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Enhanced protective efficacy and reduced viral load of foot-and-mouth disease DNA vaccine with co-stimulatory molecules as the molecular adjuvants

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Abstract

To improve efficacy of DNA vaccination, various approaches have been developed, including the use of plasmid expressing co-stimulatory molecules as molecular adjuvants. In this study, we investigated whether co-inoculation of a construct expressing either 4-1BBL or OX40L as the molecular adjuvant with FMDV DNA vaccine, pcD-VP1, can increase immune responses and protective efficacies. Compared to the group immunized with pcD-VP1 alone, the co-inoculation of either molecular adjuvant induced a higher ratio of IgG2a/IgG1, higher levels of expression of IFN-γ in $CD4^+$ and $CD8^+$ T cells and antigen-specific CTL responses, and more importantly provided an enhanced protection against the live FMDV challenge in animals. Concurrently, 4-1BBL as the molecular adjuvant dramatically reduced the viral loads of FMDV in vivo after the challenge. Together, the results demonstrate that co-stimulatory molecules 4-1BBL and OX40L can enhance the antigen-specific cell-mediated responses elicited by VP1 DNA vaccine and provide an enhanced protective efficacy with the reduced viral loads.

Keywords: FMDV DNA vaccine; Protection; Viral load; Co-stimulatory molecules; Neutralizing antibody; in vivo CTL; Cell-mediated immune responses; 4-1BBL; OX40L

1. Introduction

Foot-and-mouth disease (FMD) is a disease caused by FMD virus (FMDV) which is belongs to the genus Aphthovirus in the family Picornaviridae (Pereira, 1981) and mainly infects cloven hoofed animals appearance of vesicles on the feet and mouth. FMD is an important problem world wide, especially in many developing countries, the outbreak of FMD results in great loss of economy in those countries (Sobrino and Domingo, 2001; Grubman and Baxt, 2004). The use of killed FMDV in oil emulsion as vaccines for livestock industry represents an effective strategy to prevent the viral infections (Brown, 1992). Protective immunity to FMDV is not yet fully understood. However, the high level of neutralizing antibodies is highly effective in controlling disease and viral transmission after immunization of a killed FMDV vaccine. In spite of such vaccination and generation of neutralizing antibodies, persistent infections are detectable in those vaccinated animals (Alexandersen et al., 2002). Although cell-mediated immunities (CMI) including antigen-specific CD4+ and CD8+ T cell responses are induced upon FMDV infections (Collen, 1994; Childerstone et al., 1999; Garcia-Briones et al., 2004; McCullough and Sobrino, 2004), their roles in protection against FMDV remains largely unknown, although some reports describe at least to contribute partial protection (Sanz-Parra et al., 1999; Garcia-Briones et al., 2004). Moreover, the residual live FMDV were detected and persisted in the farm animals after they were vaccinated, suggesting an inefficiency of the inacti-

Abbreviations: RT-PCR, reverse transcriptase PCR; GAPDH, glyseraldehyde-3-phosphate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PMS, phenazine methosulfate; CFSE, carboxyfluorescein succinimidyl ester; TCID50, 50% of tissue culture infectious dose

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vated FMDV vaccine in oil adjuvant (McVicar and Sutmoller, 1969).

DNA-based vaccination is an alternative method which has been used successfully in raising protective immune responses against various pathogenic agents including HIV-1, influenza, parasites, and tumor cells (Fynan et al., 1993; Ulmer et al., 1993; Wang et al., 1993; Wang et al., 1995; Srivastava and Liu, 2003) in small animal models. On the other hand, it could effective induce both humoral and cellular immune responses in vivo. Although DNA vaccination is limited in that it often generates only weak immune response when used alone in large animals, this could be augmented by introducing of optimal adjuvants (Tacket et al., 1999; Boyer et al., 2000; Jin et al., 2004).

Adjuvants are widely used in various vaccine formulations for the enhancement of immune responses. Among the adjuvants, expressing constructs containing genes encoding cytokines or co-stimulatory molecules have been considered as molecular adjuvants and can be used to enhance immune effects of DNA vaccine (Svanholm et al., 1997; Kim et al., 1998).

Within the co-stimulatory molecules, OX40L and 4-1BBL are belong to tumor necrosis factor (TNF) receptor family and found to be expressed on the professional antigen presenting cell (APC). Their functions are believed to activate $CD4^+$ T cell functions, enhance $CD8^+$ CTL activity, and promote memory T cells. The use of OX40L and 4-1BBL proteins or expressing genes as adjuvants for vaccinations has been reported previously (Guinn et al., 1999; Gramaglia et al., 2000; Ishii et al., 2003; Serghides et al., 2005; Du et al., 2007), however, their roles in protection and clearance of viral infections are not completely clear.

In this study, we attempted to use a eukaryotic construct encoding either OX40L or 4-1BBL as a molecular adjuvant to co-inoculate with FMDV VP1 DNA vaccine and evaluated their roles in the immune efficacy and clearance of viral infection in a rodent viral challenge model. The results showed that both humoral and cellular immunities have been significantly enhanced in animals after the addition of either adjuvant with the pcD-VP1. The enhanced cell-mediated responses were apparently correlated with the levels of protection induced by FMDV DNA vaccines. The results demonstrated that the co-inoculation of OX40L or 4-1BBL construct could significantly improve the protective efficacy of FMD DNA vaccine against FMDV infection.

