

Short communication

Immunological response to porcine reproductive and respiratory syndrome virus in young pigs obtained from a PRRSV-positive exposure status herd in a PRRSV endemic area

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRS virus (PRRSV), remains a major economic threat to swine production throughout the world. The aim of this study was to investigate the humoral and cell-mediated immune responses to PRRSV in 10 PRRSV vaccinated and 10 non-vaccinated young pigs obtained from a PRRSV-seropositive herd under field conditions. On day 35 days of post-vaccination (dpv), two PRRSV seropositive mixed-litter pigs were added to each group to co-mingle the animals. Serum and whole blood samples were collected from all pigs on the first day of vaccination, as well as on the 21, 35, 49, and 63 dpv. The PRRSV-specific humoral and cell-mediated immune response was determined by ELISA and flow cytometry analysis. The PRRSV ELISA sample to positive (S/P) ratio was found to be positive at the threshold level until the age of 84 days in both non-vaccinated and vaccinated groups, whereas the IFN- γ positive staining cytotoxic (CD8⁺) cells were rapidly expressed in the early periods of vaccination and co-mingling, but were not found to be specific to PRRSV. This result might have been due to an unspecific response to stress antigens. Further studies should be conducted to obtain more immune response data over long-term observation periods and to study the effect of PRRSV endemic strain vaccinations in endemically-infected herds.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a prevalent disease in pig production worldwide and poses a major economic threat to the industry. The PRRS virus (PRRSV) is classified in the family *Arteriviridae* in the order *Nidovirales* (Kuhn et al., 2016; Lunney et al., 2016). PRRS clinical signs are variable and often reveal the symptoms of reproductive system failure in gilts and sows and respiratory failure in newborn and young pigs (Fablet et al., 2016a; Maclachlan and Edward, 2017). Moreover, PRRSV-infected pigs are frequently associated with secondary infections that involve various

pathogens due to immunosuppression. Consequently, they often develop greater pathology resulting in higher mortality rates (Kavanová et al., 2015; Schlafer and Foster, 2016). The epidemic form of PRRS is characterized by massive reproductive failure and respiratory disorders, while the infection in the endemic form is subclinical with the sporadic respiratory problems occurring in young pigs or reproductive failure occurring in naïve sows (Jeong et al., 2018; Nodelijk, 2002). Currently, PRRSV is endemic in most pig production facilities throughout the world, including Thailand.

PRRSV-naïve gilts and young pigs are highly susceptible to PRRSV infection, and this is relevant to the PRRS control effort in multi-stage

Abbreviations: CD, cluster of differentiation; dpv, days of post-vaccination; IFN, interferon-gamma; S/P, Sample to positive; SPRD, spectral red; TCID₅₀, 50% tissue culture infective dose; T, lymphocyte thymus-derived lymphocyte

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pig production farms (Pileri and Mateu, 2016). The integrative use of vaccinations as well as an acclimatization program of replacement breeding stock combined with biosafety management is a common strategy that is used to maintain the PRRSV protective immunity levels and to control PRRS outbreaks in endemic farms (Drigo et al., 2018; Toman et al., 2017; Woonwong et al., 2018). Generally, in Southeast Asia, the suckling period of piglets has ranged from 21 to 28 days, whereas the immune system in piglets is developing and remains relatively immature throughout the weaning period (Stokes et al., 2004). During this period, weaning stressors are involved, and disease susceptibility, including PRRSV infection, is then increased (Moeser et al., 2017). The PRRSV vaccination in susceptible pigs, including weaning pigs, was generally used in Southeast Asia to reduce clinical losses (Nguyen, 2013; Zhang et al., 2014). However, the maternally-derived antibodies can be an interference effect in both post-vaccination humoral and cellular immune responses in piglets (Fablet et al., 2016; Niewiesk, 2014) and the effectiveness of vaccinations at the time of weaning for the control of PRRS is also unclear in PRRSV seropositive herds. Hence, the humoral and cell-mediated immune responses to the PRRSV vaccination of young pigs in a PRRSV endemic farm under field conditions were investigated. Determination of PRRSV-specific antibodies and cell-mediated immune responses in terms of the T-lymphocyte subpopulation of peripheral blood mononuclear cells (PBMCs) throughout the early weaning period to the early stage of the growing period was evaluated in this study.

