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Hemagglutinating virus of Japan envelope (HVJ-E) can enhance the immune responses of swine immunized with killed PRRSV vaccine

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically detrimental pig pathogen that causes significant losses for the pig industry. The immunostimulatory effects of hemagglutinating virus of Japan envelope (HVJ-E) in cancer therapy and the adjuvant efficacy of HVJ-E have been previously evaluated. The objective of this study was to investigate the adjuvant effects of HVJ-E on immunization with killed PRRSV vaccine, and to evaluate the protective effects of this immunization strategy against virulent PRRSV infection in piglets. Next, the PRRSV-specific antibody response, lymphocyte proliferation, PRRSV-specific IL-2, IL-10 and IFN-γ production, and the overall protection efficacy were evaluated to assess the immune responses of the piglets. The results showed that the piglets inoculated simultaneously with killed PRRSV vaccine and HVJ-E had a significantly stronger immune response than those inoculated with killed PRRSV vaccine alone. Our results suggest that HVJ-E could be employed as an effective adjuvant to enhance the humoral and cellular responses of piglets to PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded, positive RNA virus of the *Arteriviridae* family that causes fetal abortion and respiratory disorders in swine, resulting in significant economic losses. Porcine reproductive and respiratory syndrome (PRRS) first emerged in North America in 1987 [1] and has caused serious health problems in the swine industry worldwide.

One strategy currently being used to control the clinical diseases caused by PRRSV infection is vaccination. Killed PRRSV vaccines are recommended for use in sows and gilts to reduce reproductive failure, or in sows, gilts, boars, and piglets to reduce reproductive and respiratory disorders. Unfortunately, the efficacy of killed PRRSV vaccine is not ideal [2]. To help improve the efficacy of killed PRRSV vaccine, several adjuvants, including interleukin-2 (IL-2), interferon-γ (IFN-γ) and CpG oligodeoxyribonucleotides (ODN), have been utilized to accelerate and magnify the immune responses [3,4].

Hemagglutinating virus of Japan envelope (HVJ-E) is composed of a lipid bilayer obtained from inactivated HVJ [5]. HVJ-E has been used as a safe and efficient non-viral vector for drug delivery, because it can incorporate DNA, RNA, proteins or drugs and deliver them into cells both *in vitro* and *in vivo* [6–9]. Moreover, HVJ-E has been reported to directly activate dendritic cells (DCs), and it has been used as an adjuvant in birds, in which it can activate both innate and adaptive immunity [10,11]. Therefore, we hypothesize that HVJ-E could act as a potent adjuvant for mammals due to its special immunostimulatory ability.

To further characterize HVJ-E's immunostimulatory activity and to investigate its effect on the immune response of piglets against virulent PRRSV, HVJ-E was administered to piglets along with a killed PRRSV vaccine. After simultaneous immunization with a killed PRRSV vaccine and HVJ-E, the piglets had significantly enhanced cytokine and antibody production, as well as reduced virus shedding. These results suggest that the HVJ-E could stimulate both humoral and cellular immune responses.

2. Materials and methods

2.1. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Concanavalin A (ConA) (Sigma Co., Ltd.) were used for the cell proliferation assay. A PRRS Antibody Test Kit was obtained from IDEXX Yuanheng Biotechnology Co., Ltd., China. PRRSV

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antigen and a killed PRRSV vaccine containing the JXA1-R strain at least 10^6 $TCID_{50}$/ml were gifts from Unibio Co., Ltd., China. The swine IFN-γ immunoassay kit (Invitrogen Inc.) and the swine IL-2 and IL-10 immunoassay kits (R&D Inc.) were used for the cytokine assays. Ficoll-Paque was obtained from DingGuo Biotech, Co., Ltd., China and the MiniBEST Viral RNA/DNA Extraction Kit and PrimeScript® One Step RT-PCR Kit were obtained from TaKaRa, Co., Ltd., China.

2.2. Preparation of HVJ-E

HVJ (Z strain) was preserved in our laboratory and HVJ-E was prepared as described previously [12]. The inactivated HVJ that was unable to replicate was referred to as HVJ-E.