2. Materials and methods

2.1. Animal and cell

Female Balb/c mice aged 6–8 weeks and guinea pigs weighing at 400–500 g were purchased from Animal Institute of Chinese Medical Academy (Beijing, China) and were maintained under a clear air condition with pathogen-free food and water. The Hela cell line was cultured in DMEM (GibcoBRL, NY, USA) supplemented with 10% of fetal bovine serum and penicillin–streptomycin (GibcoBRL, NY, USA) in a humidified 37 °C incubator under 5% CO_2.

2.2. FMDV vaccine and antigen

The killed FMDV vaccine (chemically inactivated FMDV and emulsified in mineral oil) was obtained from Jinyu Group Corp. (Huhhot, Inner Mongolia, China) and used as a positive control vaccine in both antibody and viral challenge studies. 146S antigen, a chemically inactivated FMDV and purified by sucrose grading at Jinyu Group Corp., was quantified by the Bradford micro-assay kit (Bio-Rad, USA) and used for the specific antigens for ELISA, T cell proliferations and intracellular staining. FMDV VP1 peptide of a T cell epitope (aa133-147, SSKYGDTSTNNVRGD) was synthesized by GL Biochem Co., Ltd. (Shanghai, China) and used for in vivo CTL assay.

2.3. Antibodies and fluorescent dye

Fluorescent conjugated rat anti-mouse monoclonal antibodies including anti-IL-4-PE, anti-IFN-γ-FITC, anti-CD4-FITC, anti-CD4-PE, anti-CD8-PE and isotype controls were purchased from BD PharMingen (San Diego, CA). CFSE was obtained from Molecular Probes (Eugene, OR).

2.4. Plasmid constructions

The plasmid pcD-VP1 encoding for the FMDV VP1 protein was constructed in our lab and used as the DNA vaccine previously (Jin et al., 2004). The gene of 4-1BBL or OX40L was respectively cloned into the downstream of a CMV promoter and hCG-β leader sequence of a provax vector constructed previously in this lab (Tu Yixian et al., 2005), and designated as provax-4-1BBL or provax-OX40L used as the molecular adjuvants (Du et al., 2007).

2.5. Immunization

All the plasmids were maxi-prepared by alkaline method and purified by PEG8000 precipitation (Sambrook et al., 1989), subsequently diluted in saline solution. For vaccination, the mice were randomly divided into 6 groups, 15 animals per group, and guinea pigs were randomly divided into 6 groups and 5 animals per group. They were immunized intramuscularly with pcD-VP1 alone or co-immunized with the respective molecular adjuvant listed in Table 1 on days 0, 14 and 28. pcD-VP1 plus provax vector were also used to eliminate the so-called "sparing effect" from the vector. The mice and guinea pigs were prebled and bled on day 7 after the third immunization.

2.6. Detection of anti-FMDV antibody

ELISA was performed following a previously published procedure (Jin et al., 2005). 96-Well plates were coated with 2 μg/ml 146S antigen per well. The serum total IgG, IgG1 and IgG2a isotypes antibody titers were defined as the highest dilution that gave an above 2:1 ratio between testing serum and the naive negative control.

Table 1
Immunization groups

Animals	Group	Vaccine	Adjuvant
MICE	1	100 μg pcDVP1	100 μg provax-4-1BBL
MICE	2	100 μg pcDVP1	100 μg provax-0X40L
MICE	3	100 μg pcDVP1	100 μg provax
MICE	4	100 μg pcDVP1	None
MICE	5	100 μg pcDNA3	None
MICE	6	100 μl Killed FMDV vaccine	Oil
Guinea pigs	1	300 μg pcDVP1	300 μg provax-4-1BBL
Guinea pigs	2	300 μg pcDVP1	300 μg provax-0X40L
Guinea pigs	3	300 μg pcDVP1	300 μg provax
Guinea pigs	4	300 μg pcDVP1	None
Guinea pigs	5	300 μg pcDNA3	None
Guinea pigs	6	200 μl Killed FMDV Vaccine	Oil

Note: (1) Animals were immunized intramuscularly on days 0, 14 and 28 with pcD-VP1 alone or plus the respective molecular adjuvant. (2) Mice and guinea pigs were immunized intramuscularly on day 0 with the killed FMDV vaccine in emulsified in oil.

2.7. T cell proliferation

Three mice of each group were sacrificed and their single lymphocyte suspensions were prepared from the spleen on day 7 after the third injection as described previously (Mosmann, 1983). Single lymphocyte suspensions were incubated in triplicates in 96-well plates at 5×10^4 cells/well, in RPMI-1640 plus 5% of fetal calf serum (FCS) at 37 °C in a 5% CO_2 incubator and stimulated for 48 h with 5 μg/ml of Con A (positive control), 2 μg/ml of the 146S (specific antigen stimulation), 2 μg/ml of BSA (irrelevant antigen), or no antigen (negative control). T cell proliferation was evaluated by Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay according to the manufacturer's instruction (Promega, USA). The mixture of MTS/PMS (20 μl each well) was added to each well to develop the color. After 4 h of incubation, the OD values of plates were read at 490 nm by a plate reader (Magellan, Tecan Austria GmbH). Data were expressed as stimulation index (SI), calculated as the mean reading of triplicate wells of antigen stimulation, divided by the mean reading of triplicate wells of negative control.

