pE2 only and those that received pE2 with either pCD40 or pCD63. Neutralizing antibodies were detected in all groups except the mock-immunized group (Fig. 3a), and neutralizing antibody titers were significantly higher in the groups that received pE2 with 25 or 50 µg of pCD40 than in those that received pE2 only ( $P < 0.01$ ). In contrast, neutralizing antibody titers did not differ between the group that received pE2 alone and those that received pE2 with pCD63.

BVDV E2-specific IgG<sub>1</sub> and IgG<sub>2a</sub> antibody titers were measured by ELISA and compared between mice that received pE2 only and those that received pE2 with pCD40 or pCD63. E2-specific IgG<sub>1</sub> antibodies were detected in all groups except the mock-immunized group (Fig. 3b), and E2-specific IgG<sub>1</sub> titers did not differ between the group that received pE2 only and those that received pE2 with pCD40 or pCD63 (Fig. 3b). However, E2-specific IgG<sub>2a</sub> titers were significantly higher in the group that received pE2 with 12.5 µg of pCD63 than in those that received pE2 only ( $P < 0.05$ ; Fig. 3c). In addition, the IgG<sub>2a</sub>: IgG<sub>1</sub> ratio did not differ between the group that received pE2 only and those that received pE2 with either pCD40 or pCD63 (Fig. 3d).

#### Cellular immune responses

To investigate the effects of CD40 and CD63 plasmids on the cellular immune responses to a BVDV E2 DNA vaccine, BVDV-specific lymphocyte proliferation was compared between mice that received pE2 only and those that received pE2 with pCD40 or pCD63. BVDV-specific lymphocyte proliferation occurred in all other groups apart from the mock-immunized group (Fig. 4a). BVDV-specific proliferation was significantly greater in the group that received pE2 with 25 µg of pCD40 than in those that received pE2 only ( $P < 0.05$ ). In addition, BVDV-specific lymphocyte proliferation was greater in the group that received pE2 with 25 µg of pCD63 than in those that received pE2 only ( $P < 0.05$ ).

We then compared BVDV-specific IFN-γ production between mice that received pE2 only and those that received pE2 with either pCD40 or pCD63. IFN-γ production did not differ between the group that received pE2 only and the mock-immunized group (Fig. 4b), but it was significantly higher in the group that received pE2 with 12.5 µg of pCD40 ( $P < 0.05$ ). In addition, IFN-γ production was significantly higher in mice that received both pE2 and 25 µg of pCD63 than in those that received pE2 only ( $P < 0.01$ ).



**Fig. 2.** *In vitro* expression of bovine virus diarrhoea virus (BVDV) E2, mouse cluster of differentiation molecule (CD) 40, and mouse CD63. Chinese hamster ovary (CHO)-K1 cells were transfected with pE2 (a), pCD40 (b), or pCD63 (c) and stained with anti-E2 (1  $\mu\text{g}/\text{mL}$ ), anti-CD40 (1  $\mu\text{g}/\text{mL}$ ), or anti-CD63 (1  $\mu\text{g}/\text{mL}$ ) mAb and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI). Scale bar, 20  $\mu\text{m}$ .