2.3. Animals and experimental design

All animal usage was approved by the China Agricultural University Research Ethics Committee. Forty 28-day-old piglets were obtained from a commercial herd source. Serum samples were collected prior to experimentation and tested for the presence of antibody titers specific to PRRSV using an IDEXX ELISA kit, to ensure that all animals used in the experiment were PRRSV-free. PCR was used to detect PRRSV [13], classical swine fever virus (CSFV), pseudorabies virus (PRV), porcine parvovirus (PPV) and porcine circovirus type 2 (PCV-2). All pigs tested negative for PRRSV-specific antibodies and PRRSV, CSFV, PPV, PRV and PCV-2 virus. Piglets were randomly divided into four groups of ten. Group A was immunized with PBS, group B was immunized with killed PRRSV vaccine, group C was co-immunized with 3×10^9 HVJ-E particles and killed PRRSV vaccine, and group D was immunized with 3×10^9 HVJ-E particles. All formulations were delivered in a total volume of 3 ml, which was administered in the musculi colli.

2.4. Sample collection

Blood samples were collected in evacuated test tubes with or without heparin lithium (150 USP units) by venipuncture of the precaval vein on days 7, 14, 21 and 28 post-immunization using sterile equipment and procedures. All blood samples collected with anticoagulant were analyzed for PBMC proliferation, IL-2, IL-10 and IFN-γ at days 14 and 28 post-immunization. On days 0, 3, 7, 14 and 21 post-challenge, all serum samples were subjected to viremia detection. All serum samples were stored at −20 °C prior to use in ELISA.

2.5. Cell preparation

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation of the anticoagulant-treated peripheral blood samples obtained from pigs as previously reported [4].

2.6. PRRSV-specific PBMC proliferation assay

PRRSV-specific PBMC proliferation assay was performed as previously reported [4]. The geometric means and standard deviations for triplicate sets of samples were calculated. Lymphocyte proliferation was expressed as a stimulation index (SI), which is defined as the mean of the experimental data divided by the mean of the unstimulated control.

2.7. Serological examination

The PRRSV-specific serum antibody responses were analyzed using an IDEXX ELISA kit according to the manufacturer's instructions. The PRRSV-specific antibody titer was reported as the *S*/*P* ratio, and the serum samples were considered positive if the *S*/*P* ratio was 0.4 or higher.

2.8. Cytokines assay

Porcine IFN-γ, IL-10 and IL-2 were assayed by culturing PBMCs (1×10^6 cells/ml) in triplicate with PRRSV antigen (10 μg/ml). The control stimuli included RPMI 1640 medium alone or ConA at 5 μg/ml. The cells were cultured for 72 h at 37 °C with 5% CO_2, after which the supernatants were harvested and stored at −70 °C until analysis. The presence of porcine IFN-γ, IL-2 and IL-10 in PBMC culture supernatants was determined using commercial swine IFN-γ, IL-2 and IL-10 immunoassay kits according to the manufacturer's instructions. The geometric means and standard deviations for triplicate sets of samples were calculated. The supplied standards were used to generate a standard curve. The detection limits of the assay were 2 pg/ml for IFN-γ and 15 pg/ml for IL-2 and IL-10, respectively.

2.9. Infection challenge

Four weeks after vaccination, all pigs were challenged intramuscularly with $10^{4.5}$ $TCID_{50}$ of the virulent PRRSV (JXA1-R strain). The swab samples and sera were collected at days 0, 3, 7, 14 and 21 post-challenge for the detection of virus excretion. The mortality rate, body temperatures, nervous symptoms, and respiratory deficiencies were observed daily for 21 days post-challenge, and weight gain was evaluated weekly. The following scores were used for nervous symptoms and respiratory deficiencies: 0 denotes the absence of nervous symptoms and respiratory deficiencies, 1 denotes mild nervous symptoms and/or respiratory deficiencies, and 2 denotes severe nervous symptoms and respiratory deficiencies.

2.10. PCR detection of viremia and virus excretion

All nasal swabs and sera samples were prepared and used for RT-PCR detection of the virus. The total viral RNA from sera or nasal swabs immersed in 400 μl PBS was purified with a MiniBEST Viral RNA/DNA Extraction Kit, after which RNA was reverse transcribed and amplified using specific primers (forward primer: 5′-GAGTTTCAGCGGAACAATGG-3′, reverse primer: 5′-GCCGTTGACCGTAGTGGAG-3′) located in PRRSV ORF6 with a PrimeScript® One Step RT-PCR Kit. The PRRSV JXA1-R strain was used as positive control, and the detection limit was 4 ng of total RNA.

2.11. Statistical analysis

Statistical analysis was performed using SPSS v.10 statistical software and the data distribution was determined using descriptive statistics. All data that were not normally distributed were transformed by ranking. Differences in the titers were investigated using ANOVA, performed on the rankings. The means of the ranks were compared using Tukey's multiple comparison tests. The differences were considered statistically significant when the *p*-value <0.05.