2.8. In vivo CTL assay

In vivo CTL assay was performed as described previously (Piriou et al., 2000), to prepare target cells for the detection of in vivo cytotoxic activity, splenocytes were isolated from naïve BALB/c spleen after erythrocytes removing, which were pulsed with VP1 peptide representing the T cell epitope (SSKYGDTSTNNVRGD) at 50 μg/10^6 cells for 16 h and labeled with a high concentration of CFSE (2.5 μM, $CFSE^{high}$ cells) as the target cells. The equal fraction of the splenocytes was labeled with a low concentration of CFSE (0.25 μM, $CFSE^{low}$ cells) without 146S pulse as a non-target control. On day 7 after the third immunization, each mouse was injected intravenously 2×10^7 cells of mixture of equal number of target and control cells. Three mice of each group were sacrificed 4 h later to isolate splenocytes, which were then analyzed on a FACSCalibur analyzer (BD Biosciences, USA). The specific lysis was calculated by the following formula: ratio = (percentage $CFSE^{low}$/percentage $CFSE^{high}$). Percentage specific lysis = [1 − (ratio unprimed/ratio primed) × 100].

2.9. Intracellular cytokine staining

Three mice of each group were sacrificed on day 7 after the third immunization. Single-cell suspensions from the spleens at 0.5×10^6 cells/200 μl were stimulated in the 96-well plates with 146S (5 μg/ml) and anti-CD28 (5 μg/ml) mAb for 12 h at 37 °C in 5% CO_2, followed by the addition of the Monensin (2 μg/ml) for 4 h, then washed two times with PBS. Cells were blocked with 1 μl of Fcγ mAb (0.5 μg/ml) for 30 min at 4 °C and fixed with 4% of paraformaldehyde at 4 °C for 15 min before the permeabilization with 0.1% saponin at 4 °C for 10 min. After one time of washing with PBS, cells were used to stain with isotype controls, or double stain with anti-CD4-FITC and anti-IL-4-PE, or anti-CD8-PE and anti-IFN-γ-FITC, or anti-CD4-PE and anti-IFN-γ-FITC for 30 min at 4 °C. The fluorescent intensities were measured by the FACSCalibur and analyzed by Cell Questpro software (BD Biosciences, USA).

2.10. FMDV-specific neutralization antibody assay

Accord to the recommend method of OIE/FAO World Reference Laboratory (WRL) for FMDV (Pirbright, UK), we detected the neutralization antibody of guinea pigs by the liquid phase blocking ELISA (LPB-ELISA) assay (Hamblin et al., 1986; Ferris et al., 1990), on 7 days after the third immunization. Briefly, the 96-wells was coated with an optimal dilution (1:4000) of rat anti-FMDV antibody in 0.05 M carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C to provide trapping antibodies. The liquid phase was carried transfer to U-bottom, 96-well plates (Nunc Inc.). A mixture of purified killed FMDV at a constant predetermined dilution (1:5) in PBS and serum samples with a serial dilution in PBS was used. After incubation overnight at 4 °C, 50 μl of serum-virus mixtures were transferred from the carrier plates to the ELISA plates and incubated at 37 °C for 1 h. The plates were washed five times by PBST and reacted with a pretitrated optimal dilution (1:2000) of rabbit anti-FMDV sera conjugated with HRP incubated the Plates 1 h at 37 °C. The colorimetric values were read after five times of washing. Moreover, 10 wells in each plate were reserved for the viral antigen control without any serum sample, the neutralizing antibody level of each serum sample was then calculated according to the following criterion: the dilution titer when 1/2 of the OD value of the viral antigen control equates with the OD value of the serum sample.

2.11. Viral challenge

On day 7 after the last immunization, each guinea pig was subcutaneously challenged with 0.2 ml of 100 ID_{50} of live viruses on left back foot, and housed separately to examine for 7 days in a BSL-3 laboratory (Jinyu Group Corp., Inner Mongolia).

Total protection was defined as no any lesion on the feet. Partial protection was defined as lesion occurred on the injected foot only. The animal was defined as no protection when lesions were found on two and more feet of the challenged animal.

2.12. Determination of viral load by real-time PCR

The footpads received the viral challenge were removed and viral RNAs were extracted on the day 7 after the challenged from guinea pigs. Virus loads were determined by real-time RT-PCR assay with the FMDV-specific primers, which are recommended by the World Reference Laboratory (WRL) for foot-and-mouth disease: SAIR89F, 5′-CTGTCTCGTAGCGGAGCATG-3′, and SAIR151R, 5′-GCCCCGTGGGTCCTTG-3′, where they are located in the internal ribosome entry site (IRES) of FMDV. cDNAs were synthesized using the AMV reverse transcriptase and a random primer used as the templates for the real-time PCR. Each PCR reaction was performed on 1 μl of cDNA mixed with 20 μl 2× Hotstart SYBR green PCR mix (Invitrogen Inc., USA), 100 nM of each primer and RNAse-free water for a final volume of 40 μl using the Applied Biosystems ABI-PRISM 7900HT detection system. Amplifications were performed as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles at 95 °C for 15 s and 56 °C for 30 s and 72 °C 30 s. The results of real-time PCR were analyzed using the SDS 2.1 software. Each PCR reaction was optimized with specific primers GAPDH, 5′-TCATGACCACAGTCCATGCCATCACT-3′ and 5′-GCCTGCTTCACCACCTTCTTGATGT-3′ for the housekeeping as described previously (Kawahara et al., 2004). Standard curve of viral concentration was obtained by real-time RT-PCR assay of a known FMDV titer previously titrated in a cell culture as TCID50. For each test sample, number of viruses was determined by means of the standard curves and expressed in number of TCID50/ml.