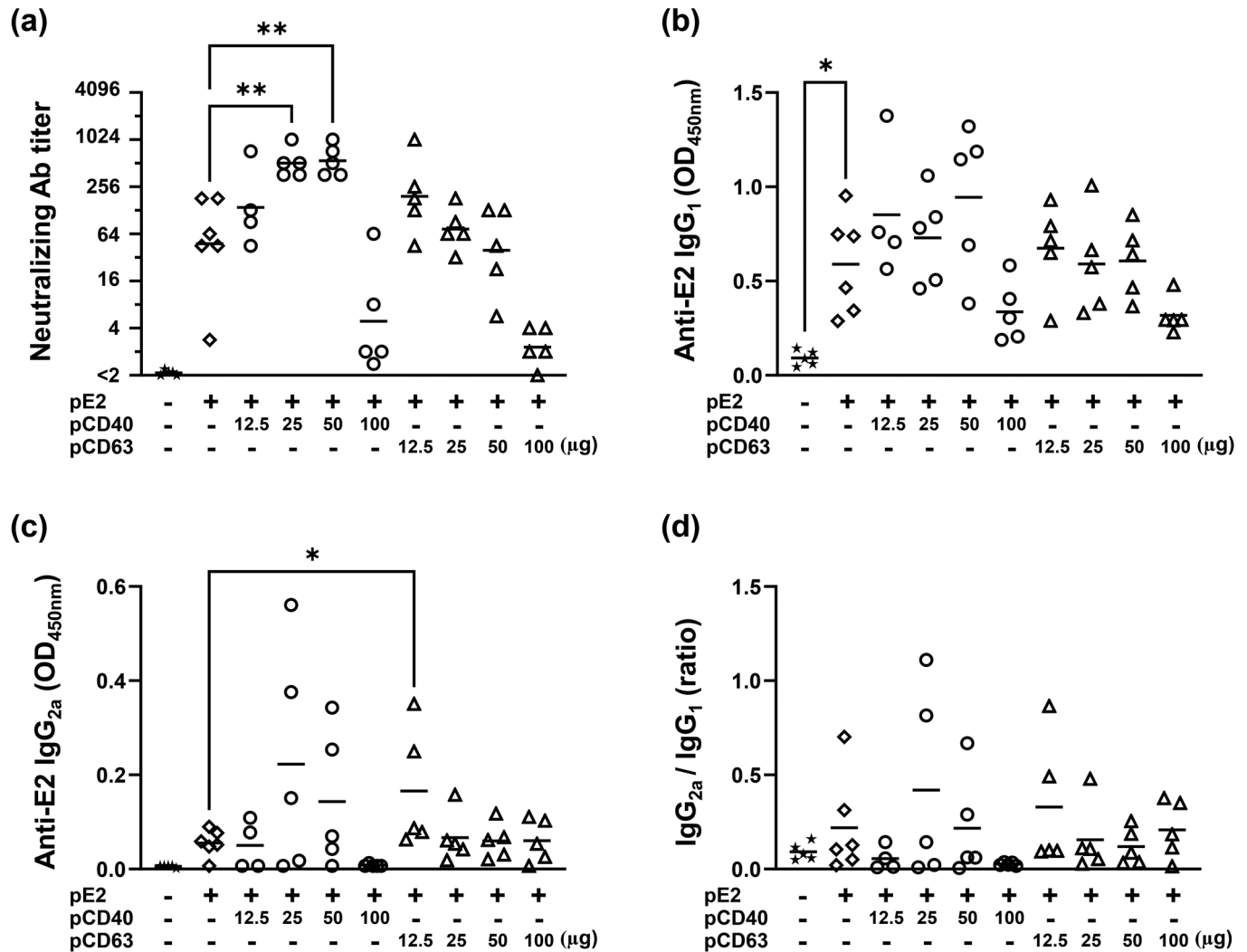
## DISCUSSION

We investigated the effects of CD40 and CD63 plasmids on the immune responses to a DNA vaccine against BVDV. First, we constructed plasmids encoding BVDV E2 protein (pE2), mouse CD40 (pCD40), or mouse CD63 (pCD63) and used Western blot analysis and immunofluorescence staining to confirm that these plasmids expressed these proteins in cultured cell lines. We then immunized BALB/c mice by using pE2 in combination with, or without, pCD40 or pCD63.

Our results showed that co-administration of pCD40 enhanced humoral and cellular immune responses to pE2. These results are consistent with those of previous studies. For example, a plasmid encoding CD40 enhanced humoral and T-cell immune responses to a DNA vaccine against foot-and-mouth disease [51]. In another study, a plasmid encoding CD40 enhanced the production of type 2 helper T (Th2) cytokines and antibody responses to a DNA vaccine against H5N1 highly pathogenic avian influenza [5]. These results suggest that co-administration of a plasmid encoding CD40 is a promising means of enhancing humoral and cellular immune responses to DNA vaccines.

In addition, our current study demonstrates that co-administration of pCD63 enhances cellular immune responses to pE2. These results are inconsistent with those of a previous study, in which co-administration of a plasmid encoding CD63 failed to increase cellular immune responses to an OVA-encoding plasmid [18]. The reason for this apparent discrepancy is unclear, but it may reflect differences in antigen (BVDV E2 or OVA), plasmid (pUMVC4a or pCI, although both use the same cytomegalovirus promoter), administration route (intradermal or intramuscular), mouse strain (BALB/c or C57BL/Cj), or other experimental conditions. Further studies are needed to clarify whether co-administration of a plasmid encoding CD63 enhances cellular immune responses to DNA vaccines.