3. Results

3.1. HVJ-E induces systemic PRRSV-specific PBMC responses

HVJ-E-mediated lymphocyte proliferation was measured using the MTT colorimetric assay method. Representative results of a proliferation study using purified PBMCs from the four groups are shown in Fig. 1. These results showed that there was no difference

in the PBMC response between group A, group B and group D. The PBMC proliferative response in piglets from group C was significantly stronger than those in piglets from the other groups on days post-immunization (DPI)14 and 28 ($p < 0.05$). In addition, PBMCs from the group C piglets on DPI 14 had a similar stimulation index (SI) to those at DPI 28. There was no significant difference between these two time points for group C piglets.

3.2. PRRSV-specific cytokines production in porcine PBMC supernatants

Higher concentrations of IFN-γ and IL-2 and a lower concentration of IL-10 were observed in the HVJ-E-added groups when compared with the control groups receiving vaccine alone or PBS ($p < 0.05$), suggesting increased Th1 polarization in the presence of HVJ-E. The levels of cytokines observed in group D were similar to those seen in group C, in which the level of IFN-γ and IL-2 were higher and the level of IL-10 was lower than in the control animals ($p < 0.05$). There was no difference in the levels of IL-2, IFN-γ and IL-10 produced by the animals in group B and group A (Fig. 2). These results indicated that the increase in cell-mediated immunity in piglets is due to the response against HVJ-E.

3.3. Antibody immune response of piglets to PRRSV in different groups

The kinetics of the development of the PRRSV-specific antibody responses in the four experimental groups as measured by ELISA is shown in Fig. 3. At DPI 7, PRRSV-specific antibodies were detectable in group C, while no antibody was detectable in the other three groups. Piglets from group C had higher antibody titers than those in group B at every time point post-immunization ($p < 0.05$). PRRSV-specific antibodies were not detectable in either the PBS or HVJ-E inoculated groups (Fig. 3).

3.4. Clinical reactions post-challenge

In the PBS group, mortality was first observed at 10 days post-challenge (DPC) and continued until 18 DPC. Only one animal from that group survived after the challenge. Six and four piglets survived in group B and group D after the challenge, respectively, whereas all animals in group C survived, suggesting the addition of HVJ-E into the killed vaccine induced obvious effects with regard to resistance to the infection (Table 1).

The piglets in group B showed anorexia and high fever (41–42 °C) at 3 DPC. Increased body temperature was also observed in group A and D piglets. The piglets in group C exhibited a high fever at 5 DPC, which dropped to below 41 °C 6 days later. Nervous symptoms and respiratory deficiencies began to appear in group B at 4 DPC, while