2.13. Statistic analysis

The data were analyzed using the one-sided Student's *t*-test. Differences were considered to be statistically significant with $p < 0.05$ and indicated as one asterisk in figures.

3. Results

3.1. Co-stimulatory molecules as adjuvants increase the titer of total IgG, IgG1 and IgG2a

To examine the effect of molecular adjuvants on the humoral responses in mice, the pcD-VP1 was inoculated alone or co-inoculated with the provax-4-1BBL or provax-OX40L into mice intramuscularly, respectively. On day 7 after the third immunization, the serum samples were collected and the titers of total IgG, IgG1 and IgG2a antibodies against FMDV were detected by the ELISA. The results showed that the animals received pcD-VP1 plus either co-stimulatory molecule produced higher titers of total IgG, IgG1 and IgG2a than in the group immunized with pcD-VP1 alone. Moreover, the provax-4-1BBL induces the highest titer of total IgG and IgG2a, compared with the provax-OX40L, although they both produced the similar serum IgG1 titers (Fig. 1). The data indicated that the co-stimulatory molecules in combination with DNA vaccine can improve the humoral response. The enhanced IgG2a titer suggests that the immunoglobulin switched under the Th1 type response was influenced by those adjuvants, particularly from the provax-4-1BBL.

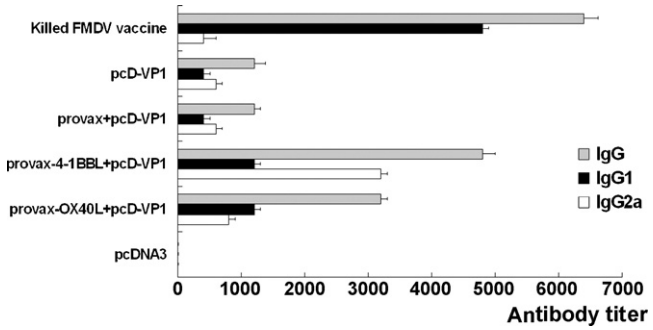

Fig. 1. Effects of the molecular adjuvants on the level of antibodies. Balb/c mice were immunized with pcD-VP1 with or without the provax-4-1BBL, or provax-OX40L, vector controls or the killed FMDV vaccine intramuscularly as indicated on *y*-axis. Serum samples were collected and diluted for detecting by ELISA on day 7 after the final boost. 146S antigen at 2 μg/ml was coated on each well in 96-well plate and titers of total IgG (▨), IgG1 (■) and IgG2a (□) were determined as indicated on *x*-axis.

3.2. Effect of co-stimulatory molecules as adjuvants on T cell proliferative response

To test for T cell response, single-cell suspensions were prepared and isolated from the spleens of the immunized mice on day 7 after the third immunization, and stimulated with 146S specific antigen. As shown in Fig. 2, the co-immunization with provax-4-1BBL as adjuvant induced the highest level ($p < 0.05$) in the proliferative response, while the co-immunization with provax-OX40L did not induce a higher level of stimulation compared to the group immunized with the pcD-VP1 alone or with the provax vector. The result suggests that the provax-4-1BBL preferentially promoted the antigen-specific T cell response significantly.

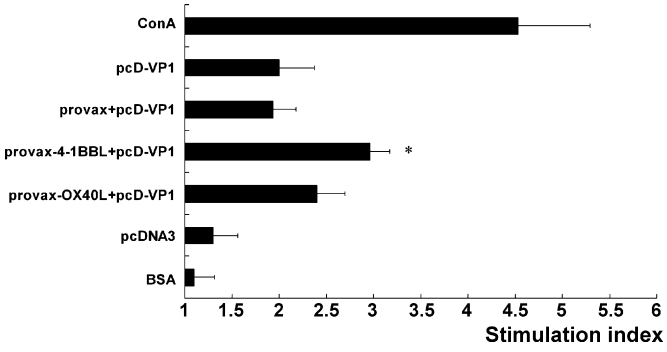

Fig. 2. Analysis of T cell proliferation in response. Balb/c mice were immunized with pcD-VP1 with or without the provax-4-1BBL, or provax-OX40L, or vector controls intramuscularly as indicated in *y*-axis. T cells were isolated from three animals per group and stimulated with 146S as the specific antigen, ConA as the positive control, and BSA as the negative control. Proliferation was analyzed by the MTS/PMS method and expressed as a stimulated index as indicated on *x*-axis.

3.3. Effect of molecular adjuvants on cytokine expressions in T cells

To detect the expression of intracellular cytokines in the T cells, splenocytes isolated from 3 mice each group on day 7 after the third immunization were double stained with anti-CD4 plus anti-IL4, anti-CD4 plus anti-IFN-γ, or anti-CD8 plus anti-IFN-γ, and detected by FACS after stimulated with the 146S in vitro. As a representative result shown in Fig. 3A–C and a sum of three independent experiments in Fig. 3D, the highest percentages of antigen induced IL-4 and IFN-γ in $CD4^+$ and IFN-γ in $CD8^+$ T cells were observed from the group immunized with the provax-4-1BBL as the adjuvant. Although mice immunized with pcD-VP1 and provax-OX40L induced the significantly higher level of IL-4 in $CD4^+$ than in the pcD-VP1 alone group, the levels of IFN-γ in $CD4^+$ and $CD8^+$ cells were not significantly different. These results indicate that provax-4-1BBL as adjuvant in DNA vaccine can enhance both Th1 and Th2 types of responses, and preferentially improves the Th1 response.