2. Materials and methods

2.1. Study animals

This study was performed on a non-cooling, evaporative, farrow-to-finish pig operation farm with a PRRSV-exposed herd (Sample to positive (S/P) ratio average ranging from 0.4 to 2.0) without clinical signs of PRRS at any stage of production for the last 6 months before the study. Cross-sectional sampling among all age categories of pigs was done on this farm twice a year to monitor the serological profile of PRRSV. The administration of PRRS modified live virus vaccine to the pregnant sows at 4–6 weeks of pregnancy and before birthing (10–12 weeks of pregnancy) (Thai Swine Veterinary Association, 2015) were used in this farm. As is shown in Fig. 1, on weaning day (twentyone-day-old), A total of 20 clinically healthy three-bred weaned piglets:

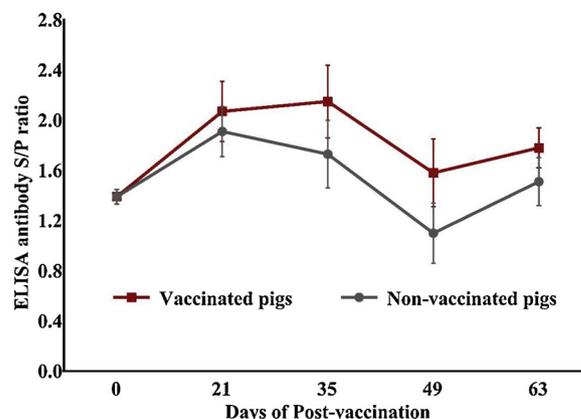


Fig. 2. Titers represent the average values of the S/P ratio, which are considered positive for PRRSV-specific antibodies (S/P value \geq 0.4). The S/P ratio values are presented as mean values \pm SEM. Statistical significance between the non-vaccinated and the vaccinated pigs at each time point was determined by a Student's *t*-test. Meanwhile, the statistical significance between the four difference time points at the 21, 35, 49, and 63 days of post-vaccination was determined by the repeated measure ANOVA.

Large White x Landrace x Duroc (Eleven females and nine males) were randomly assigned into 2 groups of 10 pigs and housed in separated pens. The PRRSV-specific antibody levels of all pigs were determined by ELISA (HerdCheck® 2XR PRRS ELISA, IDEXX, Westbrook, USA). All pigs in the vaccinated group were intramuscularly inoculated with 2 ml of a PRRS modified live virus vaccine) and the non-vaccinated pigs were inoculated with 2 ml of a sterile saline injection. Thirty-five days after vaccination (56-day-old), two 70-day-old pigs with PRRSV seropositive (S/P ratio values ranging from 1.5 to 2.0) were added to each group. PRRS clinical signs such as fever, depression, lethargy, respiratory signs, and pneumonia were observed daily. Serum and whole blood samples were collected on the 21, 35, 49, and 63 days of post-vaccination (dpv).

This study was carried out according to the guidelines for the care and use of laboratory animals (National Research Council, 2010). The study protocol was approved by the Faculty of Veterinary Medicine's Animal Care and Use Committee (Protocol number S24/2559).