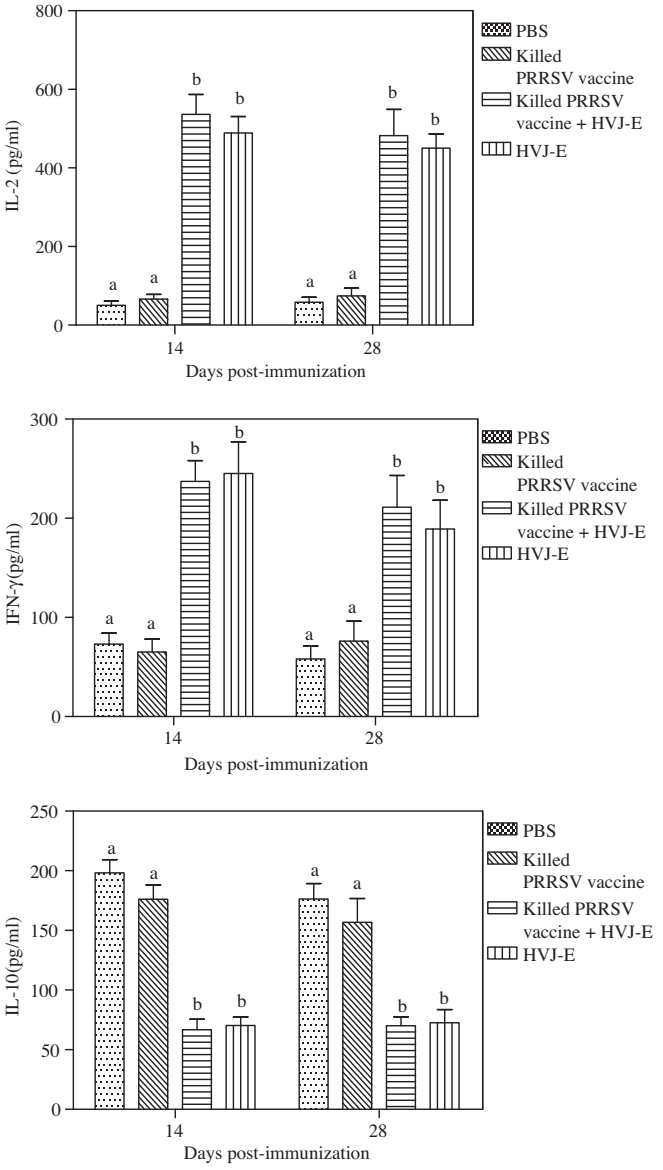

Fig. 2. The production of IL-2, IL-10 and IFN-γ at DPI 14 and 28 in porcine PBMC supernatants determined by ELISA. Each bar represents the group mean ($n = 10$) ±SEM of cytokine levels determined in triplicate. The difference between groups with different letters is significant ($p < 0.05$).

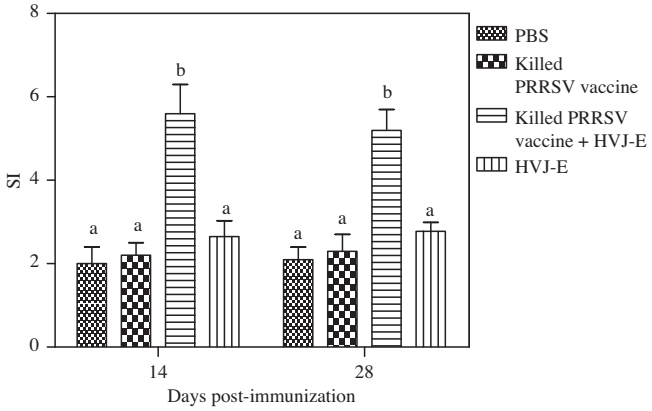

Fig. 1. Proliferation of PBMCs from the different groups. Four groups of ten 28-day-old piglets were immunized intramuscularly. Each bar represents the group mean ($n = 10$)±SEM of SI determined in triplicate. ConA served as a positive control. The difference between groups with different letters is significant ($p < 0.05$).

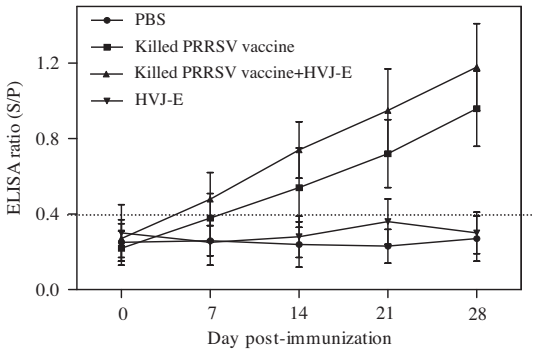

Fig. 3. PRRSV-specific antibodies detected by ELISA in pig sera samples before and after immunization.

Table 1
Clinical parameters following the challenge.

Groups	Survivors at day 21 post-challenge	Clinical score ⩾1 (number of pigs)[a]		
		DPC7	DPC14	DPC21
A: PBS	1/10	1/10	6/7	1/1
B: Killed PRRSV vaccine	6/10	10/10	6/8	0/6
C: Killed PRRSV vaccine + HVJ-E	10/10	6/10	2/10	0/10
D: HVJ-E	4/10	0/10	5/8	1/4

28 days after immunization, pigs were challenged with a virulent PRRSV strain.
[a] Clinical scores represent nervous and respiratory symptoms. 0, no symptom; 1, mild symptoms; 2, severe symptoms.

the same symptoms appeared in group A as early as 6 DPC and were irreversible. In contrast, the addition of HVJ-E to the killed PRRSV vaccine helped to reduce the respiratory symptoms in group C. Only mild clinical symptoms, such as a cough, were observed in group C piglets between 6 and 10 DPC, and these symptoms later resolved.