3.4. Effect of the molecular adjuvants on CTL in vivo

To further determine whether the molecular adjuvants influence the cellular immune responses, an antigen-specific CTL activity in vivo was assessed on day 7 after the third immunization. As a representative result shown in Fig. 4, the percentage of antigen-specific lysis of target cells in the mice immunized with pcD-VP1 plus provax-4-1BBL was at 54.5%, which reached the highest level comparing to the 31.7% and 26.7% of lysis from the pcD-VP1 plus provax-OX40L and pcD-VP1 alone group. The results suggest that the provax-4-1BBL used as a molecular adjuvant enhanced the antigen-specific CTL activities in vivo,

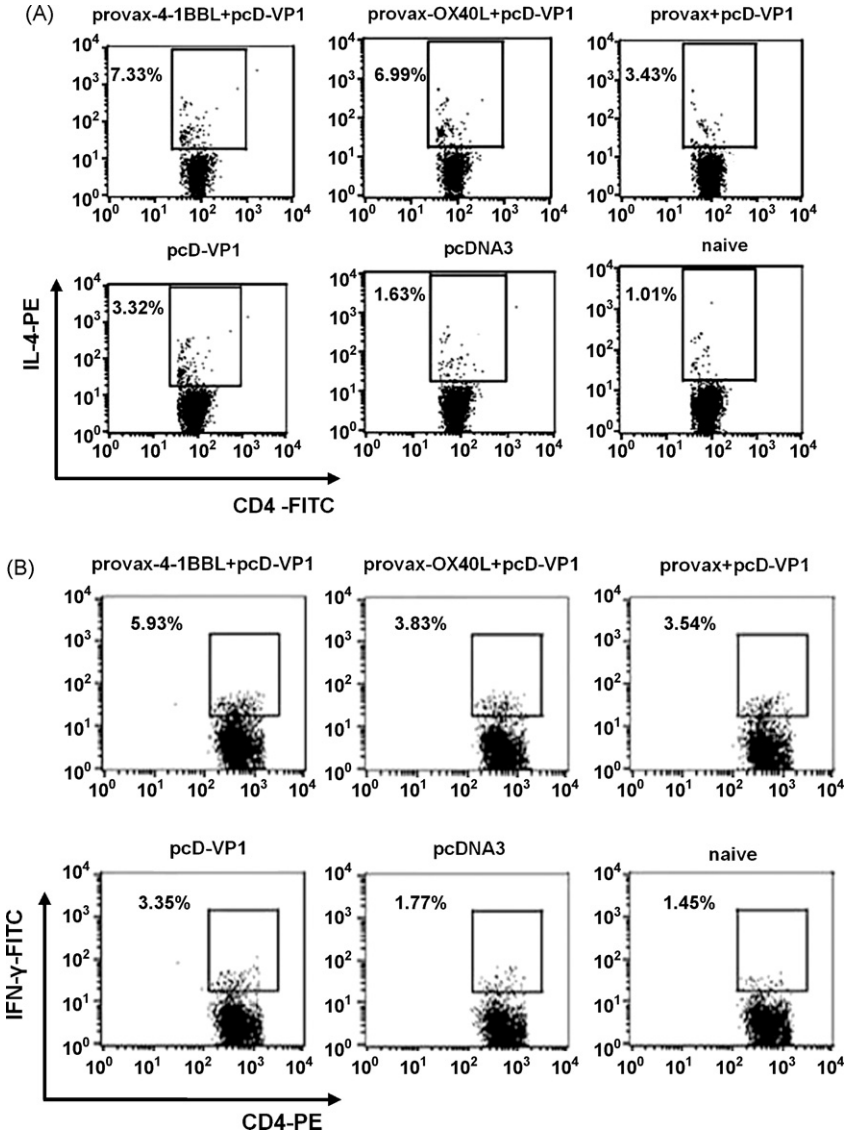

Fig. 3. Analysis of production of antigen-specific intracellular cytokines by FACS. T cells isolated and purified from the spleen of Balb/c mice after the final boost were stimulated with 146S for 6 h in vitro culture. Intracellular stainings for IL-4 in $CD4^+$ (A), IFN-γ in $CD4^+$ (B), and IFN-γ in $CD8^+$ (C) cells was performed. The percentages of positive cells were showed in each dot-plot in the gated area. Panel D is a representation of the percentage of intracellular cytokines of each group. The data shown are representative of three independent experiments.