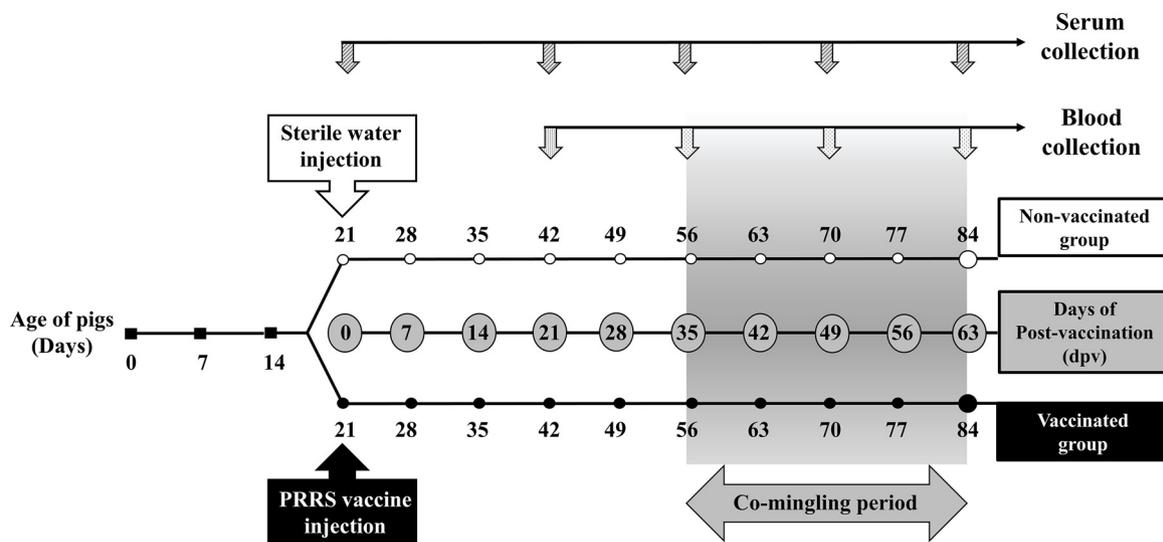


Fig. 1. Timeline of the study. Twenty-one-day-old piglets have randomly selected from PRRSV seropositive sows. On the first day of the study, serum samples were collected for PRRSV antibody testing by ELISA. Sterile water and the PRRSV-MLV vaccine was injected into pigs in the non-vaccinated and vaccinated groups ($n = 10$ each), respectively. At 35 dpv, all pigs were co-mingled with PRRSV seropositive nursery pigs. Serum and blood samples were collected at 21, 35, 49, and 63 dpv for PRRSV antibody testing and T-lymphocyte subpopulation characterization.