3.5. Viremia and virus excretion

Viremia and virus excretion were evaluated by RT-PCR on days 0, 3, 7, 14 and 21 post-challenge. The onset of viremia and virus shedding was observed at 3 DPC, where 3/10 piglets in group B tested positive in the serum and 2/10 piglets were positive for nasal swabs. At 7 DPC, all piglets displayed viremia with the exception of two piglets in group C, and the virus excretion detection showed similar results. At 21 DPC, one animal in group D exhibited a positive nasal swab, and one animal from groups A, B and D showed a positive serum test, whereas no animal of group C was positive for nasal swab or serum (Table 2).

4. Discussion

Outbreaks involving PRRSV have been reported in China since 2006. An atypical form of PRRS known as high fever disease affected approximately 2,120,000 pigs in 2006, leading to the deaths of at least 400,000 from June to September in at least ten provinces along the Southern and Eastern coasts of China. Collaborative efforts involving containment, slaughter, and vaccination were applied to control the outbreak [14], and the killed PRRSV vaccine has been employed to prevent the further spread of PRRSV and eliminate the disease in the affected regions of China. Despite millions of doses of the vaccine being dispensed to control PRRSV, the disease is still responsible for extensive production and economic losses by pig producers in China. This has resulted in strong efforts to utilize adjuvants for improving the immune responses to PRRSV vaccines.

Several vaccine adjuvants have been investigated for their ability to enhance cell-mediated immunity and antibody responses to the PRRSV vaccine. HVJ-E has been reported to induce both innate and adaptive immune responses, which makes it a potential candidate for a vaccine adjuvant. In the last decade, the immunotherapeutic effects of HVJ-E have been well documented, particularly in cancer therapy [15]. Recently, HVJ-E has also been shown to be an effective adjuvant in birds, with results indicating that HVJ-E could promote DCs maturation and cytokines production and had potent immune-enhancing potential when combined with killed H9N2 AIV [11]. However, the effectiveness of HVJ-E as adjuvant in mammals, especially in pigs, is untested. In this study, HVJ-E was co-administered with killed PRRSV vaccine to pigs to evaluate its immunostimulatory effects and observe whether it could increase the efficacy of the killed PRRSV vaccine.

Our results indicated that HVJ-E has a potent immune-enhancing effect when combined with the killed PRRSV vaccine. Specially, our results demonstrated that a combination of HVJ-E and the PRRSV antigen induced both a Th1 and Th2 response, with a strong tendency towards a Th1-type response in pigs (Figs. 1–3). After immunization, PRRSV-specific antibodies and production of the cytokines IL-10, IL-2 and IFN-γ were assayed. PRRSV-specific antibodies in Group C were detectable earlier than in Group B animals, and the level of PRRSV-specific antibodies in Group C was much higher than that in Group B, indicating that HVJ-E significantly increased the antibody response induced after PRRSV vaccination. Our results also showed that the addition of HVJ-E significantly up-regulated IL-2 and IFN-γ production and down-regulated the IL-10 production. IL-2 has been reported to significantly enhance the effectiveness of the PRRSV vaccines in reducing viremia, lung lesions and mortality in pigs after PRRSV challenge. Suradhat et al. demonstrated that increased IL-10 production might result in a poor cell-mediated response to PRRSV [16]. Inhibition of IL-10 gene expression has been considered as a strategy to enhance the cell-mediated immune responses after PRRSV vaccination. Our results indicated that HVJ-E significantly enhanced the cell-mediated responses to the PRRSV vaccine through both down-regulation of IL-10 production and up-regulation of IL-2 and IFN-γ production.

The clinical symptoms in pigs were also improved, including behavior (Table 1), body temperature and weight gain (data not shown). The addition of HVJ-E to killed PRRSV vaccine was found to enhance both the humoral and cellular responses when compared with the control group administered the killed PRRSV vaccine alone (Figs. 2 and 3). HVJ-E may be useful as an adjuvant for killed virus vaccines in light of this immunostimulatory activity. Our previous study evaluating the adjuvant efficacy and the protective effects of HVJ-E for avian influenza virus vaccination demonstrated that HVJ-E induces DCs maturation in mice and that one potential mechanism for the HVJ-E immunostimulatory ability may be induction of DCs maturation [11]. DCs are special antigen-presenting cells (APCs), and efficient priming of T cells by APCs requires both presentation of antigen peptides in the context of MHC molecules and expression of costimulatory molecules on the surface of the APCs [17]. Using bone marrow-derived murine DCs, our previous results demonstrated that MHC class II, CD40, CD80, and CD11c expression were markedly up-regulated after

Table 2
Viremia and virus excretion in animals after challenge.