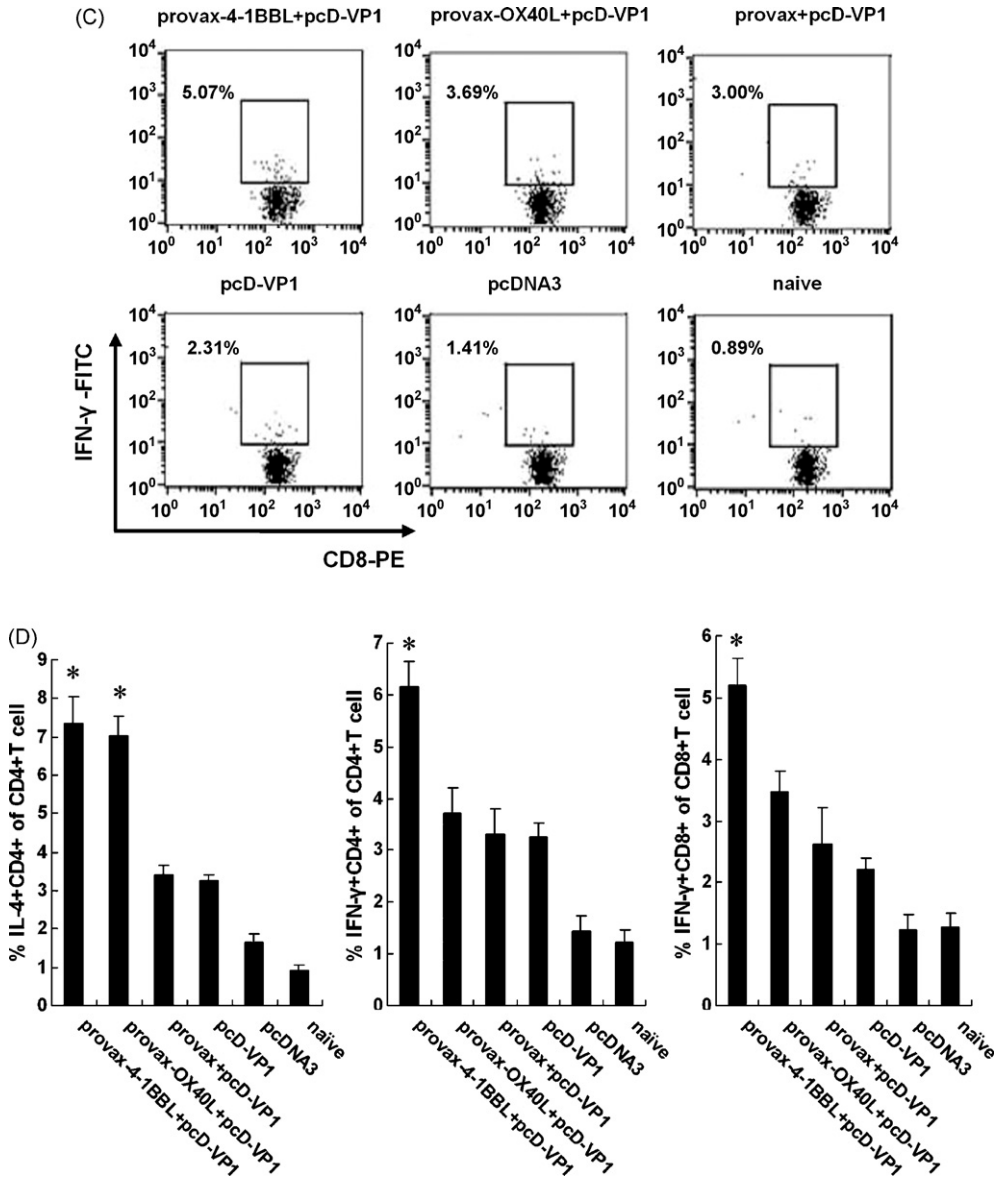

Fig. 3. (*Continued*).

which is consistent with the result of IFN-γ expressed in $CD8^+$ T cells.

3.5. Effect of molecular adjuvants on the level of neutralizing antibody

Since neutralizing antibodies are directly related to FMDV protection in animals, the titer of antiviral neutralizing antibodies from the immunized guinea pigs were detected by LPB-ELISA. As shown in Table 2, the highest level of neutralizing antibody was observed in animals receiving the pcD-VP1 plus provax-4-1BBL. The group immunized with the pcD-VP1 plus provax-OX40L also induced a higher neutralizing titer than in the group immunized with the pcD-VP1 alone or with the vector. This result suggests that the both molecular adjuvants could enhance the level of neutralizing antibody against FMDV.

3.6. Evaluation of protective efficacy by viral challenge after DNA immunization

Because mice are not susceptible to FMDV infection, the guinea pigs were used to evaluate the efficacy for vaccination strategy. To evaluate whether the adjuvants tested in the DNA vaccinations could augment the protective efficacy against viral challenge in animals, all immunized and the control guinea pigs were infected with 100 TCID50 of FMDV on day 7 after the third immunization. As shown in Table 2, the viral challenge system was validated by that five of five animals were protected in the group immunized by the chemically killed-FMDV vaccine served as the positive control and no protection in animals immunized by pcDNA3 vector as the negative control. Four of five animals were protected in the group immunized with pcD-VP1 and provax-4-1BBL, and three of five animals were protected in the group immunized with pcD-VP1 and provax-