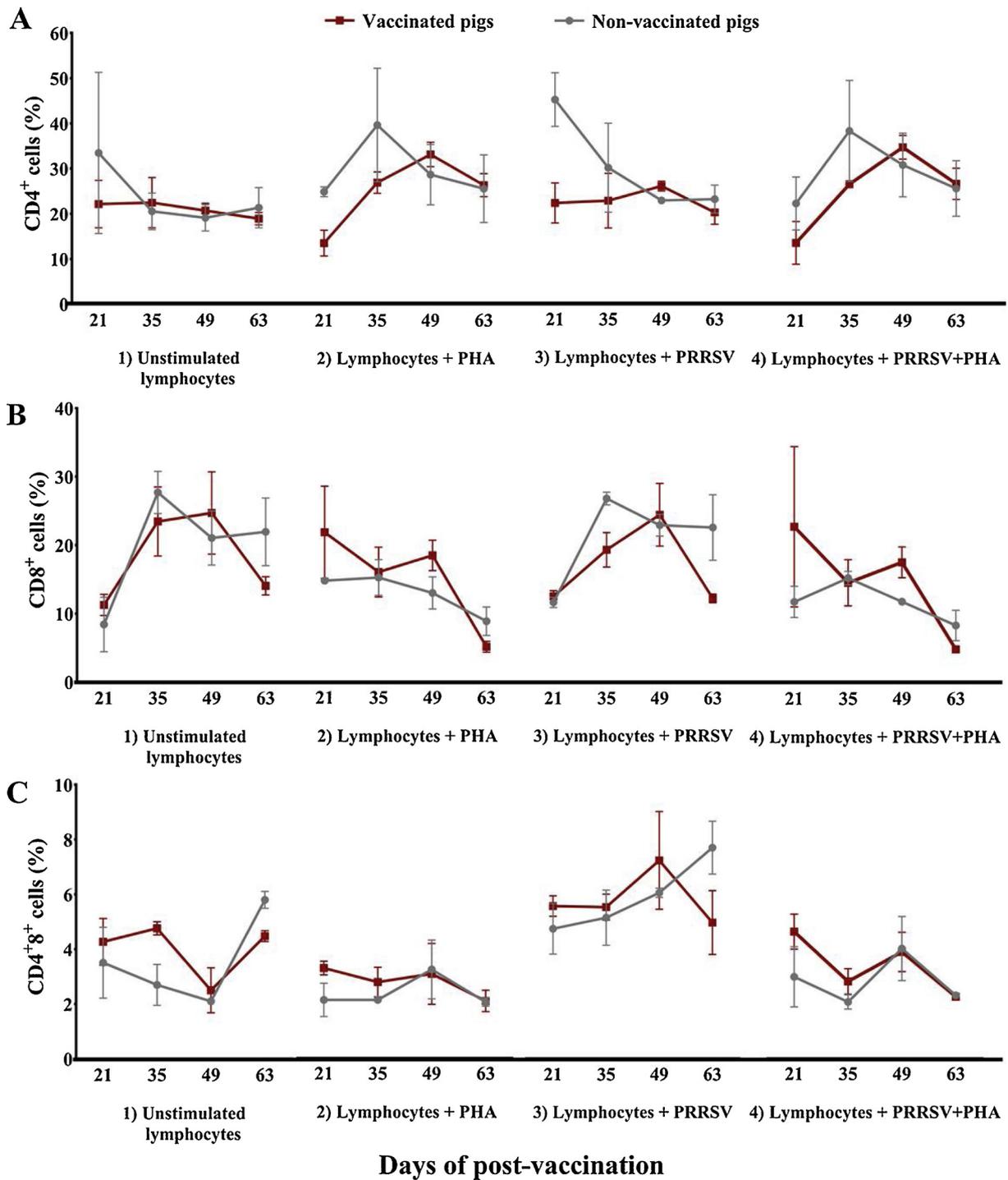


Fig. 3. Average percentages of CD4⁺ (3A), CD8⁺ (3B) and CD4⁺CD8⁺ double-positive T cells (3C) in lymphocyte presented as mean values ± SEM. Statistical significance between the non-vaccinated and the vaccinated pigs at each time point was determined by a Mann-Whitney test. Meanwhile, the statistical significance between the four difference timepoints at the 21, 35, 49, and 63 days of post-vaccination was determined by the Kruskal-Wallis one-way ANOVA.

2.2. PRRSV vaccine

The vaccine used in this study for injection into piglets and *in-vitro* cell re-stimulation was a PRRS modified live virus (MLV) vaccine (Ingelvac PRRS[®] MLV, Boehringer Ingelheim, Duluth, USA) containing 10^{4.9} TCID₅₀ of the virus.

2.3. Antibody titer evaluation

PRRSV antibodies were measured from sera using a commercial kit

(HerdCheck[®], IDEXX) according to the instructions provided by the manufacturer. The sample was defined as PRRSV positive if the S/P ratio was greater than 0.4.

2.4. Cell-mediated immunity evaluation

2.4.1. Porcine mononuclear cell isolation

Porcine lymphocytes were isolated from 10 ml heparinized whole blood using Lymphoprep[™] (Axis-Shield, Norway). The buffy coat was harvested and washed twice with sterile phosphate-buffered saline