Groups	Days post-challenge (DPC)									
	Virus excretion					Viremia				
	0	3	7	14	21	0	3	7	14	21
A: PBS	0/10	0/10	10/10	6/7	0/1	0/10	0/10	10/10	3/7	1/1
B: Killed vaccine against PRRSV	0/10	2/10	9/10	6/8	0/6	0/10	3/10	10/10	2/8	1/6
C: Killed vaccine against PRRSV + HVJ-E	0/10	0/10	6/10	2/10	0/10	0/10	0/10	8/10	1/10	0/10
D: HVJ-E	0/10	0/10	10/10	5/8	1/4	0/10	0/10	10/10	3/8	1/4

Four groups of ten 28-day-old piglets were immunized intramuscularly. 28 days later, all pigs were challenged with a PRRSV virulent strain. Viremia and virus excretion in serum and nasal swab were detected by RT-PCR.

incubation with HVJ-E [11], indicating that HVJ-E has a strong capacity for induction of DCs maturation, which would have significant implications for the development of effective vaccines. Whether the potential mechanism of HVJ-E immunostimulatory ability to PRRSV vaccine is through DCs maturation is not clear and must be further investigated.

It has been reported that the killed PRRSV vaccine can rapidly induce IgM and IgG responses, but the antibodies produced are sub-neutralizing. Four weeks post-immunization, all piglets received a virulent PRRSV (JXA1-R) challenge. The clinical symptoms, viremia and virus excretion first appeared in group B. The antibody titers detected by ELISA showed that all serum samples in groups B and C were positive, but animals from group A and D were negative (Fig. 3). These results indicated that the immunized piglets exhibit clinical symptoms after virulent PRRSV infection earlier than the non-immunized piglets. Sub-neutralizing antibodies have been shown to enhance viral replication in macrophages, a phenomenon referred to as antibody-dependent enhancement (ADE) [18], and the early development of neutralizing antibodies may play a significant role in the development of PRRS. However, compared with group B, animals in group C alleviated the phenomena possibly by the HVJ-E induced cytokines. ADE is considered to be a significant obstacle in developing effective vaccines for many viruses like PRRSV, because virus-specific antibodies that are not neutralizing facilitate the entry of the virus into target cells. This may explain why the non-immunized piglets were slower to show clinical symptoms. However, neutralizing antibodies, which play an important role in humoral immunity against PRRSV, are not detectable after 28 DPI with European and American-type strains of PRRSV [19]. Precautions should be taken to minimize the ADE-associated risks when formulating vaccines for controlling PRRSV.

Over the course of this study, piglets in the group receiving HVJ-E in combination with the killed PRRSV vaccine also presented clinical symptoms, viremia and virus excretion, but all piglets in this group recovered quickly and none of these piglets died. Although the difference was not significant, antibody titers in piglets receiving HVJ-E with the killed PRRSV vaccine were higher than those in piglets receiving the killed PRRSV vaccine alone, suggesting that HVJ-E might increase the levels of neutralizing antibodies, which is a major component of humoral immunity and provides protection against PRRSV. Piglets from group C had concentrations of the cytokines IL-2 and IFN-γ that were significantly higher than those seen in groups A and B ($p < 0.05$), and the concentration of the cytokine IL-10 in piglets from group C was significantly lower than those seen in groups A and B ($p < 0.05$). HVJ-E alone promoted IL-2 and IFN-γ production and decreased IL-10 production, suggesting that HVJ-E enhances the Th1 mediated cellular immune response. Taking into account the protection rate, clinical symptoms and viremia, the cellular immune response induced by HVJ-E may play a key role in protection against PRRSV infection.

HVJ-E contains single-stranded RNA fragments of approximately 300 bases [20]. These RNA fragments can be recognized by RIG-I and MDA5 in DCs, which results in DCs maturation and type I interferons and cytokines production [21]. Furthermore, the biological effect of HVJ-E was not diminished with repeated injections, although anti-HVJ specific antibody was generated [8]. Another merit of HVJ-E as adjuvant is the simplicity of preparation compared with the existing adjuvants such as IFN-γ, CpG and IL-2. Meanwhile, HVJ is not a swine pathogen, in addition, HVJ-E was derived from the UV-inactivated HVJ particles, therefore, HVJ-E has the potential to be a good adjuvant with good biosafety for killed PRRSV vaccine. However, the appropriate dose of HVJ-E and usage method for killed PRRSV vaccine is further to be investigated. Nevertheless, HVJ-E can be used as a good adjuvant because its robust ability to improve the immune responses.

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