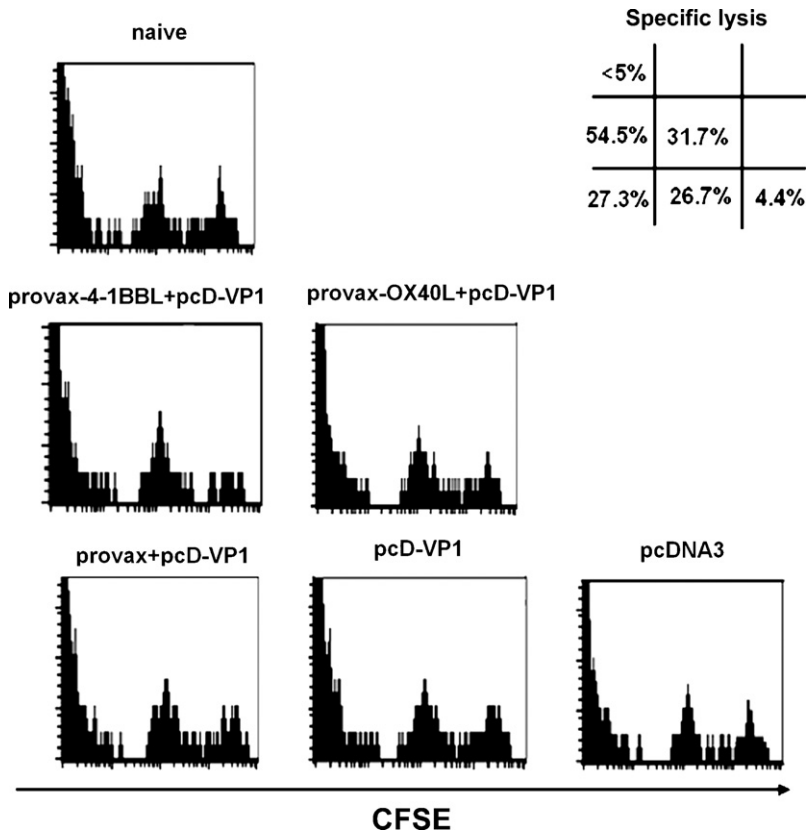

Fig. 4. Effects of the molecular adjuvant on in vivo CTL responses. The splenocytes from naïve mice as target cells were pulsed with or without a VP1 T cell epitopic peptide as described in Section 2 and subsequently labeled with either high or low concentration of CFSE respectively. A 1:1 mixture of 10^7 cells of each target cell population was transferred i.v. into the naïve syngeneic mice or mice previously immunized with pcD-VP1 plus provax-4-1BBL, or provax-OX40L, 7 days after the third immunization. After 4 h, the mice were killed and the T cells were analyzed for the presence of $CFSE^{high}$ and $CFSE^{low}$ target cell. The percentage of specific lysis of each group was calculated and indicated on the upper right panel. The data shown are representative of three independent experiments.

OX40L; whereas only two out five animals were protected in the group immunized with the pcD-VP1 alone or with the pcD-VP1 and provax (Table 2). Although both provax-4-1BBL and provax-OX40L as the molecular adjuvants augmented the protective efficacy against FMDV infection, the provax-4-1BBL provided a higher ratio of protection.

3.7. Viral loads in guinea pigs

Having demonstrated the enhanced protective efficacy from both provax-4-BBL and provax-OX40L served as the adjuvants, it would be interested to test whether the viral loads in each group of animals were affected and consistent with the challenge

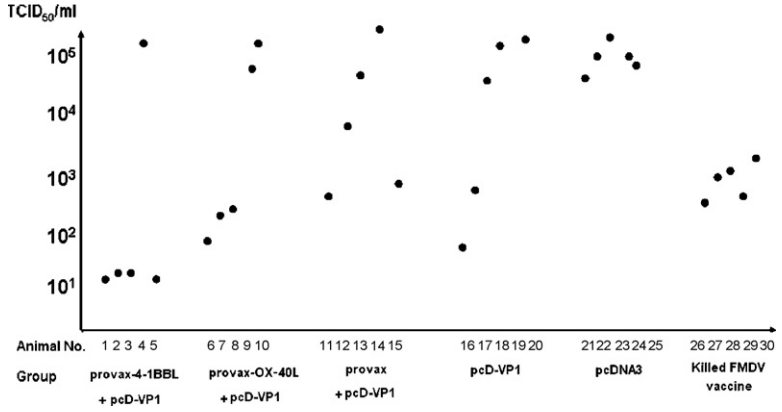

Fig. 5. Effects of the molecular adjuvant on the virus load in the challenged guinea pigs. Guinea pigs (5 animals/group) immunized with pcD-VP1 and different adjuvants or controls were challenged with 100 TCID50 of FMDV on day 7 after third immunization. On day 7 after the challenge, the guinea pigs were sacrificed and total RNAs from their feet where the viruses injected were extracted and isolated to generate cDNAs by RT. The cDNAs were amplified by real-time PCR. Viral concentration was determined against the number of viruses pre-determined at TCID50/ml as showed on *y*-axis and vaccines for each individual animal are indicated on *x*-axis.

result. Viral loads were determined on the day 7 after challenged by real-time RT-PCR assay with the FMDV-specific primers. As shown in Fig. 5, FMDV concentration in the group immunized with pcD-VP1 with provax-4-BBL was almost reduced 2 logs compared to the positive control vaccine, except one animal which showed none protection after the challenge. Viral loads in three animals in the group immunized with pcD-VP1 plus provax-OX40L were lower compared with the positive control vaccine, and the other two showed higher viral loads were the two failed in the challenge. The two animals shown at low viral loads in the pcD-VP1 and pcD-VP1 plus provax vector groups were also the animals protected after the viral challenge.