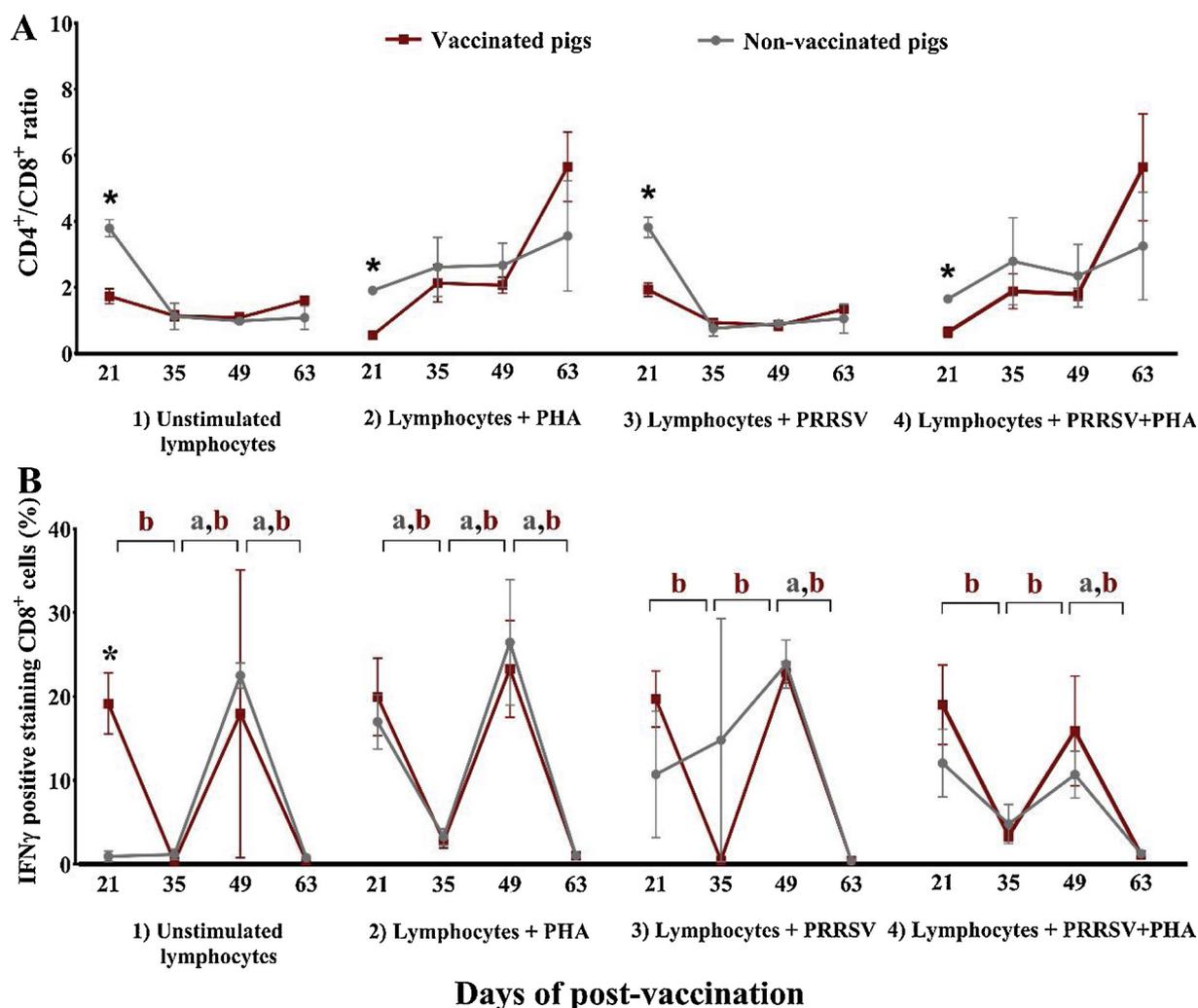


Fig. 4. The CD4⁺/CD8⁺ ratio, which was calculated as the percentage of CD4⁺ cells divided by the percentage of CD8⁺ cells (4A), and percentages of IFN- γ positive staining CD8⁺ cells in lymphocytes of pigs (4B) are presented as mean values \pm SEM. Statistical significance between the non-vaccinated and the vaccinated pigs at each time point was determined by a Mann-Whitney test. Meanwhile, the statistical significance between the four difference time points at the 21, 35, 49, and 63 days of post-vaccination was determined by the Kruskal-Wallis One-way ANOVA with a Mann-Whitney test. An asterisk indicates a statistically significant difference between the non-vaccinated and vaccinated groups at a specific time point ($p < 0.05$). The letters 'a' and 'b' indicate significant differences of the CD4⁺/CD8⁺ ratio and the IFN- γ positive staining CD8⁺ cells in a comparison between the 2-week interval in the non-vaccinated and vaccinated pigs ($p < 0.05$), respectively.

(PBS) supplemented with 1% fetal bovine serum (FBS, Invitrogen, Thermo Fisher Scientific, Waltham, USA). Erythrocytes were lysed with a red blood cell lysis buffer for 2 min (1 g/L KHCO₃ and 0.87 g/L NH₄Cl in deionized water) and centrifuged at 400 \times g for 5 min at 4 $^{\circ}$ C (Allegra X-15R centrifuge; Beckman Coulter, Brea, USA). Lymphocyte pellets were washed with PBS + 1% FBS and then centrifuged at 700 \times g for 5 min at 4 $^{\circ}$ C and re-suspended in 3 ml of cRPMI medium (RPMI-1640 medium + 10% FBS, 2 mM L-glutamine, 100 μ M non-essential amino-acids, 50 μ M 2 β -mercaptoethanol, 100 U/ml penicillin G, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B). Trypan blue staining was performed to determine lymphocyte viability. Lymphocyte counts were performed by manual differential white blood cell count with 200-cell counting throughout 4 h. Live cells were adjusted to 2 \times 10⁶ cells/ml in cRPMI for immunophenotypic assays.