We found that pCD40—but not pCD63—enhanced the neutralizing antibody responses induced by pE2. This apparent difference may reflect the varied mechanisms through which these factors exert their effects. In previous studies [5, 51], the effect of pCD40 was, at least in part, mediated by agonistic anti-CD40 antibodies which enhance the activation of the CD40/CD40L signaling pathway in dendritic cells and helper T cells upon antigen presentation [51]. In contrast, it has been previously shown that the expression

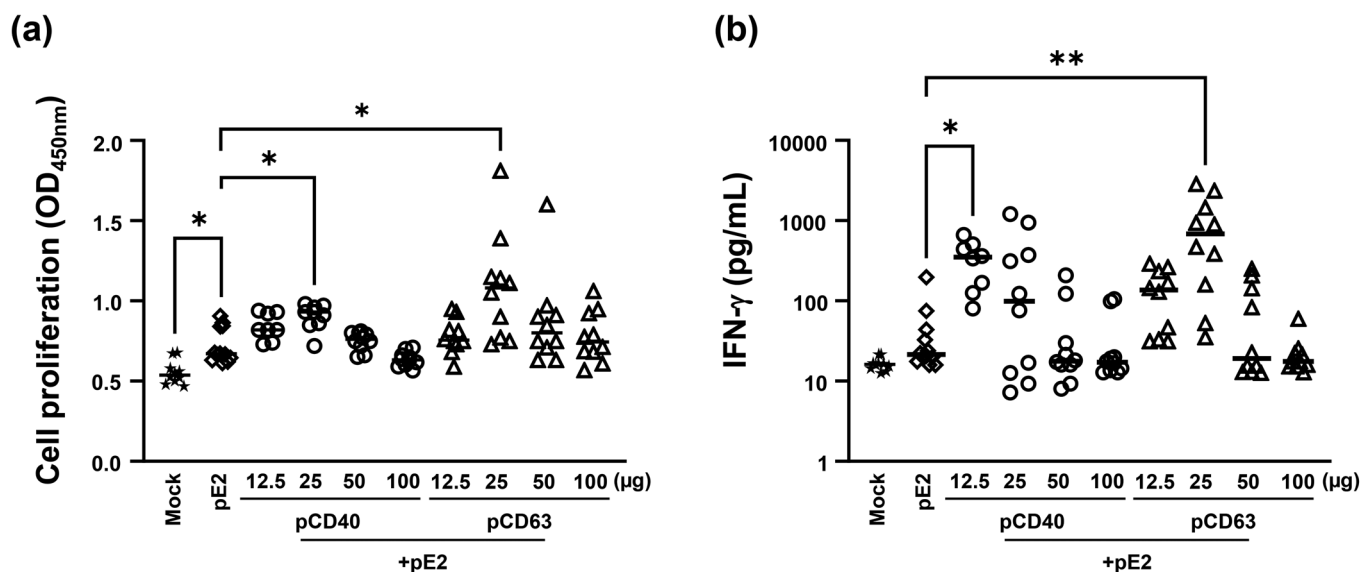


**Fig. 3.** Adjuvant effects of cluster of differentiation molecule (CD) 40 and CD63 on humoral immune responses to a bovine virus diarrhoea virus (BVDV) E2 DNA vaccine. BALB/c mice were intradermally immunized twice with pE2 in combination with, or without, pCD40 or pCD63 (3 weeks between doses). (a) Virus neutralizing antibody titers. Horizontal bars indicate geometric mean values. (b) Anti-E2 IgG<sub>1</sub> titers. Horizontal bars indicate mean values. (c) Anti-E2 IgG<sub>2a</sub> titers. Horizontal bars indicate mean values. (d) IgG<sub>1</sub>:IgG<sub>2a</sub> ratio. Horizontal bars indicate mean values. Differences between groups were assessed by one-way analysis of variance followed by Bonferroni's multiple comparison test,  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*). The presented data are representative of two independent experiments. ★, pUMVC4a; ◇, pE2; ○, pE2 and pCD40; △, pE2 and pCD63.

of an antigen and CD63 as a fusion protein produced extracellular vesicles containing the antigen at the site of administration and efficiently delivered the antigen to dendritic cells [18]. However, the mechanisms how the co-administration of pCD63 affect the immune responses to DNA vaccines are totally unclear in this study.