Table 2
Level of neutralizing antibodies and rate of protections

Groups animals no.	NAb titer[a]	Protection[b]	Severity of symptom[c]
pcDVP1+PROVAX-4-1BBL			
1	45	Total	None
2	128	Total	None
3	90	Total	None
4	22	None	Severe
5	64	Total	None
pcDVP1 + PROVAX-0X40L			
6	64	Total	None
7	64	Total	None
8	45	Total	None
9	<16	None	Severe
10	45	Partial	Mild
pcDVP1 + PROVAX			
11	64	Total	None
12	<16	None	Severe
13	<16	None	Severe
14	22	None	Severe
15	45	Total	None
pcDVP1			
16	45	Total	None
17	45	Total	None
18	<16	None	Severe
19	22	None	Severe
20	22	None	Severe
pcDNA3			
21	<16	None	Severe
22	<16	None	Severe
23	<16	None	Severe
24	<16	None	Severe
25	<16	None	Severe
Killed FMDV vaccine			
26	90	Total	None
27	128	Total	None
28	90	Total	None
29	180	Total	None
30	90	Total	None

[a] Serum samples were taken on days 0 and 28 before challenge with live FMDV and the titers of neutralizing antibody (NAb) were measured as described in Section 2.

[b] Severity of symptom was scored as: none, no lesion on any foot; mild, lesions on injection foot; sever, lesions on two and more feet.

[c] Total protection was defined as complete absence of lesions; partial protection was scored as lesions restricted on challenge foot; none protection was defined as lesionson both or more feet (Guo et al., 2005).

Although, all five animals were protected in the group immunized with the killed FMDV vaccine, their viral concentrations were at the level of 10^3 TCID50/ml, suggesting a considerable amount of viruses could not be cleared after such vaccination. The result further suggests that provax-4-BBL as a molecular adjuvant could significantly inhibit viral replication in the challenged animals, implying that a potent cell-mediated immunity mediated the viral clearance.

4. Discussion

Immunization with DNA vaccines is an alternative approach which is being investigated against FMD by eliciting both humoral and cell-mediated responses including antigen-specific CTL. Additionally, the effect of co-stimulatory molecules 4-1BBL and OX40L on the enhancement of immune responses has been reported previously. For instance, Bukczynski et al. (2004) demonstrated that 4-1BBL could be used as an efficient adjuvant for human antiviral cytotoxic T cell responses. Ishii et al. (2003) reported that OX40 and OX40L interaction could plays an adjuvant role during Th2 responses in vivo. Recently, we have also showed that OX40L was a more potent adjuvant over the 4-1BBL when used a molecular adjuvant for HBsAg DNA vaccine (Du et al., 2007). In the present study, we showed that the use of 4-1BBL as the molecular adjuvant for FMD DNA vaccine induced the highest levels of IgG production with a higher ratio of IgG2a/IgG1, T cell proliferation and CTL responses among all the groups tested. Although, animals immunized with pcD-VP1 plus provax-OX40L could enhance the specific humoral response, a lower level of cell-mediated responses was exhibited when compared with the same responses elicited by 4-1BBL as the adjuvant.

Neutralizing antibody plays a key role in protecting animals from the acute FMDV infection (Cedillo-Barron et al., 2001). We first showed that the immunization of pcD-VP1 plus the constructs expressing the co-stimulatory molecules produced higher titers of neutralizing antibodies specific against FMDV, indicating that improved efficacy in protection may be achieved against FMDV infection in animals. Furthermore, the improved protective efficacy of such strategy was confirmed in the guinea pigs by challenging live FMDV. As consistent with the level of neutralizing antibodies, a higher protective rate was observed in the animals immunized with pcD-VP1 plus 4-1BBL followed by plus OX40L, suggesting that the higher immune responses enhanced by the two adjuvants were correlated with the protections. Although neutralizing antibody is an important element in protection against FMDV, it is well documented that the protective immunity against viral infections is also associated with the induction of cell-mediated responses (Garcia-Briones et al., 2004). To further understand if the higher level of cell-mediated responses elicited by using the two adjuvants correlated with the viral clearance of in vivo, viral concentrations were assessed by real-time RT-PCR after the viral challenge the correlations were established. Although the full protection was achieved in the group received the killed FMDV vaccine, their viral concentrations were stayed round 1000 TCID50, versus the 10–30 or 80–500 TCID50 in animals immunized pcD-VP1 plus 4-1BBL

or pcD-VP1 plus OX40L, respectively. This result suggests that the higher cell-mediated responses elicited by DNA vaccine plus adjuvants, the lower viral replications could be obtained. It further implies that the killed FMDV vaccine provides less ability to stop the viral replications in vivo, which might be one of the reasons for persistent infections in animals after such vaccinations (McVicar and Sutmoller, 1969).

4-1BBL and OX40L as molecular adjuvants enhanced the level of FMDV-specific cell-mediated response in several important parameters including their proliferation, CTL and cytokine expressions in T cells. All those may contribute to the inhibition of viral replication in vivo observed in the study, even though in general, antigen-specific CTL is considered an essential component of the immune response for the control of viral replication and persistency, and leads to eradicate the established virus infection in the host (Akahata et al., 2003).

In summary, our study is the first time to demonstrate that the enhanced protective efficacies and reduced viral loads by 4-1BBL and OX40L used as the molecular adjuvants for FMD DNA vaccine against FMDV challenge in animals. However, whether such strategy used in this study can provide the similar protective efficacy in cattle or swine should be determined in the future.

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