2.4.2. Lymphocyte culture and in vitro re-stimulation

Lymphocytes from each pig were seeded in duplicate sets. In each set, 2 \times 10⁵ lymphocyte cells were cultured in 4 wells of a 24-well flat-bottomed plate (Corning[®] Costar[®], Merck, Darmstadt, Germany). Each well was incubated with 1) cRPMI as a negative control, 2) mitogen phytohemagglutinin (PHA), (Capricorn Scientific, Ebsdorfergrund, Germany) as a positive proliferation control, 3) PRRSV vaccine, and 4)

PRRSV vaccine + PHA. The third and fourth wells received PRRSV (10^{4.9} TCID₅₀ from MLV vaccine. The final concentration of 5 μ g/ml of PHA was added in the second and fourth well, while the first well was an unstimulated control receiving cRPMI. The first set was incubated at 37 $^{\circ}$ C in a 5% CO₂ incubator for 48 h for cell surface staining. For the intracellular IFN- γ staining set, the cell medium was aspirated out 3 h before cell harvest. Subsequently, 1 ml of 10 μ g/ml Brefeldin A (BioLegend, San Diego, USA), the protein transport inhibitor, was added to all of the samples (Ferrari et al., 2013; Martelli et al., 2016)

2.4.3. Cell staining

After re-stimulation, the lymphocytes were harvested and washed with cold PBS + 1% FBS and spun at 370 \times g for 3 min at 4 $^{\circ}$ C. Lymphocytes in each treatment were re-suspended in 100 μ l of a cell staining buffer (1 \times PBS + 2% FBS + 0.1% sodium azide) and treated with 100 μ l of 2% pig serum that had been supplemented with a cell staining buffer for 20 min at 4 $^{\circ}$ C. The specimens were then centrifuged at 370 \times g for 3 min at 4 $^{\circ}$ C. After which, the supernatant was discarded.

Afterward, a 3-color antibody combination consisting of mouse anti-porcine CD3 ϵ -FITC (#4511-02, SouthernBiotech, Birmingham, USA), mouse anti-porcine CD4 α -SPRD (#4515-13, SouthernBiotech) and

mouse anti-porcine CD8 α -PE (#4520-09, SouthernBiotech) diluted in a cell-staining buffer, was added to each sample and the samples were incubated for 30 min at 4 °C in the dark. After that, surface stain cells in the first set were washed twice with the cell-staining buffer and re-suspended in an ice-cold cell staining buffer containing 1% paraformaldehyde (1% PFA). Meanwhile, at the end of the incubation period of the intracellular IFN- γ staining set, cells were harvested and treated with 2% pig serum in a cell-staining buffer for 20 min at 4 °C. All samples were surface stained with mouse anti-porcine CD8 α -PE and incubated for 30 min at 4 °C in the dark. They were then centrifuged and subsequently fixed in a fixation buffer (#420801, BioLegend) for 20 min at room temperature in the dark. Lymphocytes were washed twice in 1 ml of 1 \times diluted permeabilization wash buffer (PWB, #421001, BioLegend) and incubated with Alexa Fluor[®] 647 Mouse Anti-Pig IFN- γ (cloneP2G10, BD Pharmingen, San Jose, USA) that had been diluted in PWB for 30 min at 4 °C in the dark. Permeabilized and stained cells were washed twice with 1 \times diluted PWB and fixed in 1% PFA in a cell staining buffer at 4 °C in the dark until flow cytometry analysis.

2.5. Flow cytometry

Approximately 50,000 cells were evaluated on the flow cytometer (CyAn[™] ADP flow cytometer, Beckman-Coulter, Brea, USA). The FITC, SPRD, and PE fluorescence levels were excited by a 488 nm excitation laser, and the Alexa Fluor[®] 647 level was excited by a 633 nm excitation laser. Compensation control was performed using sets of each individually stained beads (BD[™] CompBeads, BD Biosciences, San Jose, USA), and compensation matrices were calculated using Summit[™] software. Flow cytometric data were analyzed using the FlowJo v.10 software package (FlowJo, Ashland, USA).

2.6. Statistical analyses

A statistical comparison of the data sets was performed using the Statistical Software SPSS version 22.0 (IBM corp., New York, USA), and data collected from each group and treatment are shown as mean values \pm SEM. Parametric methods were applied when data follow the normal distribution using Shapiro-Wilk test, otherwise non-parametric methods were used to compare the treatment groups. A Student's *t*-test was used to compare the PRRSV S/P ratio between the non-vaccinated and the vaccinated pigs meanwhile the repeated measure ANOVA was used on four difference time points at the 21, 35, 49, and 63 days of post-vaccination. Significant differences in the percentages of the CD4⁺, CD8⁺, CD4⁺CD8⁺ double staining cells, CD4⁺/CD8⁺ ratios and the IFN- γ -positive staining CD8⁺ cells in lymphocyte between the non-vaccinated and the vaccinated pigs at each time point was determined by a Mann-Whitney test. Meanwhile, the statistical significance between the four different time points at the 21, 35, 49, and 63 days of post-vaccination was determined by the Kruskal-Wallis One-way ANOVA with a Mann-Whitney test. A difference with a *p*-value of less than 0.05 was considered statistically significant.