In the current study, we did not observe dose-dependency in the adjuvant effect of pCD40 or pCD63. For example, the highest dose (100 µg) of neither pCD40 or pCD63 enhanced the immune responses to pE2, whereas 25 or 50 µg of pCD40 induced the greatest neutralizing antibody responses, and 25 µg of pCD63 induced the greatest lymphocyte proliferation and IFN-γ responses. The reason that the highest doses of pCD40 and pCD63 failed to induce the highest immune responses is unclear. In our preliminary *in vitro* experiments, the expression of E2 protein in the culture supernatant of HEK 293T cells co-transfected with pE2 (3 µg) and pCD40 or pCD63 (3 µg) was lower than that in the culture supernatant of HEK 293T cells transfected with pE2 (3 µg) alone (data not shown). This result suggests that co-administration of an excessive dose of pCD40 or pCD63 may have competitively diminished the expression of E2 from pE2 *in vivo*. In addition, the excessive dose of pCD40 or pCD63 may have hampered the expression of CD40 or CD63, respectively, consistent with the inhibitory effect of excess doses of plasmids on the expression of foreign genes [12, 41]. The optimal dose of a plasmid encoding CD40 or CD63 for use as a DNA vaccine adjuvant will be needed to be determined in the target animal species.

Intradermal administration of pE2 encoding a secreted form of BVDV E2 protein induced IgG<sub>1</sub> antibody responses, and co-administration of pCD40 or pCD63 did not significantly change the IgG<sub>2a</sub>: IgG<sub>1</sub> ratio in our current study. In contrast, intramuscular



**Fig. 4.** Adjuvant effects of cluster of differentiation molecule (CD) 40 and CD63 on cellular immune responses to a bovine virus diarrhoea virus (BVDV) E2 vaccine. BALB/c mice were immunized intradermally twice with pE2 in combination with, or without, pCD40 or pCD63 (3 weeks between doses). **(a)** BVDV-specific lymphocyte proliferation. Splenocytes were stimulated with BVDV for 48 hr. Then, WST-8 was added and the absorbance at a wavelength of 450 nm was measured. **(b)** BVDV-specific interferon (IFN)- $\gamma$  production. Splenocytes were stimulated with BVDV for 72 hr. IFN- $\gamma$  in the cell culture supernatants were measured by ELISA. Horizontal bars indicate median values. Differences between groups were assessed by using the Kruskal-Wallis test followed by Dunn's multiple comparison test,  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*). The data presented are representative of two independent experiments.  $\star$ , pUMVC4a;  $\diamond$ , pE2;  $\circ$ , pE2 and pCD40;  $\Delta$ , pE2 and pCD63.

administration of a plasmid encoding a secreted form of BVDV E2 predominantly induced IgG<sub>2a</sub> antibody responses in previous studies [27, 28]. The reason for this apparent discrepancy is unknown. The cellular localization of antigens encoded by DNA vaccines affects the Th1/Th2 bias: administration of plasmids encoding secreted antigens induces IgG<sub>1</sub> responses, whereas administering plasmids encoding cytosolic antigens induces IgG<sub>2a</sub> responses [13, 39]. The route of administration of DNA vaccines also affects the Th1/Th2 bias: intradermal administration of DNA vaccines induces IgG<sub>1</sub> responses, whereas intramuscular administration induces IgG<sub>2a</sub> responses [24, 30]. Therefore, our current induction of IgG<sub>1</sub> antibody responses by the intradermal administration of pE2 encoding a secreted form of E2 protein is consistent with these previously established patterns.

In conclusion, the results that we have presented indicate that co-administration of a plasmid encoding CD40 or CD63 enhances humoral and cellular immune responses to a DNA vaccine encoding BVDV E2 protein in mice. Further studies are needed to investigate whether the plasmid encoding CD40 or CD63 enhances immune responses to, and protection by, the BVDV E2 DNA vaccine in cattle. Our current results further suggest that these adjuvant plasmids have the potential to compensate for the weak immunogenicity of DNA vaccines—not only for BVDV but also for various other applications, including other infectious diseases, therapy of cancer, allergies, and autoimmunity.

**CONFLICT OF INTEREST.** The authors declare that no conflict of interest exists.

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