3. Results and discussion

From the primary clinical observation, there were no PRRS clinical signs observed in both the vaccinated and non-vaccinated piglets throughout the course of this study.

3.1. Detection of PRRS antibody response

In Southeast Asia, the PRRSV vaccination is generally used in the endemic area for the purpose of herd protective level maintaining and minimizing clinical losses (Nguyen, 2013; Thai Swine Veterinary Association, 2015). The PRRSV vaccination is usually administrated in gilts and sows in farrowing units, whereas the additional PRRSV

vaccination in piglets at the early stage of weaning period (21 days of age) is used in some area. PRRSV-specific antibodies could be passively transferred from sows to the piglets via colostrum. Afterward, the maternally-derived antibodies were found to be present in the piglets' serum for varying durations from the date of birth until the age of 42 days (Chung et al., 1997; Silva et al., 2015). However, the maternally-derived antibodies and maternally-derived neutralizing antibodies can interfere immune response to the PRRSV vaccination in piglets (Fablet et al., 2016; Renson et al., 2019). In the current study, all pigs were found to be PRRSV-seropositive by an ELISA test on the first day of vaccination (3-week-old of age) and remained positive throughout the study with no significant differences between each time point. Likewise, there were no significant differences observed in the S/P ratio between the vaccinated and non-vaccinated pigs (Fig. 2). The results suggested that the PRRSV vaccination in 3-week-old pigs, which were delivered from PRRSV vaccinated sows in an endemic farm under stabilized immunity conditions, were not accelerate the PRRSV humoral immune response. In accordance with previous study, the immune response may have been interfered by the effect of the maternal-derived antibodies (Fablet et al., 2016; Renson et al., 2019). In addition, PRRSV-specific antibodies were prolonged at the threshold level (S/P value \geq 0.4) over a period of 84 days of age in both vaccinated and non-vaccinated group. Hence, the analysis of the degree of the immune response that occurs under farm conditions over a more extended period should be evaluated in future studies.

3.2. Detection of PRRS cell-mediated immune response

T-lymphocytes play a major role in the defense mechanisms to control many porcine pathogens. T-lymphocytes in swine and other species are defined by the expression of either α or β TCR (Pescovitz et al., 1998; Yang and Parkhouse, 1996). The CD4⁺ T cells or T helper cells are the subsets of α T cells and play a vital role in the activation of cytotoxic T cell as well as the maturation of B cells and antibody production (Burrell et al., 2017). Regularly, CD4⁺ cells in pigs increase with age, exposure to foreign antigens, or repeated exposure to the same antigen over time; this could induce CD4⁺ cell generation and proliferation. Meanwhile, CD8⁺ T-cells are able to recognize and eliminate virally infected cells (Bakshi et al., 2014; Charerntantanakul and Roth, 2006). In contrast to human and murine, the CD8⁺ cells in swine blood were higher than the CD4⁺ cells approximately twice as much as was found in blood (Lunney and Pescovitz, 1987; Yang and Parkhouse, 1996). Likewise, CD4⁺CD8⁺ double-positive T cells are unique in that they can be found in swine peripheral blood, whereas the CD4⁺CD8⁺ T cells in other species are found in the thymus (Pescovitz et al., 1998). The CD4⁺CD8⁺ double-positive T cells act as a memory Th cells and increase with age and prolonged exposure to pathogen or antigens (Charerntantanakul and Roth, 2006).

In the present study, the T-lymphocyte responses between PRRSV vaccinated pigs, and non-vaccinated pigs were observed and presented in term of CD4⁺, CD8⁺, CD4⁺CD8⁺ double-positive T- cells sub-population and the CD4⁺/CD8⁺ ratios. As shown in Fig. 3, the percentages of CD4⁺ and CD8⁺ and CD4⁺CD8⁺ double-positive T- cells were not found to be significantly different between the non-vaccinated and vaccinated pigs in all treatments through the study period.

During the PRRSV post-exposure period, the declined of CD4⁺ cells and the proliferation of CD8⁺ cells was reportedly seen in several previous studies (Li et al., 2016; Samsom et al., 2000; Shimizu et al., 1996). The CD4⁺/CD8⁺ ratios of the present study in both study groups are shown in Fig. 4A. The higher values of the CD4⁺/CD8⁺ ratios with a statistically significant difference between the study groups were observed in each treatment of the non-vaccinated pigs at 21 dpv (42-day-old piglets). However, the CD4⁺/CD8⁺ ratio values in the present study revealed that the CD4⁺ cells outnumbered the CD8⁺ cells in non-vaccinated pigs at the initial day of the study, which was inconsistent with the previous studies as described. These results could

have possibly occurred as a consequence of some other stimulants such as bacterial infections, which can cause a marked increase in CD4⁺ cells (Salles et al., 2002; Sarradell et al., 2003).

3.3. Detections of IFN- γ positive staining cytotoxic cells

IFN- γ is an essential cytokine and play an important role in host cell-mediated immunity (CMI) response as viral eliminator by various functions. A large amount of IFN- γ is produced by cytotoxic (CD8⁺) cells such as natural killer (NK) cells and several different types of T-cells, including cytotoxic CD8⁺ T-cells (Kak et al., 2018; Sarradell et al., 2003). As is shown in Fig. 4B, significantly higher levels of IFN- γ positive staining CD8⁺ cells in the unstimulated lymphocytes were found in the PRRS vaccinated pigs at 21 dpv compared with the non-vaccinated pigs. Thereafter, IFN- γ positive staining CD8⁺ cells rapidly decreased to very low levels within 14 days. The IFN- γ positive staining CD8⁺ cells were higher detected than the later time points at 21 and 49 dpv (42 and 70-day-old piglets) with a significantly higher result in recall to respond to all treatments from the vaccinated pigs. However, repeated changes in the high-and-low patterns of IFN- γ positive staining CD8⁺ cell expression were also found in the *in vitro* re-stimulation of lymphocytes in non-vaccinated pigs suggesting that the IFN- γ expression is not specific to PRRSV, but it might be an unspecific response to stress antigens as has been previously reported (Amadori and Zanotti, 2016). However, several previous studies have indicated that the initial IFN- γ response in PRRSV inoculation and vaccination trials was low and delayed. IFN- γ levels were expected increased within 2–3 weeks after the modified live attenuated PRRSV vaccination was administered, but could reach a maximum level in 32 weeks, which was considered an enormous delay when compared with some other RNA viruses (Li et al., 2014; Meier et al., 2003; Zuckermann et al., 2007). High levels of IFN- γ expression in the serum was found in highly pathogenic PRRSV (HP-PRRSV) infected pigs. The coincidence of these findings with the high percentage of NK cells present in the blood might be a result of the innate immune response of the antigen-stimulated NK cells (Wesley et al., 2006). Nevertheless, previous studies have reported that higher levels of IFN- γ in the serum were not associated with virus reductions in the circulating blood. However, IFN- γ function in PRRSV protection at this stage is still unclear (Li et al., 2014). Hence, the role of IFN- γ in pathogenesis and the host immune response to PRRSV needs to be further explored.

The results of this study revealed that the levels of PRRSV-specific antibodies remained high in all piglets to the end of the study, regardless of whether they were vaccinated or not. There were no observed PRRSV-associated clinical signs in the piglets which were the offspring of PRRSV seropositive sows. Nevertheless, the IFN- γ positive staining CD8⁺ cells were found to be impermanent increased in vaccinated piglets at 21 dpv and 14 days after co-mingling (49 dpv). The results suggested that the PRRSV vaccination in 3-week-old pigs, which were delivered from PRRSV vaccinated sows in an endemic farm under stabilized immunity conditions, might not accelerate the PRRSV immune response within 84 days of age. However, the immune response to PRRSV is known to vary according to a range of factors such as virus strains, duration of infection, population size, intervention variations, and management strategies. Previous studies have indicated that the cell-mediated immune response to PRRSV is significantly delayed following vaccination (Li et al., 2016; Samsom et al., 2000). Thus, further studies on the long-term immune response to PRRSV vaccination should be conducted to determine the relevant time points from farrowing to the end of the finishing stage among gilts, sows, and boars. In particular, the effect of PRRSV endemic strain vaccinations on endemically-infected herds for clinical relevance in order to develop an effective and suitable control program that fits the conditions of a designated herd.

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